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Comparisons of antifeedancy and spatial repellency of three natural product repellents against horn flies, *Haematobia irritans* (Diptera: Muscidae)

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Abstract

BACKGROUND: Horn flies are among the most important biting fly pests of cattle in the United States. Horn fly management is largely dependent upon pesticides, which ultimately leads to the rapid development of insecticide resistance. Alternative control strategies, including repellents, have shown promising results in reducing fly biting. In the present study, we examined the efficacy and longevity of recently identified natural product repellents against horn flies.

RESULTS: Catnip oil, geraniol and C8910 acids reduced horn fly feeding in a laboratory bioassay and also exhibited spatial repellency in the olfactometer. Residual activity was observed for up to 3 days in laboratory assays; however, 24 h of residual effectiveness was observed from the two repellents when applied on cattle in the field. The limited residual effectiveness was correlated with the high volatility of the major active repellent compounds.

CONCLUSION: All three natural product repellents effectively repel biting horn flies, exhibiting both feeding deterrence and spatial repellency. They may be used for developing an effective push-pull strategy with a slow release matrix that can prolong their effectiveness for horn fly management.

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Keywords: *Haematobia irritans*; repellent efficacy; catnip oil; geraniol; fatty acids

1 INTRODUCTION

Horn flies, *Haematobia irritans*, are among the most important pests of pastured cattle in the United States.¹ The species was introduced into North America from Europe between 1884 and 1886.^{2,3} Both sexes feed on cattle, causing annoyance, alteration of grazing behavior, reduction in feed conversion efficiency and reduced milk production and weight gain.^{4–8} Kunz *et al.*⁹ reported that the damage caused by horn flies costs US cattle producers \$US 1 billion annually. Furthermore, horn flies have been implicated in the transmission of *Staphylococcus aureus* mastitis and cause bovine teat atresia.^{10,11}

Horn fly management in pastures is largely dependent upon chemical control methods, such as pour-ons, insecticide-impregnated ear tags, back-rubbers and feed-through. However, the low costs of generic insecticide formulations have enabled producers to use them frequently, which has led to widespread resistance.^{12–15} Additionally, some of them are not labeled for use on lactating dairy cattle either in conventional or in organic farming systems. In Nebraska, Prolate/Lintox-HD (a sprayable phosmet pesticide) has been suggested for horn fly control on pasture cattle owing to the very low repellency observed.

Although insecticides remain the main horn fly management tool, alternative management methods are under development. Natural product repellents have been used for hundreds of years

to protect humans and their animals from arthropod attack.¹⁶ Recently, several reports have demonstrated the repellency of plant oils and fatty acids, including essential oils of catnip (*Nepeta cataria*) and geranium, as well as short-chain-length fatty acids, against biting fly.^{17–19} The present paper reports the efficacy and longevity of repellent and antifeeding activity of three natural products in laboratory bioassays and on cattle under field conditions.

2 MATERIALS AND METHODS

2.1 Horn flies

Laboratory bioassays were conducted using an insecticide susceptible laboratory strain of horn flies maintained at the USDA-ARS Knipling-Bushland US Livestock Insects Research Laboratory in

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Kerrville, Texas. Horn flies were maintained at $23 \pm 2^\circ\text{C}$ with variable humidity (30–50%) and a light:dark photoperiod of 12:12 h. Adult horn flies were fed citrated bovine blood ($3.7\text{ g sodium citrate L}^{-1}$) twice daily by soaking feminine hygiene pads (Stayfree®; McNeil-PPC Inc., Skillman, NJ) in blood and placing them inside the cages.

2.2 Natural product repellents and insecticides

Catnip oil was purchased from Bramble Berry Inc. (Bellingham, Washington, DC). The chemical composition was determined by gas chromatography–mass spectrometry (GC-MS) analysis to be 85% (*Z,E*- and *E,Z*-nepetalactone.²⁰ Geraniol was purchased from FASST Products (Rockville Centre, NY), and the oil contained >90% (*2E*)-3,7-dimethylocta-2,6-dien-1-ol. C8910 acids, a 1:1:1 mixture of octanoic, nonanoic and decanoic acids, was purchased from Stratacor Inc. (Richmond, CA). Prolate/Lantox-HD containing 11.75% phosmet was purchased from Valley Vet Supply (Marysville, KS).

The two nepetalactones were accumulated and purified (>95%) from the purchased catnip essential oil, following the method described in Peterson.²¹ Synthetic standards of (*2E*)-3,7-dimethylocta-2,6-dien-1-ol, octanoic acid, nonanoic acid and decanoic acid were purchased from Sigma-Aldrich (St Louis, MO) with a purity of >98%.

2.3 Laboratory feeding bioassay

The laboratory bioassay for testing antifeeding activity used a six-cell apparatus similar to that described by Klun *et al.*²² (K&D module), with modifications for horn flies.²³ Test flies (3–4 days old) were starved for 24 h prior to testing. Three doses of repellents (0.2, 2 and 20 mg) were dissolved in 300 μL of hexane (high-purity solvent; Burdick & Jackson, Muskegon, MI) and then evenly applied to the outer layer cut from a sanitary pad ($4 \times 5\text{ cm}$). After the solvent evaporated (2–3 min), the repellent-impregnated layer was placed on top of a blood-soaked sanitary pad in the K&D module. A control sample was treated with 300 μL of hexane only. Test flies were transferred into each of the six testing cells (3–5 flies cell⁻¹) by using a glass/rubber tube pooter. After 4 h, horn flies were anesthetized with CO_2 and checked for feeding status by squashing their abdomen and examining for the presence of blood. Flies in the repellent bioassay were exposed to randomized treatments (repellent candidates at various dosages) until at least ten replicates were completed (fresh flies and layers were used for each test). During the experiments, we recorded the time to knockdown at the 20 mg dosage, which was defined while flies were dead or lying on the floor of the box unable to fly.

Results of the feeding bioassay were analyzed by logistic regression. LS means were separated by *t*-tests (Proc Genmod, SAS 9.3; SAS Institute, Inc., Cary, NC). Antifeedancy is reported as backtransformed LS means with 95% confidence limits.

2.4 Spatial repellent assay in the single-cage olfactometer

A single-cage, dual-port glass olfactometer was used to assess spatial repellency of the three repellents against horn flies.²³ Horn flies (3–4 days old) were starved for 24 h prior to testing. Flies were released into the olfactometer individually and given 3 min to respond; their presence in the repellent-treated or control port (>10 cm inside the port) was recorded. Normally, one set of twelve tests (three repellent products at four doses) was performed each day. Repellents were first dissolved in hexane to make solutions at concentrations of 0.01, 0.1, 1 and 10 $\mu\text{g } \mu\text{L}^{-1}$. A quantity of 10 μL

of repellent solution was applied to a piece of filter paper (cut as a small triangle, 2 cm of each side). For the control, 10 μL of hexane was applied. The filter paper was air dried, fixed to an insect pin and placed in the middle of a test port of the olfactometer. Within each set of tests, the order of ports, repellents or control was randomized. All three ports (including the releasing port) were cleaned with acetone followed by hexane before each test. A test consisted of the sequential introduction of five flies into the olfactometer. Each test was replicated 20 times. New sets of five flies were used for each replicate.

Responses were recorded as the 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.

2.5 Electroantennal responses to repellents

Electroantennograms (EAGs) were recorded as indicated in Zhu *et al.*²³ Three dosages (1, 10 and 100 μg) of each repellent dissolved in redistilled HPLC-grade hexane (10 μL) were prepared. The prepared solutions were applied to filter paper strips ($0.5 \times 2.5\text{ cm}$, Whatman No. 1; Whatman International Ltd, Maidstone, Kent, UK). Air-dried filter paper strips were inserted into 15 cm long Pasteur pipettes. A 5 mL puff (Auto-Puffer; SYNTECH, Kirchzarten, Germany) containing odorant compound was blown through the pipette and directed across the antennae to elicit an EAG response. Control puffs (hexane only) of air were applied after each puff of a test stimulus. The EAG response for each stimulus was recorded as the mean amplitude of each of the six replicated measurements. The sequence of exposure of each stimulus to each antenna was random. The significances of differences of horn fly relative EAG responses (absolute EAGs – controls) were determined by multi-way ANOVA followed by a Scheffe test (PASW Statistics 18, SPSS Inc., Chicago, IL), and Student's *t*-test was used for comparisons of EAG recordings between male and female antennae.

2.6 Longevity of repellency of the three natural product repellents in the laboratory and field

Antifeedant longevity tests were first conducted under laboratory conditions. The three natural products were tested at a 20 mg dose. Samples were prepared as in Section 2.5. Repellent-impregnated layers were aged by hanging from a metal stand placed inside a fume hood (Air Sentry, New York, NY) with continuous ventilation at 27 m min^{-1} . Antifeedancy was tested on horn flies (3–4 days old) starved for 24 h on samples aged for 0 (freshly made), 24, 48, 72 and 96 h. Each age class was replicated a minimum of 10 times.

Repellency was tested on cattle under field conditions during the summers of 2011 and 2012 at the University of Nebraska, West Central Research and Extension Center, North Platte, Nebraska. Tests were conducted using criteria specified by the American Society for Testing and Materials (ASTM, 1980) and protocols approved by the Institutional Animal Care and Use Committee of the University of Nebraska (IACUC Protocol No. 06-12-053C). Test cattle were restrained in a chute, and 250 mL of repellent (15% catnip oil, or 30% geraniol in light mineral oil, or C8910 acid formulation) was sprayed evenly over the entire body, except for the face area, using a compressed-air hand gun (J.E. ADAMS Industries Ltd, Cedar Rapids, IA) with 241 kPa air pressure. This dose was estimated to be equivalent to approximately 0.01 mL cm^{-2} . Control cattle were treated with 250 mL of mineral oil. Prolate/Lantox-HD sprayable solution was diluted as per label instructions (1:200 in

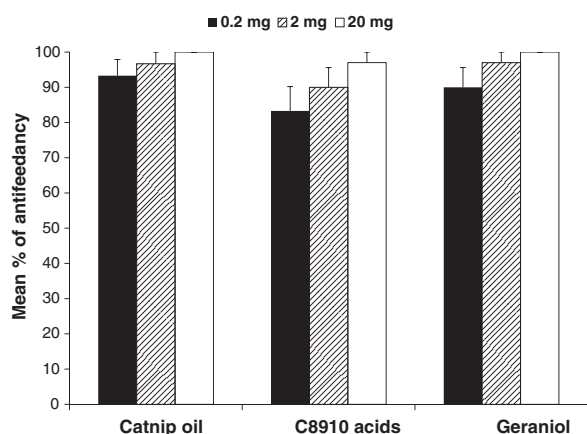


Figure 1. Comparisons of antifeedancy from three natural product repellents at 4 h against horn flies at three dosages (0.2, 2 and 20 mg) in laboratory bioassays compared against control. There were no significant differences among repellent treatments ($P > 0.05$), and only slight differences among dosages ($P < 0.05$).

water). Five animals were used for each treatment. Cattle were placed in pens separated by >20 m by treatment. Treatment pens were >50 m downwind from the control pen. Horn fly density on animals was recorded by digital photography at 1, 3, 6 and 24 h after treatment. The number of flies in each image was counted using GIMP Image Editor (v.2.8; <http://www.gimp.org>) and doubled to express the total number of flies per animal.

The number of flies per animal was analyzed relative to treatment and time interval with ANOVA. Means were separated with Tukey's HSD when the overall F -value was significant.

2.7 Release rates of the three repellents

The release rates of the repellents were estimated by measuring the absorption rates of their major compositional compounds [(*Z,E*)- and (*E,Z*)-nepetalactone for catnip oil, (*2E*)-3,7-dimethylocta-2,6-dien-1-ol for geraniol and octanoic acid, nonanoic acid and decanoic acid for C8910 acids] with solid-phase microextraction (SPME) fibers (100 μ m of polydimethylsiloxane for catnip oil and geraniol and 70 μ m of polydimethylsiloxane/carboxen for C8910 acids; Supelco, Bellefonte, PA). Cattle hide (Turkey Creek Furs & Recycling, Crete, NE) was cut into 10 cm \times 10 cm samples, and 1 mL of repellent solution was applied to each sample. Repellent-treated hide samples were placed outdoors in an open field, separated by ≈ 10 m among treatments in July. SPME fibers were placed 2–3 cm from the hide samples for collecting released volatiles (five hide samples per treatment) for 5 min. Collections were conducted at 0–1, 3–4, 6–7 and 23–24 h after repellent applications.

The relative concentrations of the compositional compounds were analyzed with an Agilent GC system equipped with a DB-FFAP column (30 m \times 0.25 mm i.d.; Agilent Technologies Inc., Palo Alto, CA, USA). Helium was used as the carrier gas, and the flow rate was maintained at 2.5 mL min^{-1} . Samples were injected under the splitless mode. The temperature program for GC analyses was 50 $^{\circ}\text{C}$ for 3 min, rising by 10 $^{\circ}\text{C} \text{ min}^{-1}$ to 230 $^{\circ}\text{C}$. The quantities of the compositional compounds were assessed by the external standard method. Synthetic standards were weighed and dissolved in hexane. Calibration curves to determine linearity were obtained for each standard at 5, 10, 50, 100 and 500 ng μL^{-1} with three replicates per concentration. Linearity was assumed when the regression coefficient provided an R^2 value of >0.98 . The

quantities of the compositional compounds were obtained by integrating the areas of the standards' peaks and calculating the concentrations based on the standard curves.

The amount of compositional compounds absorbed on SPME fibers as a function of time since application was analyzed with ANOVA ($\alpha = 0.05$), followed by Tukey's HSD if the overall F -value was significant.

3 RESULTS

3.1 Antifeedant assay

Differences were observed among the three repellents and the control ($F = 389.2$; $df = 3, 125$; $P < 0.05$) and among the three repellent doses ($F = 7.7$; $df = 2, 125$; $P < 0.05$). The interaction term was non-significant ($F = 8.9$; $df = 6, 125$; $P = 0.18$). Fewer than 15% of the flies fed when exposed to 0.2 or 2 mg of repellent whereas $93.3 \pm 1.9\%$ of the control flies fed (Fig. 1). When exposed to 20 mg of repellent, 2% or less of the flies fed. Among the repellents,

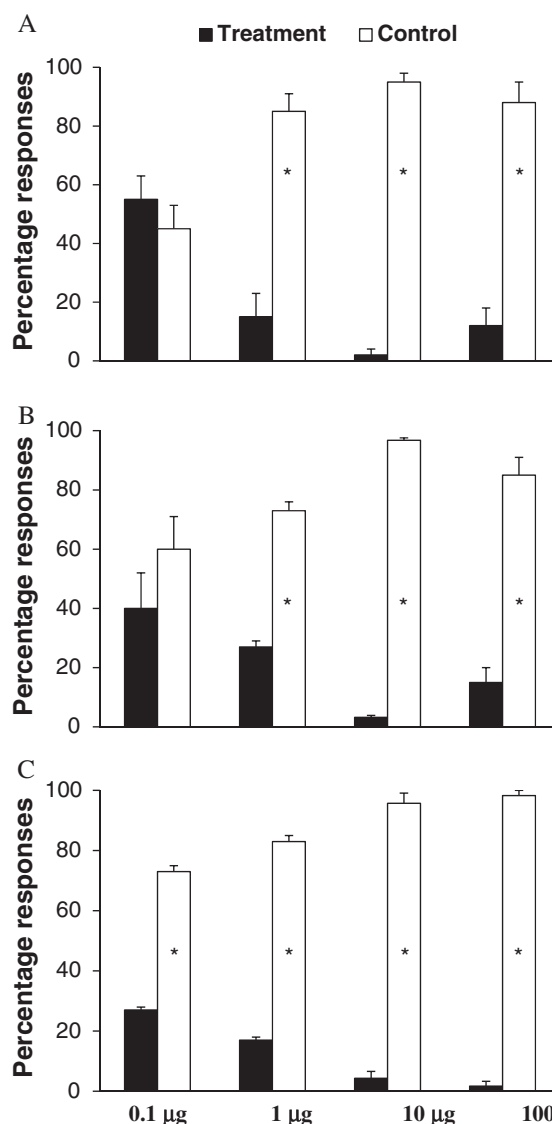


Figure 2. Behavioral responses of horn flies to the four dosages of three natural product repellents (A, catnip oil; B, C8910 acids; C, geraniol) tested in a single-cage olfactometer. Asterisks inside the columns indicate significant differences between the treatments and control ($P < 0.05$).

horn fly feeding did not differ significantly between catnip oil and geraniol ($Z = -0.24$; $P = 0.81$), but the antifeedancy was slightly higher with C8910 ($Z = 9.7$ for catnip and $Z = 9.6$ for geraniol; $P < 0.05$).

Horn fly knockdown time at 20 mg dosage was 2.58 ± 0.28 min for catnip oil, 9.38 ± 1.68 min for geraniol and 130.20 ± 20.03 min for C8910 acids respectively.

3.2 Spatial repellency test

During the course of the laboratory antifeedant bioassay, we observed that horn flies placed in the repellent-treated cells tended to fly away from the treated surface, which indicated spatial repellency. The single-cage olfactometer demonstrated

spatial repellency for all three repellents (Fig. 2). Horn flies were strongly repelled from treated ports with repellent with dosages of 1–100 μg ($t = 2.43$ – 2.78 ; $P < 0.05$). However, at the lowest dose tested (0.1 μg), only geraniol repelled flies ($t = 2.44$; $P < 0.05$) (Fig. 2C). Overall, >80% of horn flies tested in the olfactometer responded to either treatments or the control.

3.3 Antennal response

Olfactory sensilla of horn flies responded strongly to each of the repellents (Fig. 3), compared with the controls ($222 \pm 13 \mu\text{V}$ for females and $159 \pm 5 \mu\text{V}$ for males, absolute responses). Responses were similar for the three repellents at each of the doses tested. However, significant differences were observed among doses and

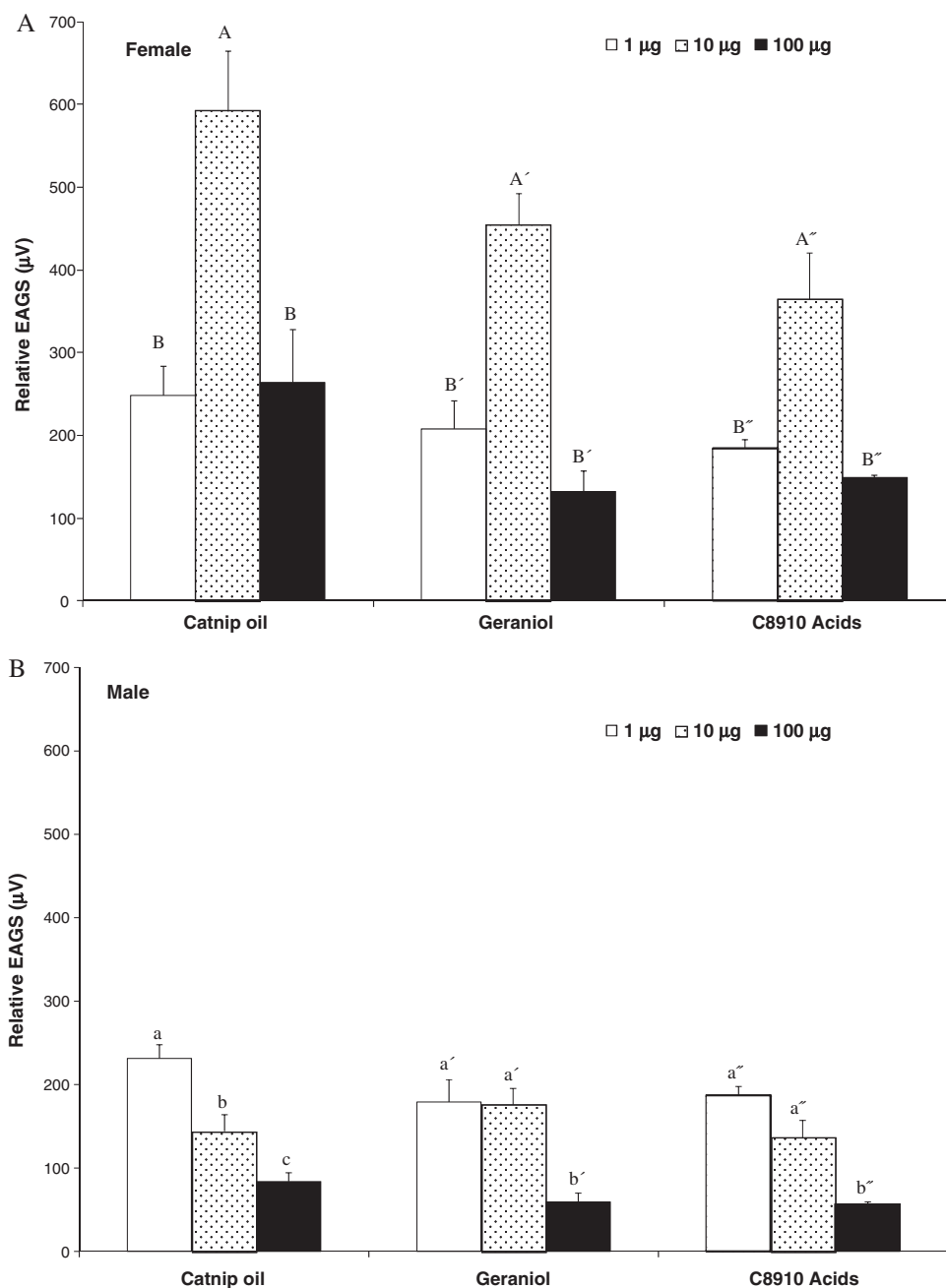


Figure 3. Relative electroantennal responses of female (A) and male (B) horn flies to three dosages of the natural product repellents. Values are means \pm SE; different letters on top of the bars denote significant differences ($P < 0.05$).

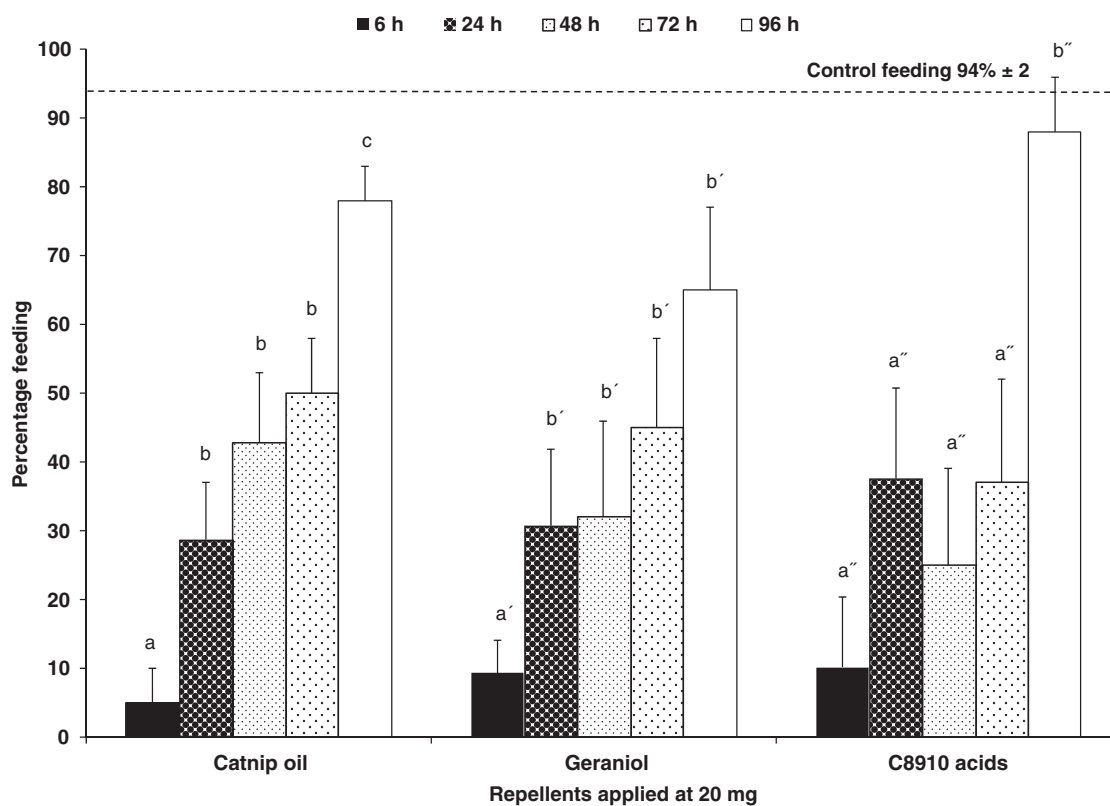


Figure 4. Longevity of antifeedancy effects of three natural product repellents against horn flies at a dosage of 20 mg applied to sheets aged for variable periods in laboratory bioassays. Different letters on top of the bars indicate significant differences ($P < 0.05$).

between the sexes. For female horn flies, significantly higher EAG responses were elicited to the higher dosages (10 and 100 μg) of geraniol and C8910 acids ($t = 2.35\text{--}3.18$, $P < 0.01$), compared with those of males. Among females, responses were highest when responding to the intermediate 10 μg dose ($F = 4.46\text{--}37.45$; $df = 2, 15$; $P < 0.001$). Antennae of males responded strongly to the lowest dose ($F = 9.80\text{--}34.26$; $df = 2, 15$; $P < 0.01$).

3.4 Effectiveness and longevity of antifeedancy

Under the laboratory conditions, the three repellents retained more than 90% antifeedancy during the first 24 h (Fig. 4). The efficacy of antifeedancy of catnip oil and geraniol decreased significantly after 6 h ($F = 3.15\text{--}3.31$; $df = 4, 45$; $P < 0.05$), but they still retained more than 50% of their antifeeding activity at 72 h. All three repellents lost more than 70% of their antifeeding activity after 4 days. Residual activity was similar among the three repellents during the course of the 4 day trial. Average feeding of control flies was $94 \pm 2\%$.

During the field trials, all three repellents strongly repelled horn flies. A significantly lower number of flies per animal was observed up to 6 h after application ($F = 12.72\text{--}454.15$; $df = 4, 35$; $P < 0.001$) (Fig. 5). Geraniol and C8910 acids retained repellency up to 24 h ($F = 13.11\text{--}36.65$; $df = 4, 35$; $P < 0.01$). Prolate was not repellent. Overall, the number of horn flies on all of the animals, including the controls, decreased 2 days after treatment.

3.5 Release rates of the repellent compounds

Differences were found from all three repellents, but only significant in two repellents (catnip oil and geraniol: $F = 7.51$; $df = 4, 20$

$P < 0.001$ and $F = 5.95$; $df = 4, 20$, $P < 0.01$). Geraniol had the highest release rate with more than 100 \times decreases found at 6 h, compared with the other two repellents with 2.8–4.8 \times losses. Almost zero absorption was found after 48 h from catnip oil and geraniol.

4 DISCUSSION AND CONCLUSION

In the United States, for horn fly control, insecticides are often applied at least 2–3 times per month to manage them on pastured cattle.⁶ Such frequent and high-dose uses of insecticides rapidly lead to the development of insecticide-resistant flies. Resistance to the two most commonly used insecticide groups, organophosphates and pyrethroids, have been reported.⁸ Therefore, novel, appropriate control strategies that minimize resistance are needed.^{6,8} Among the alternative pest controls, biopesticides, including repellents, are of growing importance, with a global market value of \$US 3 billion annually.²⁴ These products can effectively control arthropod pests by acting as repellents or feeding deterrents of biting insects. The results from this study on the three natural product feeding deterrents may provide useful information towards an alternative method for control of flies on pasture cattle.

Catnip, *Nepeta cataria* (Lamiales: Lamiaceae), is a herbaceous mint native to Eurasia and North Africa, well known for its pseudonarcotic effects in cats.^{25,26} Topical application of catnip oil on human skin can prevent biting by several mosquito species.^{27–29} Recently, catnip oil has also been reported as an effective antifeedant against several species of muscoid flies.¹⁷ Geraniol is a primary component of rose oil, palmarosa oil and citronella oil (the Merck Index). Barnard and Xue²⁹ demonstrated its repellency against several mosquitoes. Geraniol at 30% tested

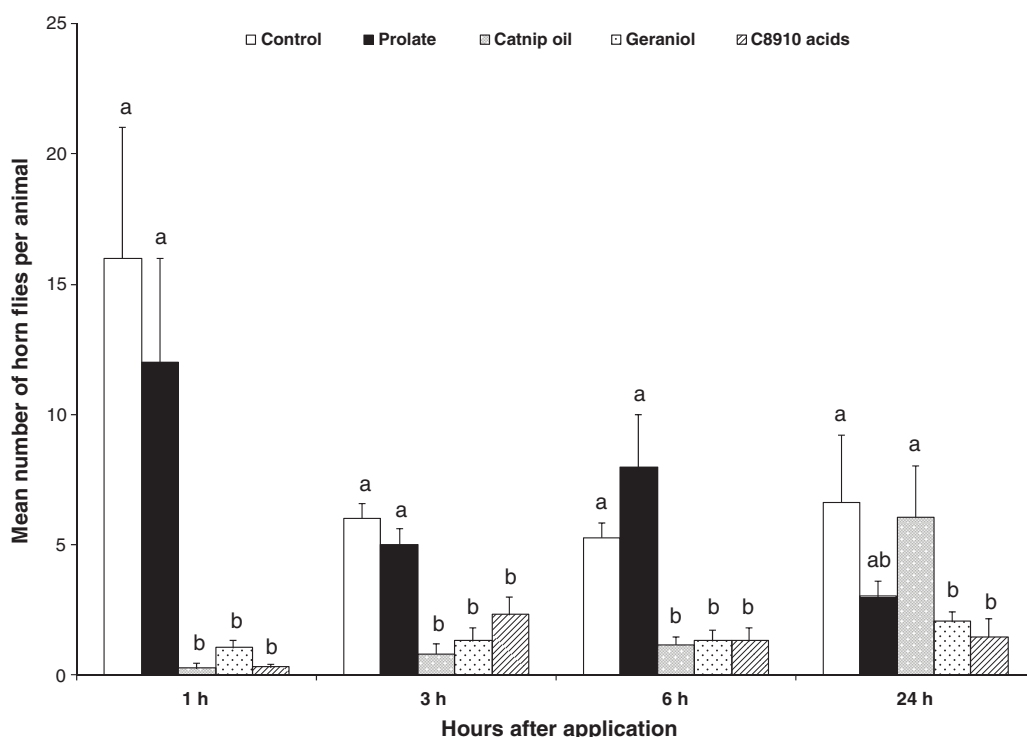


Figure 5. Number of horn flies per animal after treatments with repellents and an insecticide at different hours after application. Different letters on top of the bars indicate significant differences within a time period ($P < 0.05$).

on cattle as a horn fly repellent effectively reduced fly density under the economic threshold (200 per animal; Watson W, private communication). Short-chain fatty acids (C_8 , C_9 and C_{10}) tested in laboratory trials showed strong antifeedancy against horn flies.¹⁷ The present study using the modified K&D module to test these three natural product repellents demonstrated

strong antifeedancy against starved horn flies, with over 90% efficacy even from the lowest dose (0.2 mg) tested (approximately $8 \mu\text{g cm}^{-2}$). An average of 90% antifeedancy was obtained by the three test products during the first 4–6 h of testing; however, the effect declined to about 50% from day 2 to day 3, probably owing to volatilization.

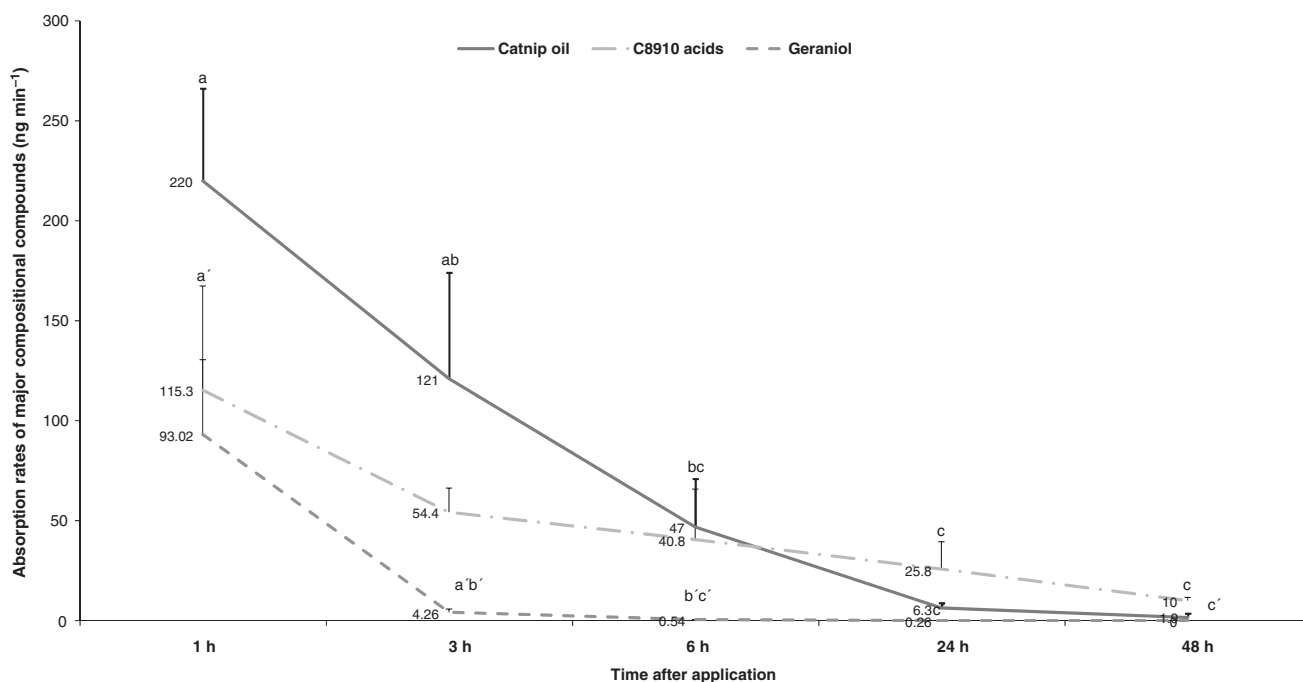


Figure 6. SPME absorption rates of their major compositional compounds from three topically applied repellents on cattle hides measured at 1, 3, 6, 24 and 48 hours. Different letters on top of the SE bars indicate significant differences ($P < 0.05$).

In general, the definition of a natural product repellent is a substance found in nature that elicits an avoiding reaction. Repellents can be further characterized as contact repellents (antifeedants in most biting insects) and spatial repellents. All three repellents tested in the present study demonstrated a strong antifeedancy in the laboratory feeding assay. In addition, tested flies were also observed being driven away from membranes treated with repellents, which indicated spatial repellency. Our single-cage olfactometer assay showed that as little as 1 µg of catnip oil or C8910 fatty acids repelled horn flies. The strong electroantennal responses elicited from horn fly olfactory sensilla to the three repellents are evidence of a physiological response to the repellents as well. Among the three repellents, the vapor pressures of their major constituent compounds [nepetalactones, (*E*)-3,7-dimethyl-2,6-octadien-1-ol and three short-chain fatty acids] are all less than 1.0 mmHg (25 °C), which indicates that they exist solely in a vapor in the atmosphere. A reduction in EAG responses from both sexes of horn flies from the repellents at highest dose may be induced by adaptation in the olfactory receptors, which has been commonly found in moth sex pheromone communication.³⁰ However, the highest EAG responses to 10 µg of all three repellents observed among female horn flies and differences in EAG responses between the two sexes to higher dosages of geraniol and C8910 acids remain unexplained.

In the present study, all three repellents seem to operate in the vapor phase. Repellents with high vapor pressure may offer protection at low concentrations, but because of rapid volatilization, they may only provide a limited residual activity. The results from SPME absorption analyses supported the notion that decreases in repellency are correlated with their fast evaporation in the field. However, the number of horn flies in the field was relatively low by day 2. It is uncertain that the decrease in horn fly population in the field was due to our testing or to other causes. Catnip oil has been reported to have contact and fumigant toxicity against biting flies.³¹ Geraniol and C8910 acids also possess low toxicity.^{18,19,32} Our laboratory antifeedancy assays (20 mg treatment) demonstrated that the tested horn flies were knocked down in less than 10 min from catnip oil and geraniol, which indicate their toxic effects as well. During the course of field trials, a commonly used organophosphorous insecticide (Prolate/Lintox-HD) on beef/dairy cattle against horn flies was also tested, and no repellency was observed. However, owing to the windy conditions in the field, no dead horn flies were found around the treated cattle, even with relatively high landings on the cattle.

All three natural products demonstrated strong antifeedancy and repellency against horn flies, but with relatively short effective periods. More efforts need to be focused on prolonging residual repellent activity if these compounds are to be used for practical applications. Reifenrath *et al.*³³ suggested mixing several repellents together for extended longevity. They discovered that evaporation rates are slightly less and that repellency decayed less rapidly from mixing repellents relative to individual compounds at the same dose. More studies are under way to evaluate the extended longevity and effectiveness of blends of the three repellents against horn flies in the field.

Push-pull strategies have been proposed for confined livestock systems employing on-animal repellents and attractant-baited traps.^{34,35} A modified push-pull methodology employing repellents on the majority of the animals and a few insecticide-treated 'trap' animals may be more effective. Interestingly, Prolate/Lintox-HD insecticide did not exhibit repellency, making its use on 'trap' animals a viable option. The relatively strong

toxicity of geraniol and catnip oil may further help to kill the flies, while flies contact residues left on treated cattle hide after the loss of spatial repellency.

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