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ARTICLE

Repellency of a Wax-Based Catnip-Oil Formulation against Stable Flies

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Stable flies, *Stomoxys calcitrans* (L.), are one of the most serious livestock pests, which cause significant economic loss in the cattle industry. Current practices for managing stable flies are limited to costly sanitation techniques and unsustainable insecticide applications. The present study reports the initial efforts using catnip essential oil as a spatial repellent and the results of field trials using a wax-based formulation to repel stable flies in the cattle feedlot. Electroantennograms showed that catnip oil and its ingredient compounds elicit significant antennal responses from both sexes of stable flies. Catnip oil and *ZE*- and *EZ*-nepetalactone showed repellent activity in a single cage olfactormeter study. No behavioral activity was observed from another ingredient compound, caryophyllene. A laboratory dispersal bioassay also showed that stable flies avoided areas treated with catnip oil. Using a solid phase microextraction (SPME) method, the atmospheric concentration of catnip active ingredient compounds (nepetalactones) absorbed by SPME fiber in treated areas was detected at 4 times higher than those in control areas. Application of wax-based catnip pellets in cattle feedlots resulted in >99% repellency of stable flies in treated areas, compared with that in nontreated areas. However, the repellent efficacy of the formulation only lasted 3 h. This is the first study demonstrating the potential application of a plant-based repellent formulation that may be used as an alternative method against stable flies.

KEYWORDS: *Stomoxys calcitrans*; *Nepeta cataria*; spatial repellency; wax formulation; atmopheric concentration

INTRODUCTION

The stable fly, *Stomoxys calcitrans* (L.), is a serious pest that feeds on many livestock animals, especially on bovines and equines (*1*). Fly-feeding on grazing hosts has led to reproductive failure and reduction of meat and milk yields, with an estimated annual economic loss of up to two billion dollars for the cattle industry (*2*−*4*). Furthermore, stable flies are also capable of transmitting a large variety of pathogens including helminths, protozoans, bacteria, and viruses, some of which are primary agents of mortality in cattle (*1*, *5*, *6*).

Stable flies can utilize decomposing vegetation (fermenting) as their breeding sites (*7*−*9*). The area along the soil-to-concrete interface of the feed apron in the feedlot pens can generate about 80% of immature flies at confined cattle facilities (*10*). Stable fly management involves the use of insecticides and cultural sanitation as primary control methods. The direct application of insecticides results in only marginal control (*11*, *12*). Cultural control including the removal and dispersal of substrates that could serve as potential breeding sites can be tedious and costly. Zumpt (*1*) suggested that spraying cattle with repellents or applying contact insecticides to fly resting areas could suppress the development of fly densities.

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Plant derivatives or botanical-based insecticides and repellents have been used against arthropods for at least two millennia in ancient China, Egypt, and India (*13*, *14*). Even in Europe and North America, the documented practice of using botanicals extends back more than 150 years, predating the discovery of the major classes of synthetic chemical insecticides (*15*). Recent studies have confirmed the repellency effectiveness of plant essential oils against Dipteran blood-sucking insects, particularly in mosquitoes (*16*−*19*). Zhu et al. (*20*) reported that catnip (*Nepeta cataria* L.) essential oil acts as an extremely effective antifeedant/repellent against several filth fly species (including stable flies) in laboratory assays. They have further demonstrated that catnip oil is a relatively safe repellent with an extremely low toxicity in rabbits and rats. The use of repellents could be an effective strategy for reducing the impact of stable flies on livestock. There is a great deal of interest in developing botanical-based repellent formulations that serve a valuable function in integrated management against stable flies. Development of effective repellent formulations for livestock application would build a strong base for stable fly integrated management involving a push−pull strategy. Similar strategies have been developed successfully for other agricultural and urban pests (*48*).

The present article reports (1) evidence of antennal responses of stable flies to catnip and its ingredient compounds; (2) spatial

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repellency of the essential oil of catnip against stable flies in laboratory bioassays; and (3) evaluation of the effectiveness and longevity of a wax-based catnip formulation in repelling flies in cattle feedlot pens.

MATERIALS AND METHODS

Insects. Stable flies used for laboratory bioassays were from colonies maintained at the United States Department of Agriculture, Agricultural Research Service, Agroecosystem Management Research Unit (Lincoln, Nebraska). The flies were maintained at 23 ± 2 °C with variable humidity (30−50% RH) and a 12 L/12D photoperiod. Adults were fed with citrated bovine blood (3.7 g sodium citrate/liter) from a blood soaked absorbent pad (Stayfree, McNeil-PPC Inc., Skillman, New Jersey) placed on top of the screened cage.

Catnip Essential Oil. Catnip essential oil was purchased from Bramble Berry Inc. (Bellingham, Washington, USA). The chemical composition was determined by gas chromatography−mass spectrometry (GC-MS) analysis to be 90% of *ZE*- and *EZ*-nepetalactone, and 10% of caryophyllene (*19*). The two nepetalactones were accumulated and purified (>95%) from the purchased catnip essential oil following the method described by Peterson (*37*). Caryophyllene (>98%) was purchased from Sigma-Aldrich (St. Louis, MO). *N*,*N*-Diethyl-3-methylbenzamide (DEET) was purchased from Morflex Inc. (Greensboro, NC) with >98% purity.

Electroantennogram (EAG) Responses. EAG recordings were made by connecting an electrogel-filled (Spectro 360, Parker Laboratory, New Jersey) glass pipet Ag-AgCl electrode to the excised head of a stable fly. A recording electrode filled with the same electrogel was placed in contact with the tip of the fly funicle. Antennal responses to repellent stimuli were recorded from 10 μg of catnip oil and its ingredient compounds, and DEET as well, and then a series of three dosages of catnip oil ranging from 1 to 100 μg/10 μL. Serial dilutions (10×) were made in hexane (Burdick & Jackson High Purity Solvent, Muskegon, MI). The tested repellents were applied to filter-paper strips (0.5 × 2.0 cm, Whatman No. 1) in 10 μL of solvent. The filter-paper strips were inserted into Pasteur pipettes (15 cm long). Control puffs of controls (hexane only) were applied after each puff of a test stimulus. The EAG system consisted of a high-impedance DC amplifier with automatic baseline drift compensation (SYNTECH Equipment and Research, Kirchzarten, Germany). An EAG program (SYNTECH EAG-Pro 4.6) was used to record and analyze the amplified EAG signals. The order of exposure of each stimulus to each antenna was randomly defined and at a 3-min interval for antennal sensitivity recovery. A total of 12 antennae were tested in the first EAG test (6 males and 6 females since no differences in responses were found; therefore, EAG data were pooled). For the dose−response EAG test, a total of 20 antennae were used (8 males and 12 females).

Spatial Repellency Assays. The first laboratory spatial repellency assay (dispersal test) was conducted inside a green house using a large screen cage (1 m long × 0.5 m wide × 0.5 m high) under a temperature of ∼27 °C and 50% relative humidity. Approximately 150 mixed sex stable flies (3−4 day-old, with >50% females already mated, but no egg-laying behavior observed) were released into the screened cage. The cage was divided into two zones for recording fly distribution (0.5 m-left and 0.5 m-right). Two Petri dishes, each contained one filter paper (one with solvent as control and another catnip oil in solvent as treated) were placed on stands at a height of 5 cm above the cage floor and set at 0.5 m apart (0.25 m from each end of the cage). One Petri dish contained a 10-cm diameter Whatman No. 1 filter paper impregnated with 100 mg of catnip oil (treated) in hexane (0.5 mL), and the second Petri dish was treated with hexane (0.5 mL) only and served as the control. Solvent was allowed to evaporate at room temperature before the Petri dishes were placed inside the cage. The positions of treated and control dishes were alternated between each test to avoid heterogeneity. Fly distribution within the cage was determined by counting the number of flies in each of the two zones for 5 min before introduction of the Petri dishes and then for 5 min beginning 1, 2, 3, and 4 h after introduction of the Petri dishes. The spatial repellent bioassay was repeated 6 times with 2−3 day intervals between replicates to ensure that catnip oil residuals had dissipated.

Atmospheric concentrations of catnip volatiles in the treated and control zones were sampled using solid-phase microextraction (SPME) during the bioassay. The 100 μm polydimethylsioxane (PDMS) fibers (Supelco, St. Louis, MO) were conditioned in a GC inlet held at 250 °C for 30 min before sampling. SPME holders were placed 2 cm above each Petri dish (parallel). SPME fibers were exposed for a 5-min time period at the beginning of each hour (3 collections were made from the control and catnip-treated dishes, respectively, at every hour during the 4-h experiments). Relative concentrations of volatile samples were analyzed in an Agilent GC system equipped with a DB-5 column (30 m × 0.25 mm i.d., J & W Scientific, Agilent). Helium was used as the carrier gas and the flow rate maintained at 1.5 mL/min. Samples were injected under the splitless mode. The temperature program for the GC analyses was set at 50 °C for 3 min, rising by 10 °C/min to 280 °C. Quantities of the active ingredient compounds (*ZE*-nepetalactone and *EZ*-nepetalactone) were assessed by the external standard method. The two nepetalactones were weighed using an analytical balance with a readability of 0.01 mg (Mettler, Toledo AL104) and then dissolved in hexane. Calibration curves to determine linearity were obtained from each identified nepetalactones at four concentrations (10, 50, 100, and 1000 ng/μL) with three replications per concentration. Linearity was assumed when the regression coefficient provided an $R^2 > 0.97$. Quantities of catnip ingredient compounds were obtained by integrating the two peak areas and calculating concentrations based on the established standard curves.

The second repellency assay was conducted using a single cage, dual port olfactometer to assess the spatial repellency of catnip oil, its ingredient compounds, and DEET against stable flies. This system was constructed from clear glass (4 mm), which was modified from a triple cage olfactometer described in Posey (*21*). The whole dimensions of the olfactometer were 96 cm long by 50 cm wide and 25 cm high, and placed on top of a metal tool table painted white. The front of the test cage was connected with a pair of glass ports (30 cm long and 10 cm in diameter) that were set at 10 cm apart. The rear of the test cage was connected with a similar sized glass port covered with a 1.0 mm mesh window screen in frame that allowed the flies to acclimatize to the air being used in the tests. Two small table fans were set in front of the dual ports to generate wind speeds measured at 0.35−0.4 m/s, which were regulated by separate voltage regulators. All experiments were conducted at ∼27 °C and 55% relative humidity.

Stable flies used for testing were about 3−4 days old and were starved for 24 h prior to testing. The flies were released individually. Each fly was given 3 min to respond, and their presence in either repellent/attractant-treated or control ports (at 10 cm inside the port) was recorded. Normally, 1 set of 6 tests (6 treatments) was performed in one day. Within each set of tests, the order of ports with either attractants/repellents or the controls was randomized. All three ports were cleaned first with acetone and then hexane before new tests began. Each experiment was replicated 3−4 times with 20 flies, and different (new) flies were used in each replication.

Catnip oil and other repellent compounds were tested at 100 μg dosage. They were first dissolved in hexane to make a solution of 10 μg/μL, then 10 μL of the solution was impregnated onto a filter paper (cut as a small triangle piece). The filter paper then was air-dried and fixed to an insect pin, which later was placed in the middle of the testing ports. For the control port, only hexane was applied (10 μL).

Wax Pellet Formulation. Wax pellets were produced by combining catnip oil (10% w/w) with a molten mixture of soy wax (9% w/w) and paraffin wax (81% w/w) at approximately 60 °C. The mixture was transferred to a heated syringe pump (New Era Pump Systems, Inc., Wantagh, NY) and maintained at 65 °C. The molten wax mixture was introduced dropwise into the bottom of a column of water with a 22 ga needle at a rate of 1.5 mL/min. The column of water was 1 cm in diameter and 30 cm long maintained at 73 °C, slightly above the melting point of the wax solution. The released drop floated through the column until it reached a second column of water, 2.5 × 100 cm that was maintained at 50 °C, below the melting point of the wax solution. The droplet solidified during accent before it reached the reservoir at the top of the second column. Two jacketed columns were joined one on top of the other with the temperature of each column controlled via a recirculating water bath. The pellets were removed from the reservoir and dried with paper towels. The columns were jacketed glass cylinders obtained from Ace Glass inc. (Vineland, NJ). Similar strategies have been previously used to make wax pellets (*22*, *23*). High melting point Soy wax (Ecosoya PB) was obtained from a local distributor. Paraffin wax (mp. 58−62 °C) was obtained from Sigma-Aldrich (St. Louis, MO).

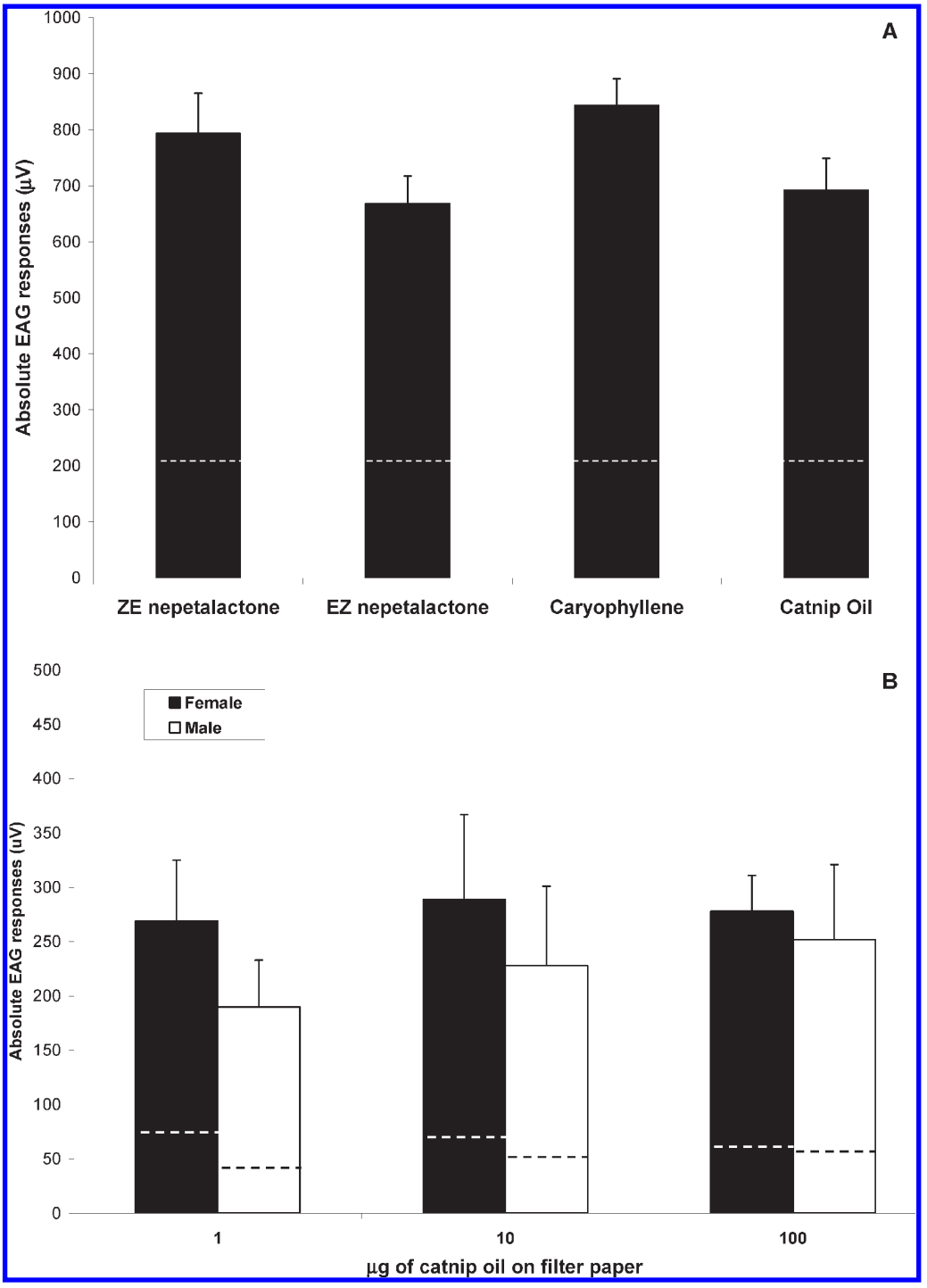

Figure 1. EAG recordings from stable flies responding to catnip oil and its ingredient compounds (10 μg) (**A**, n = 12, 6 antennae of each sex), and stable flies responding to different dosages of catnip oil (**B**, antennae of males (n = 8) and females (n = 12)). The dashed lines inside the bars are the mean EAG responses to control puffs. No significant differences were found among the treatments, dosages, and two sexes, $P > 0.05$, ANOVA.

Repellency of Catnip Wax Formulation against Stable Fly in Feedlot Pens. Field trials were conducted in cattle feedlots at the Agricultural Development and Research Center (Mead), University of Nebraska (Ithaca, NE). Eight feeding pens (16 m × 36 m) were selected, with 7−10 cows in each pen. Stable flies were observed to rest either on the cement walls of the feeding bunks or on adjacent areas containing manure mixed with soil and feeding materials. Stable flies landing on these resting areas were counted for 5 min before the treatment and 5 min after the treatment. Subsequently, fly numbers were recorded every hour for 3 h and finally 24 h after treatment. About 9 g of formulated wax pellets (~545 pellets containing 32 mg of catnip oil) were spread evenly in manure-soil areas (0.5 m × 0.3 m). These areas were observed to be used as resting areas by stable flies after their blood meals. Another 6 pens located at upper wind (to avoid the catnip odor drifting from the treated pens) were selected as controls treated with the same amount of wax pellets made without catnip oil. The number of stable flies was also recorded as described above.

Atmospheric concentrations of active ingredient compounds was determined in a similar method described for the lab repellent bioassay using a field sampler of SPME equipped with PDMS fiber (Supelco, St. Louis, MO). The fiber was placed in the center of the treated areas, 5 cm above the ground, for 3 min of sample collection. The samplers were brought back to the lab for quantitative analyses by gas chromatography as described above.

Statistical Analysis. The mean percentage of fly population reduction from the treated zone in cage studies was calculated as follows:

$$\%R = (T_a - T_b)/T_a \times 100$$

where T_a is the total number of stable flies observed in the treated zone before introducing the repellent, and T_b is the number of flies in the treated zone after introducing the repellent. Percentage data were transformed using square root ($X + 1$). The significances of differences among individual means (population reduction, catnip concentrations, EAG dosages, and fly counts) were determined by multiway ANOVA followed by the Scheffe test (PASW Statistics 18, SPSS Inc.), and Student's *t*-test was used for comparisons of the EAG recordings. For the single cage olfactometer study, data were recorded as percentage of flies inside the treatment or control ports. After checking the homogeneity of variance and normality of data, they were analyzed using Student's *t*-test. Log transformation was done when necessary. Results with $p < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Antennal Responses of Stable Flies to Catnip Oil. Sensory organs on the antennae of insects are known to be used in locating mates, hosts, habitats, and oviposition sites (*22*, *24*−*27*). Most sensory organs in muscoid flies used for the perception of chemical odorants are located on the funicle of antennae (*28*−*30*). These sensory organs have been reported to respond to physical and chemical stimuli including warmth, humidity, host odors, ammonia, and carbon dioxide (*31*−*34*). EAG responses were detected from stable fly antennae when tested with a range of host animals and odorants associated with oviposition sites, with demonstrated attractiveness in the wind tunnel (*35*). However, the present study is the first report that stable fly antennae are also capable of detecting active repellent compounds (nepetalactones of catnip oil) (**Figure 1A**). No differences in EAG responses to catnip oil were found between the two sexes of stable flies and the three dosages tested (**Figure 1B**). The dose−response EAG data from this study suggested that the stable fly antennae were able to detect lower doses of catnip oil repellent compounds (**Figure 1B**). The laboratory olfactormeter study has further shown that stable flies avoided entry into the glass ports treated with catnip oil and two nepetalactones (**Table 1**). More interestingly, in the feedlot field studies, stable flies were observed to avoid the catnip treated areas by flying away abruptly (∼5 cm from the treated area). Such a behavior may indicate a spatial repellent nature of catnip oil. Our EAG tests further showed that another ingredient compound, caryophyllene, and a less volatile DEET could also elicited antennal responses (**Figure 1A**). A relatively lower antennal response was recorded from DEET at 0.38 ± 0.03 mV (compared with catnip oil and nepetalactones) but still significantly higher than the control at 0.20 ± 0.01 mV ($t = 2.2$, $P < 0.05$).

Table 1. Spatial Repellency of Catnip Oil and Its Ingredient Compounds in a Simple Cage Olfactormeter against Stable Flies, *Stomoxys calcitrans*[a]

treatments	significance
control (hexane) 74 ± 1 vs catnip oil (100 μg) 26 ± 1	$P < 0.001$
control (hexane) 83 ± 2 vs *ZE*-nepetalactone (100 μg) 17 ± 1.5	$P < 0.005$
control (hexane) 73 ± 2 vs *EZ*-nepetalactone (100 μg) 27 ± 2	$P < 0.005$
control (hexane) 52 ± 8 vs caryophyllene (100 μg) 48 ± 8.4	$P = 0.85$
control (hexane) 25 ± 5 vs 1-octen-3-ol (100 μg) 75 ± 4	$P < 0.01$
control (hexane) 62 ± 4 vs 1-octen-3-ol + catnip oil (100 μg) 38 ± 3	$P < 0.05$
control (hexane) 60 ± 11 vs DEET (100 μg) 40 ± 11	$P = 0.16$

[a] Results were the mean % of stable flies observed into treated or control ports (± S.E., $n = 60-80$). Significance was measured using Student's *t*-test.

Catnip Oil Spatial Repellency. Catnip oil has been known to repel up to 13 families of insects (*36*). During the last 5 years, several studies have shown catnip, as an alternative insect repellent, that strongly repels several disease-transmitting urban insect pests including house flies, mosquitoes, and cockroaches (*37*−*39*). Recently, Hieu (*40*) reported the contact repellency of female stable

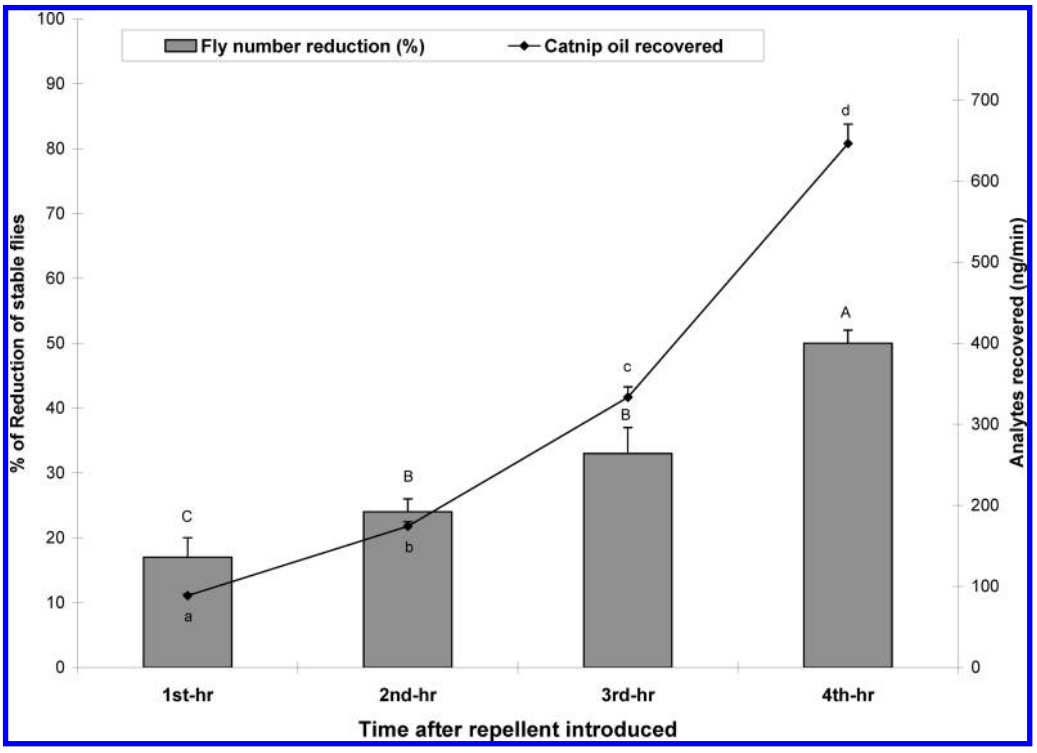

Figure 2. Reduction of stable fly numbers after catnip oil application at 4 μg/cm^3 concentration to filter paper in the screen cage (bars) and catnip oil volatiles recovered by solid phase extraction (5 min atmospheric sampling). Different letters on top of bars and lower case letters on the line are significantly different at the level of $P < 0.05$ according to ANOVA, separated by the Scheffe test.

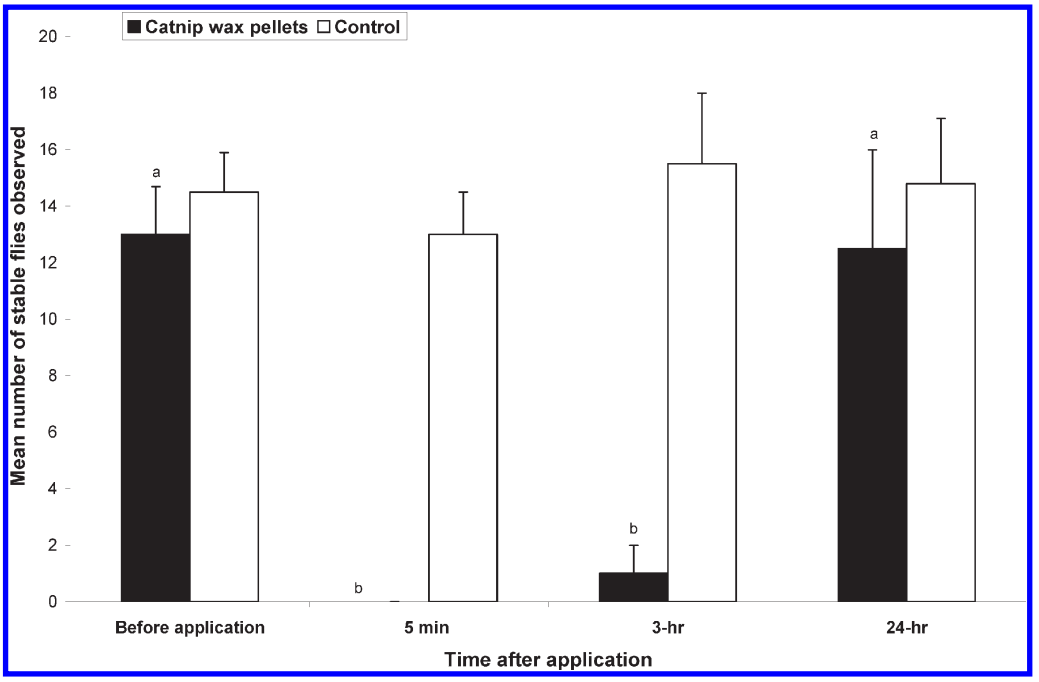

Figure 3. Mean number of adult stable flies observed landing on catnip oil-treated areas and the untreated control areas of cattle feeding lots. The means for each sample time with different letters are significantly different at the level of $P < 0.05$ ANOVA, separated by the Scheffe test.

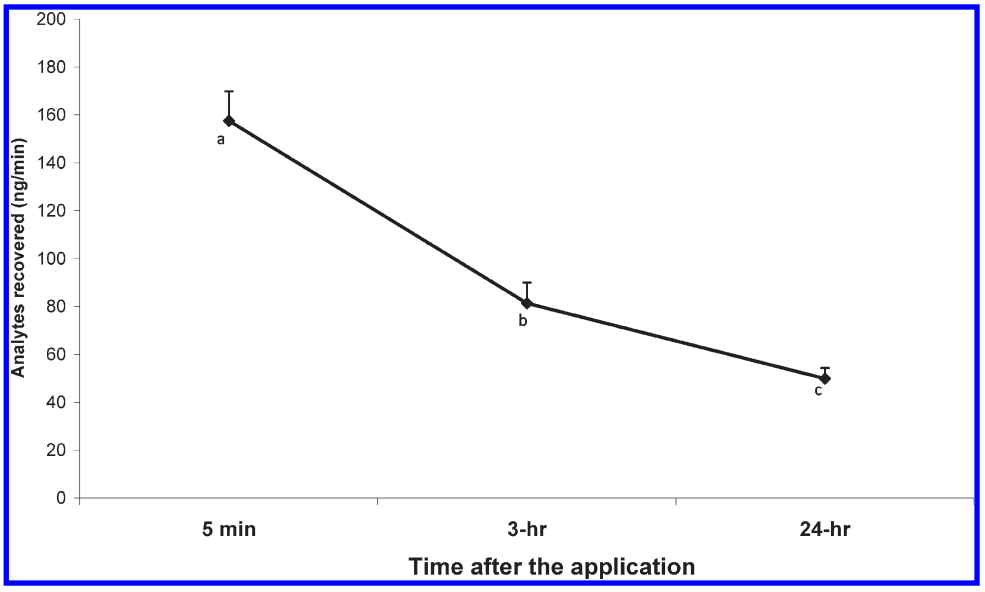

Figure 4. Catnip oil volatiles (analytes) recovered by solid phase extraction (5 min atmospheric sampling) in feedlot areas after treatment with a wax-pellet formulation of catnip oil. Means with different letters are significantly different at the level of $P < 0.05$ ANOVA, separated by the Scheffe test.

flies on humans treated with 21 essential oils applied alone or in combination with *Calophyllum inophyllum*. During the course of our catnip antifeedancy studies conducted in modified K&D modules (*20*), we observed that tested stable flies in the catnip-treated cells tried to fly away from the repellent-treated surface. This indicated that catnip oil may also have a spatial repellency against stable flies. The current dispersal study and the single cage olfactormeter assay showed that catnip significantly repelled stable flies from the catnip oil-treated areas. Percentiles of repellency ranged from 18% to 50% observed from the dispersal study during the 4-h experimental period (**Figure 2**, bars). Further analyses of the accumulative, atmospheric concentrations of two nepetalactones absorbed by SPME fibers in the catnip-treated areas revealed a 6-fold increase of catnip atmospheric concentration 4 h after initial exposure (**Figure 2**, line). These results suggest that the atmospheric concentration of catnip oil contributed significantly to the spatial repellency, with a positive correlation found between the concentration of catnip oil and the fly number reduction with a linear regression coefficient at $r^2 = 0.99$. Bernier et al. (*27*) demonstrated that catnip oil acts as a spatial repellent against female *A. aegypti* mosquitoes. Our single cage olfactometer study further showed that over 70% of flies were repelled from the catnip oil treated ports, compared with the control (**Table 1**). Stable flies were observed to be highly attracted to 1-octen-3-ol (a ruminant odorant found in animal breath) with an observed 75% of attractancy in the olfactometer study, but the attractiveness was reduced significantly when catnip oil was added (*35*, *41*, *42*).

The spatial repellency of catnip oil against stable flies is attributable to its vapor phase released in the atmosphere; similar cases were also found with DEET against mosquitoes (*43*, *44*).

Spatial repellency of DEET against mosquitoes has been reported with an effective repelling distance of 38 cm from treated hands in an olfactormeter (*38*). Although our EAG test showed that stable fly antennae can detect DEET, it was not found to be an effective repellent of stable flies in our antifeedancy study (with 50% repellency observed, personal communication), nor a good spatial repellent in the olfactometer assay from the present study (**Table 1**).

Repellency Efficacy of Catnip Wax Pellets in the Field. Repellent chemicals, including some insecticides with residual repellency activity, operate in the vapor phase, where volatiles can be detected by insect olfactory sensilla to keep them at a distance (*45*). Repellents, such as catnip oil, which have a high vapor pressure may offer protection at low concentration, but lose repellency in a relative short time (*37*). Wax-based formulations have previously been shown to be effective slowing the release of volatile compounds (*46*). Behle recently reported the effective use of wax-based pellets for the controlled release of sex pheromones for mating disruption of Oriental beetles (Coleoptera: Scarabacidae) in turf grass (*47*). However, wax pellet formulation with 10% catnip oil only repelled stable flies effectively from their common resting areas up to 3 h with no repellency after 24 h (**Figure 3**). The atmospheric concentrations of catnip repellent compounds measured by SPME in treated areas are shown in **Figure 4**. Three hours after the application, there was a 50% reduction in the measured concentration of the repellent compounds from catnip wax pellets-applied areas and a further 50% decrease after 24 h. At a mean recovered catnip repellent compound of 81 ng, the repellency of catnip wax pellets provided with >95% protection within 3 h. However, the protection level was <4% after 24 h. The rapid loss of effectiveness could be partly due to the high temperature conditions during the field trials, with 33 °C in the air and more than 60 °C on the ground where wax pellets were applied. Control pellets without catnip oil did not reduce stable fly numbers in the feedlot areas, compared with pretreatment fly counts. More work is needed for developing formulations for extended longevity of repellency for use with a push−pull strategy for the control of stable flies. It is not known if a 3-h repellent "push" period is sufficient to move flies to a treated "pull" site for control.

More than 10 successful cases using push−pull strategies in the control of agricultural, horticultural, veterinary, and urban pests have been reported (*48*). The push components repel or deter pests away from a resource by using stimuli that mask the resource's appearance (repellent or deterrent). For stable fly control, catnip oil has already been proven to be a very effective antifeedant in the laboratory bioassay (*20*), with further evidence as a spatial repellent from the current study. However, the longevity of the catnip formulation for field applications would need to be improved significantly for commercial acceptance. The pull components simultaneously attract the pests using highly apparent and attractive stimuli to other areas where they are concentrated for elimination (lure−kill techniques). Formulations of the identified attractant (1-octen-3-ol) or other attractive components need to be developed and combined with mass trapping devices for final proof of concept. This method of stable fly management may also offer additional benefits, such as being environmentally nonpersistent and having lower mammalian toxicity (*15*, *49*).

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