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Behavioural responses of stable flies to cattle manure slurry associated odourants

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Abstract. Stable flies (*Stomoxys calcitrans* [Diptera: Muscidae] L.) are blood-feeding synanthropic pests, which cause significant economic losses in livestock. Stable fly antennae contain olfactory sensilla responsive to host and host environment-associated odours. Field observation indicated that the abundance of stable flies increased significantly in grasslands or crop fields when cattle manure slurry was applied. Major volatile compounds emanating from manure slurry were collected and identified. Behavioural responses of stable flies to those compounds were investigated in laboratory bioassays and field-trapping studies. Results from olfactometer assays revealed that phenol, *p*-cresol and *m*-cresol were attractive to adult stable flies. When tested individually, attraction was higher with lower dosages. Stable flies were most attracted to blends of phenol and *m*-cresol or *p*-cresol. Traps with binary blend lures caught more stable flies in field trials as well.

Key words. *Stomoxys calcitrans*, attractants, cresol, phenol, traps.

Introduction

Gibson & Torr (1999) investigated the visual and olfactory responses of several haematophagous dipterans to host stimuli. By comparing their behaviour, olfactory and visual responses, they found that biotic and environmental constraints resulted in unique host-seeking behaviours in different species of biting dipterans, which primarily relied on olfactory and visual stimuli. Several other studies have also shown that various attractant odourants are used for host seeking by biting flies [Cilek, 1999; Kristensen & Sommer, 2000; International Atomic Energy Agency (IAEA), 2003; Birkett *et al.*, 2004].

Stable flies, *Stomoxys calcitrans* (L.), are blood-feeding flies that can lower weight gain and milk production of cattle (Catangui *et al.*, 1997; Campbell *et al.*, 2001). They are considered to be among the most important arthropod pests of livestock with economic losses in the U.S. cattle industry estimated to

exceed \$2 billion per year (Taylor *et al.*, 2012). Similar to other biting flies, the host-seeking behaviour of stable flies is partially mediated by host and host environment-associated odourants (Gatehouse & Lewis, 1973; Holloway & Phelps, 1991). Recent studies have shown various host-associated volatile compounds evoke electrophysiological and behavioural responses from adult male and female stable flies (Jeanbourquin, 2006; Tangtrakulwanich *et al.*, 2011). Stable flies are diurnal, typically biting during late morning or earlier afternoon, which indicates that visual cues may contribute to their host-seeking behaviours.

During the course of routine monitoring of stable fly populations in eastern Nebraska (Taylor *et al.*, 2007), we observed high numbers of stable flies caught from traps placed in proximity to grasslands and crop fields on which cattle manure slurry (a mixture of cattle manure and urine, water and accumulated organic waste) had been recently applied (Schils & Kok, 2003). Volatile compounds isolated from bovine manure slurry may be useful

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for improving the efficacy of trap catches by the deployment of mass trapping technologies with attractant-baits for stable flies (Meifert *et al.*, 1978).

In the present study, we: (a) identified volatile compounds emanating from cattle manure slurry; (b) characterized the behaviour responses of adult stable flies to selected identified compounds; and (c) evaluated the effectiveness of those compounds as trap baits under field conditions.

Materials and methods

The effect of manure slurry application on stable fly catches

The effect of manure slurry on stable fly trap catches was examined in several harvested wheat and grass fields (~200 ha) at the University of Nebraska Agricultural Research and Development Center, Ithaca, NE (3 August to 8 September 2011). Manure slurry was collected in the dairy barn by a barn cleaner daily, and stored into a slurry tank. Manure slurry was applied three times (3, 17 and 30 August) by a John Deere tractor-hauling slurry tank equipped with a mechanical liquid manure spreader in the centre of the field (at a rate of applying 4400 L/ha with a 3.5 m wide pass area, soil broadcast). Aلسynite cylinder sticky traps covered with 10-mL Sur-Flex plastic sleeves (Flex-o-glass, Inc.; Chicago, IL, U.S.A.) coated with Tangle-Trap (The Tanglefoot Co., Grand Rapids, MI, U.S.A.) diluted 1:1 with low-odour paint thinner (Sunnyside Corp., Wheeling, IL, U.S.A.) were placed at 1.6-km intervals (Fig. 1). The placement of these traps was a part of our stable fly monitoring programme, where three traps placed in the area that was sprayed with liquid manure and the rest of six traps set in near-by fields. Traps were checked twice a week starting 1 week before application and ending 1 week after. Sleeves were replaced each time traps were checked. Stable flies were identified and their capture numbers were recorded from traps (all sexes combined).

Volatile collection and analysis of manure slurry

Cattle manure slurry (24–48 h old) was collected from cattle production pens (dairy farms and feedlots) at the University of Nebraska Agricultural Research and Development Center, Ithaca, NE, U.S.A. The manure slurry was placed in two 50-mL Corning screw cap centrifuge tubes with a conical bottom (no. 430290, Corning®) and transported to the laboratory for odour collection and analysis. Samples were analysed within 2 h of collection. Ten milliliters of slurry was transferred to a glass vial (20-mL Micro Liter Analytical Sample Vials) and sealed with parafilm (Pechiney Plastic Packaging PM992-Parafilm M®). Solid-phase microextraction (SPME, 75 µm PDMS/Carboxen, Supelco; Sigma-Aldrich®, St Louis, MO, U.S.A.) was used for volatile collection. SPME fibres were pre-conditioned for 1 h in the inlet of the gas chromatograph (GC) at 300 °C with a continuous helium stream before the collection. SPME fibres were inserted through the parafilm into the collection vial, positioned 1–2 cm above the manure slurry, and allowed to absorb volatiles for 30 s. A total of six vials with manure slurry were used for odour collection, and each vial was

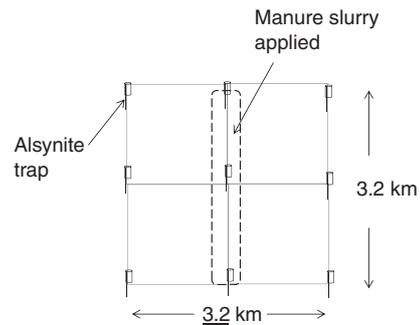


Fig. 1. Scheme showing the application of cattle manure slurry in stable fly monitoring fields.

designated with its own SPME fibre for the duration of the analyses. Volatiles were analysed using an Agilent 6890 GC with a 5973 Mass spectrometer (Agilent Technologies, Palo Alto, CA, U.S.A.). SPME fibres were thermo-desorbed at 200 °C and analysed with either DB-Wax or FFAP columns (30 m × 0.25 mm diameter, with 0.25 µm film thickness; J&W Scientific, Agilent). Helium (1.5 mL/min) was used as the carrier gas. Samples were injected under the splitless mode. The GC temperature programme was starting at 50 °C for 3 min and then raised to 240 °C at a rate of 10 °C/min. Mass spectra were recorded from 30 to 550 amu with electronic impact ionization at 70 eV. Compounds were identified by comparisons of their retention times and mass spectra with those of synthetic standards using Wiley MS Library Database (Agilent).

Olfactometer trials

Attraction of stable flies to odourants from cattle manure slurry was assayed with a single cage, dual port olfactometer (Zhu *et al.*, 2010). Stable flies from a 4-year-old colony maintained at the U.S. Department of Agriculture, Agricultural Research Service, Agroecosystem Management Research Unit (Lincoln, NE, U.S.A.) were reared at 23 ± 2 °C with variable humidity (30–50%) and a LD 12:12 h photoperiod. Adults were fed citrated bovine blood (3.7 g sodium citrate/L) in an absorbent pad (Stayfree®; McNeil-PPC Inc., Skillman, NJ, U.S.A.).

Volatiles were tested individually or as two compound blends. Solutions of single compounds (phenol, *p*-cresol, *m*-cresol, 1-octen-3-ol; > 95% purity; Sigma-Aldrich) were prepared in hexane at three concentrations, 1, 10 and 100 µg/10 µL, and the mixtures containing phenol: *m*-cresol or phenol: *p*-cresol with the same dosages at a 25:1 ratio (with each compound applied on separate filter papers). Hexane alone was used for the control. 1-Octen-3-ol was used as a positive control. Samples were prepared by applying 10 µL of test solution to a small triangle of Whatman No.1 filter paper (0.5 cm²) (General Laboratory Supply, Pasadena, TX, U.S.A.). Hexane was allowed to evaporate under a fume hood for 10 min. Samples were suspended on a wire 3 cm from the end of each collection port.

Flies used were 3–4 days old (post-eclosion) and mixed sexes (~1:1). They were fed with blood one time before starving for 24 h prior to testing. Flies were released individually into the

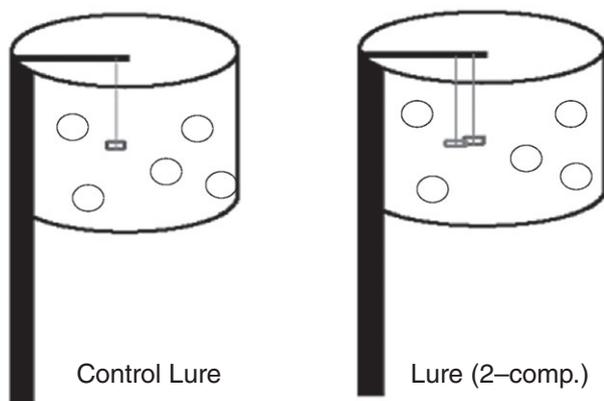


Fig. 2. Drawings of the modified Broce traps baited with attractant lures.

introduction port and their position was recorded 5 min later. If after 5 min, the fly had not entered either collection port it was recorded as a 'no-response'. The fly was then removed and a new fly was introduced. This process was repeated until 10 flies were exposed to one treatment. The olfactometer was then cleaned with acetone, and rinsed with 70% ethanol, and air-dried in preparation for another repetition of testing. Control and treatment ports were reversed between sets of 10 flies. Testing of each treatment volatile was repeated 4–5 times, with at least 50 flies for each set of test volatiles (doses and blends).

As no differences in behavioural responses in attraction or number of flies entering either port was observed, data on responses between male and female stable flies to testing chemicals were combined. 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 analysed using Student's *t*-test. Log transformation was done when necessary. Results with $P < 0.05$ were considered to be statistically significant. All analyses were conducted using SAS, version 9.1 (SAS Institute Inc., Cary, NC, U.S.A.).

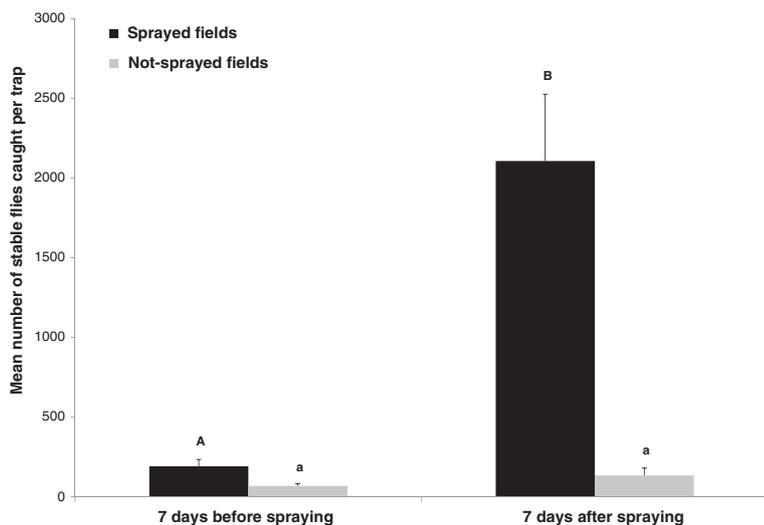


Fig. 3. Comparisons of stable fly catches in traps between the manure/slurry sprayed fields and those placed in fields not being sprayed at the University of Nebraska farms at MEAD. Different capital letters indicate significant differences in trap catches between the two types of fields, and same lower cases letter suggest no differences found in between (Student's *t*-test, $P < 0.05$).

Field experiments

Trapping sites for testing lures were approximately 100 m from cattle pens at the animal science complex of the University of Nebraska (Lincoln, NE, U.S.A.). Attractant lures were pairs of volatile compounds: phenol (2500 μg) and *m*-cresol (100 μg), phenol (2500 μg) and *p*-cresol (100 μg), and hexane control (one cotton roll only). Lures consisted of 5-cm-long cotton rolls impregnated with individual test compounds and were suspended about 0.5 cm apart in the centre of modified Broce traps (Fig. 2). Traps were modified by drilling a hole at a diameter of 2 cm through the Alsynite and plastic sleeve material to allow volatiles released from the lures to escape. Traps were maintained daily (trap catches checked every day, and sticky panel replaced after check). The experiment was arranged in a randomized completed block design with five replications and trapping tests were conducted during the summer of 2011 (6–17 September).

Data analysis for field experiments

One-way factorial analysis of variance (ANOVA) was used to analyse trap catches (number of flies collected per trap) in field tests. Means were compared using the least square difference (LSD) test. Values of $P < 0.05$ were considered significant. Analyses were performed using SAS, version 9.1 (SAS Institute Inc.).

Results

Effect of manure slurry application on stable fly trap catches

Stable fly mean captures from three traps placed in the field sprayed with cattle manure slurry were significantly greater (almost 10 times) 1 week after the application than those in the same field a week before the slurry application (Fig. 3; $t = 4.69$, $P < 0.001$). In contrast, stable fly trap catches from non-sprayed

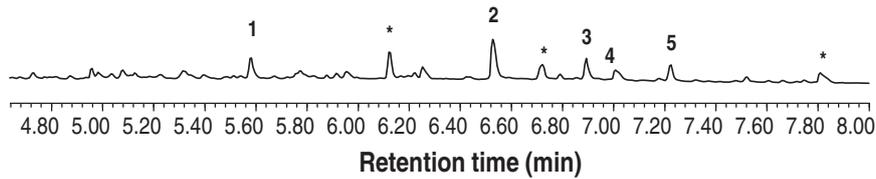


Fig. 4. Gas chromatographic traces of fresh cattle manure slurry volatiles (1) methoxy-phenyl oxime, (2) phenol, (3) *m*-cresol, (4) *p*-cresol and (5) 4-ethylphenol. *Background peaks or unidentified compounds.

fields showed no significant changes during the period before and after the manure slurry application ($t = 1.42$, $P > 0.05$).

Chemical analyses of volatiles of manure slurry

Five volatile compounds were detected by gas chromatographic analyses of SPME collections from cattle manure slurry (Fig. 4). By comparing their retention times and mass spectra to those of synthetic standards, they were identified as methoxy-phenyl oxime, phenol, *m*-cresol, *p*-cresol and 4-ethylphenol. Methoxy-phenyl oxime and 4-ethylphenol were identified based on the matching profiles of the MS library standard (>91%), but they were not observed consistently, only appeared in two out of six samples and were not further considered for behavioural tests.

Behavioural responses of adult stable flies

Olfactometer calibration trials detected no bias for either of the collection ports. At the lowest concentration tested (1 μg), more flies responded to all four test compounds, compared with the hexane control (Fig. 5; $t = 3.35$ – 4.31 , $P < 0.05$). At higher concentrations, *m*-cresol and *p*-cresol were not attractive, even acting as repellants ($t = 2.92$ and 4.13 , $P < 0.05$). Mixtures of phenol and either *m*-cresol or *p*-cresol at 100 μg dosages were more attractive to stable flies than the control (Fig. 6; $t = 3.18$ and 3.53 , $P < 0.01$). No behavioural preferences were found from tests when comparing individual compounds or with lower concentrations. Overall, 46.3% of the flies tested were scored as no-response as they failed to enter either treatment or control port.

In the field, traps baited with lures containing the mixtures of phenol and *m*-cresol or *p*-cresol captured 16 ± 3 and 13 ± 2 stable flies per trap per day, respectively, compared with only 5 ± 1 flies with alsynite traps only (hexane control), which was significantly different ($F = 6.08$, d.f. = 2,87, $P < 0.01$). The number of stable flies caught by the two blends did not differ.

Discussion

Ruminants are preferred hosts for haematophagous insects as a result of their habit of herding and their sessile nature (Birkett *et al.*, 2004). Jeanbourquin & Guerin (2007) analysed volatiles from the rumen digesta of cattle and found that dimethyl trisulfide, butanoic acid, *p*-cresol, skatole and especially 1-octen-3-ol

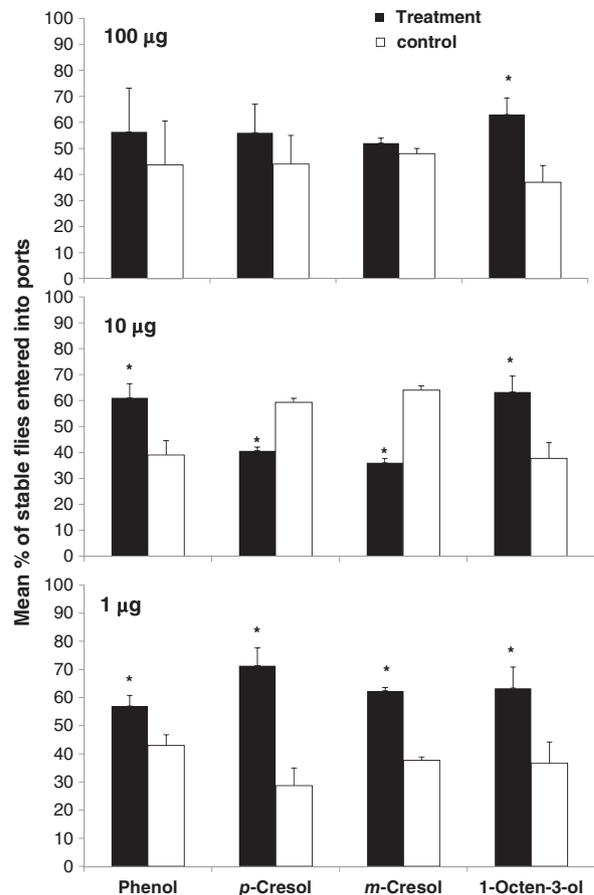


Fig. 5. Mean percentiles of stable flies observed into treatment or hexane control ports of a single cage olfactometer within 5 min of exposure to host-associated volatiles at three concentrations (\pm SE, $N = 180$ – 200). Asterisks above the bars indicate significant differences (Student's *t*-test, $P < 0.05$).

were attractive to stable flies. 1-Octen-3-ol has been identified from aged cattle urine and emanations of other terrestrial mammals (Hall *et al.*, 1984). Manure also has been found to attract stable flies, especially gravid females searching for oviposition sites containing aged cattle and horse manure (Broce & Haas, 1999; Jeanbourquin, 2006; Romero *et al.*, 2006). Our trapping study suggested that stable flies were strongly attracted to odours associated with cattle manure slurry applied to fields. Such odourants associated with the applied manure slurry also may provide chemical cues that indicate the presence of cattle

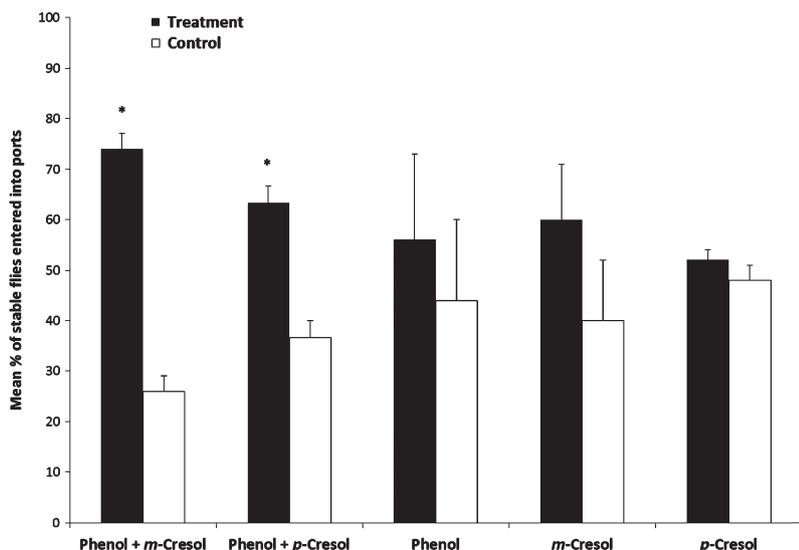


Fig. 6. Mean percentiles of stable flies observed in treatment and hexane control ports of a single cage olfactometer within 5 min of exposure to host associated volatiles (mixtures and individual compounds at 100 µg dosage) (Means ± SE, $N = 180\text{--}200$ in each comparison). Asterisks above bars indicate significant differences (Student's *t*-test. $P < 0.05$).

herds, which elicit a stable fly host-seeking behaviour. Our previous study on stable fly sensory physiology showed that various olfactory sensilla from stable fly antennae responding strongly to those odourants (Tangtrakulwanich *et al.*, 2011). Traps placed in fields with application of manure slurry caught over 10 times more flies than those placed away from sprayed sites. Gas chromatography analysis of cattle manure slurry identified three compounds phenol, *p*-cresol and *m*-cresol consistently. These compounds are among the odourants emitted from fresh cattle manure (Miller & Varel, 2002) as well.

1-Octen-3-ol, phenol, *m*-cresol and *p*-cresol have been reported to evoke strong electroantennogram (EAG) responses from stable flies, with no differences in responses between males and females (Tangtrakulwanich *et al.*, 2011). 1-Octen-3-ol has been documented as a strong attractant for various haematophagous insects (Hall *et al.*, 1984; French & Kline, 1989; Gibson & Torr, 1999). In our laboratory behavioural assay, we used 1-octen-3-ol as a positive control, and found at all tested dosages that it elicited significant stable fly upwind movement compared with the hexane control. However, Alzogaray & Carlson (2000) and found that 1-octen-3-ol at the same dosage tested was not attractive to stable flies in a triple cage olfactometer study, which could be due to the use of flies at different ages, or different release rates from the tested dispensers (filter paper in the present study versus glass tube/vial cap). Phenol is reported as another attractant to haematophagous insects such as stable flies (Mihok *et al.*, 1995) and tsetse flies, *Glossina morsitans morsitans* and *G. pallidipes gambiensis* (Diptera: Glossinidae) in Zimbabwe (Vale *et al.*, 1988; Mihok *et al.*, 2007). Results from our olfactometer study showed that phenol, *p*-cresol and *m*-cresol at the lowest dosages (1 µg) elicited significant behavioural responses, but at higher dosages they appeared to be not attractive, and even as behavioural antagonists for stable flies (Fig. 5). More stable flies were attractive to mixtures of phenol and *p*-cresol or *m*-cresol at a 100-µg dose in the olfactometer, relative to that of the control (Fig. 6). In contrast, no preferences were found from stable flies while tested with individual compounds. The effect of combining compounds to enhance

fly captures also was observed by Kyorku *et al.* (1990) with *Glossina longipennis* Corti (Diptera: Muscidae) in south-west Kenya. In addition, Mihok & Mulye (2010) demonstrated that traps baited with 4-methylphenol (*p*-cresol) or 3-*n*-propylphenol alone were ineffective in catching horse flies, *Hybomitra lasiophthalma* (Diptera: Tabanidae) (Macquart). But when traps were baited with cattle urine (mixtures of more than phenol) or cattle urine and 1-octen-3-ol, horse fly capture increased 1.5–2.6 folds.

Phenol, *p*-cresol and *m*-cresol are three major cattle-associated volatile compounds (Miller & Varel, 2002) and they were recovered from aged horse manure as well (Zhu *et al.*, personnel communication). Alsynite traps baited with mixture blends of these compounds have shown strong attractiveness to stable flies, with 2–3 times more flies captured as compared with unbaited alsynite panels. Phenol and *p*-cresol also have been demonstrated to attract tsetse flies (IAEA, 2003). Traps baited with attractants, such as CO₂ and 1-octen-3-ol from cattle breath and urine, have been reported to increase the trapping efficacy for control of biting flies (Schofield *et al.*, 1995). Cilek (1999) had shown a six-fold increase in catches with traps baited with a 4:1:8 mixtures of 1-octen-3-ol, propylphenol and methyl phenol. Holloway & Phelps (1991) and Mihok *et al.* (1995) demonstrated that adding 1-octen-3-ol to traps significantly increased stable fly catches relative to those without attractant lures added. However, Mullens *et al.* (1995) and Cilek (1999) reported that 1-octen-3-ol failed to increase the captures from traps with lures. Discrepancies among these various studies could be due to trap materials and design, geographical variation in trapping sites (Africa vs. North America), and bait concentration and carrier used (different release rates), as well as other environment parameters (background odors). We did not compare the attractiveness of 1-octen-3-ol with the mixture blends in our field trials, because 1-octen-3-ol was not found from the manure/slurry collection.

In conclusion, attractant baited traps can be used to improve monitoring and management of stable flies. The synergism between cresols and phenol found in stable fly trap catches may enhance mass trapping efficacy in a Push-Pull control

strategy. However, additional studies are needed to extend the bait persistence in traps and reduce the cost of using the alsynite trap.

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