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Acquired Smell? Mature Females of the Common Green Bottle Fly Shift Semiochemical Preferences from Feces Feeding Sites to Carrion Oviposition Sites

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Abstract We investigated foraging decisions by adult females of the common green bottle fly, *Lucilia sericata*, in accordance with their physiological state. When we gave female flies a choice between visually occluded, fresh canine feces (feeding site) and a CO_2-euthanized rat (carrion oviposition site), 3-d-old "protein-starved" females responded equally well to feces and carrion, whereas protein-fed gravid females with mature oocytes responded only to carrion, indicating resource preferences based on a fly's physiological state. Dimethyl trisulfide (DMTS) is known to attract gravid *L. sericata* females to carrion. Therefore, we analyzed headspace from canine feces by gas chromatographic-electroantennographic detection (GC-EAD) and GC/mass spectrometry. In bioassays, of the 17 fecal odorants that elicited GC-EAD responses from fly antennae, a blend of indole and one or more of the alcohols phenol, *m-/p*-cresol and 1-octen-3-ol proved as attractive to flies as canine feces. Unlike young females, gravid females need to locate carrion for oviposition and distinguish between fresh and aging carrion, the latter possibly detrimental to offspring. Gravid female *L. sericata* accomplish this task, in part, by responding to trace amounts of DMTS emanating from fresh carrion and by discriminating against carrion as soon it begins to produce appreciable amounts of indole, which is also the second-most abundant semiochemical in fresh canine feces, and apparently serves as an indicator of food rather than oviposition resources. Our results emphasize the importance of studying foraging choices by flies in accordance with their physiological stage.

Keywords *Lucilia sericata* · Diptera · Calliphoridae · Feces · Carrion indole · Dimethyl trisulfide · Behavioral shift · Foraging · GC-EAD

Introduction

Insects undergo significant morphological and physiological changes as they develop. Depending on the nutritional need of a stage, insects may seek and exploit different resources (Hochuli 2001; Stockhoff 1993). For example, mosquito females seek floral nectar and obtain energy (Gary and Foster 2006; Smith and Gadawski 1994). blood hosts that mature their ovaries (Qiu *et al.* 2011). and standing water for oviposition (Navarro-Silva *et al.* 2009; Osgood 1971).

Adult blowflies (Diptera: Calliphoridae) rely on floral nectar, animal feces, and carrion for survival and reproduction (Erzinclioglu 1996). Each resource presents a specific nutritional or reproductive benefit to blowflies: floral nectar provides carbohydrates (Grinfel'd 1955; Norris 1965), feces provide protein (Hanski 1987). and carrion commonly serves as an oviposition site (Byrd and Castner 2010; Norris 1965). Whether young or gravid flies respond similarly to each type of resource and readily distinguish between them based on odor profiles has rarely been investigated (but see Brodie *et al.* 2014).

Animal feces and manure are particularly odiferous protein sources. For example, >140 volatiles emanate from pig manure (O'Neill and Phillips 1992; Yasuhara and Fuwa 1979), three of which [3-methylbutanoic acid, indole, dimethyl trisulfide (DMTS)], in combination, are attractive to houseflies, *Musca domestica* (Cossé and Baker 1996). Similarly, of the many volatiles that emanate from bird droppings, five (ammonia, methylamine, dimethylamine, trimethylamine,

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and 1-pyrroline) attract tephritid fruit flies (Epsky *et al.* 1997; Robacker *et al.* 2000).

Blowflies frequently visit animal feces and obtain nutrients, but oviposit only on fresh carrion (Hanski 1987; Norris 1965). Gravid, but not recently eclosed, females of the common green bottle fly, *Lucilia serricata*, orient toward fresh carrion in response to DMTS (Brodie *et al.* 2014). but DMTS also emanates from feces, indicating that there must be additional semiochemicals that help blow flies distinguish at long range between these different resources. The composition of semiochemicals in a resource may change over time, indicated by older carrion being rejected by gravid blowflies as a suitable oviposition site (Archer and Elgar 2003; George *et al.* 2012; Hall and Doisy 1993). The semiochemical(s) that mediate the foraging shift by maturing flies from feces to fresh carrion, or those that render older carrion unsuitable for oviposition, are not known. Identifying them and demonstrating their effect on the behavior of flies were the major objectives of this study.

We worked with *L. sericata* as a representative blowfly species of the northern hemisphere (Hall 1948; Hall and Townsend, 1977) and as an early responder to animal carrion (Byrd and Castner, 2010; Hall and Doisy 1993). We focused on the response of female flies, because females undergo significant physiological changes as they mature and develop their oocytes, and, unlike males, must find opposition sites. We used recently deceased Norway rats, *Rattus norvegicus*, and mice, *Mus musculus,* as oviposition sites because they are commonly exploited by *L. sericata* and are sufficiently small to be deployed as baits in laboratory and field bioassays. We selected canine feces as a proven attractive protein source to flies (Lawson and Gemmell, 1990) because it could readily be collected immediately after defecation.

To unravel the mechanism(s) that underlie the foraging shift by maturing flies from fresh feces to fresh carrion, and the rejection of aged carrion by females, our specific objectives were to: (1) compare the attractiveness of fresh canine feces and fresh rat carrion to young and gravid flies; (2) obtain head space volatile (HSV) extract from fresh feces and bioassay its attractiveness to flies; (3) identify candidate semiochemicals in bioactive HSV extracts; (4) determine the key semiochemical(s) in HSV extracts; (5) monitor time-related changes in the odor profile of aging mouse carrion; and (6) test the effect of key semiochemical(s) on the acceptance or rejection of fresh mouse carrion.

Methods and Materials

Source of Rodents, Canine Feces, and Flies Norway rats and mice were purchased by, and housed in, the Animal Care Facility of Simon Fraser University (ACF-SFU) or were purchased from a retailer (CTC Predator Feed, Duncan, BC, Canada). Rodents were CO_2-euthanized in the ACF-SFU (Permit # 1042-12) and were incised *post-mortem* (Brodie *et al.* 2014) to simulate the death of an injured animal. Both rats and mice were used in our study because blowflies seek both for oviposition (Byrd and Castner, 2010) and because both became available from a rodent pheromone research project, when the animals were nearing the natural end of their life. Moreover, only mice were sufficiently small to fit as bait in Oak Stump traps (see below).

Dog feces served as an analytical resource and test stimulus, and originated from two healthy, mixed-breed, 5- and 9-yr-old dogs. The dogs' diet consisted primarily of kibble (Science Diet® Lamb Meal and Rice Formula; Nutro®), and occasionally of table scrapes and grass. Fresh feces from both dogs was collected daily, combined, and homogenized to standardize odor cues, and tested within 6 h of defecation.

Experimental flies were reared in SFU's insectary under a L16:D8 photoperiod, at 30–40 % RH and 23–25 °C. Every 12 mo, the colony was re-started using 50 wild-captured gravid female flies, increasing the colony to about 5000 flies at specific times, depending on experimental requirements.

General Bioassay Procedure Two groups of flies, differing in age and reproductive status, were tested in bioassays: (1) young, 3–5-d-old females, and (2) aged, 10–12-d-old (gravid) females. Considering that recently eclosed young flies primarily seek nectar (carbohydrates; Brodie, unpublished data) and may have had only ephemeral access to animal feces, and that gravid flies will consume considerable protein for maturation of eggs (Barton Browne 2001). the group of young flies was given access only to water and sugar, whereas the group of aged, gravid flies was provided with proteinaceous milk and liver powder (Sigma Aldrich, St. Louis, MO, USA) in addition to sugar and water.

For each experimental replicate, 50 cold-sedated females (Table 1) were placed into an aluminum wire mesh cage (61 × 61×61 cm; Fig. 1a) and given 24 h to acclimate before the bioassay. Taking into account that the propensity of flies to respond can vary among days, and thus make experimental treatment effects less apparent, the same numbers of replicates of each treatment in experiments 1–4, 7–12, 13–18, and 19–22 were run concurrently (in parallel) on any given day, with treatments being applied to each cage in random order. Flies were tested only once for their response to a particular experimental treatment.

The test stimuli for laboratory experiments 1–4 are described in objective 1 (see below). For laboratory experiments 5–22, each test stimulus was placed on the bottom of a white paper Solo cup (0.5 l; 9×8.5 cm; Solo®, Lake Forest, IL, USA), which was then covered with 2-ply white cheesecloth (VWR, Radnor, PA, USA) to standardize visual cues (Fig. 1a). Each semiochemical stimulus (including control) was prepared in pentane and pipetted in 30-μl aliquots onto white

Table 1 List of objectives/experiments and stimuli tested in laboratory and field experiments for the response of *Lucilia sericata* flies

Exp. no	Location	Test stimuli (**T**)	Type of flies & No of replicates				Time
			Protein-hungry (3–5-d-old)		Protein-fed (10–12-d-old)		
			♀	♂	♀	♂	
Objective 1: Compare the attractiveness of fresh canine feces and fresh rat carrion to young and gravid females							
1-4	Laboratory	**T_1**: Canine feces (10 g; fresh); **T_2:** Rat carrion (fresh)	10	10	10	10	5 min
Objective 2: Obtain head space volatile (HSV) extracts from fresh canine feces and bioassay its attractiveness to flies							
5	Laboratory	**T_1:** HSV[a] extract of canine feces; **T_2:** Solvent control	14	–	–	–	5 min
Objective 3: Identify candidate semiochemicals in bioactive HSV extracts							
Objective 4: Determine the key semiochemical(s) in bioactive HSV extracts							
6, 7, 13, 19	Laboratory	**T_1:** SB[a,b]; **T_2:** Solvent control	10	–	–	–	5 min
8	Laboratory	**T_1:** SB *minus* acids; **T_2:** Solvent control	10	–	–	–	5 min
9	Laboratory	**T_1:** SB *minus* aldehydes; **T_2:** Solvent control	10	–	–	–	5 min
10	Laboratory	**T_1:** SB *minus* alcohols; **T_2:** Solvent control	10	–	–	–	5 min
11	Laboratory	**T_1:** SB *minus* ketones; **T_2:** Solvent control	10	–	–	–	5 min
12	Laboratory	**T_1:** SB *minus* [indole + DMTS][b]; **T_2:** Solvent control	10	–	–	–	5 min
14	Laboratory	**T_1:** SB *minus* phenol; **T_2:** Solvent control	10	–	–	–	5 min
15	Laboratory	**T_1:** SB *minus* 1-octen-3-ol; **T_2:** Solvent control	10	–	–	–	5 min
16	Laboratory	**T_1:** SB *minus m-/p*-cresol; **T_2:** Solvent control	10	–	–	–	5 min
17	Laboratory	**T_1:** SB *minus* DMTS; **T_2:** Solvent control	10	–	–	–	5 min
18	Laboratory	**T_1:** SB *minus* indole; **T_2:** Solvent control	10	–	–	–	5 min
20	Laboratory	**T_1:** Alcohols; **T_2:** Solvent control	10	–	–	–	5 min
21	Laboratory	**T_1:** Indole; **T_2:** Solvent control	10	–	–	–	5 min
22	Laboratory	**T_1**: Alcohols plus indole; **T_2**: Solvent control	10	–	–	–	5 min
Objective 5: monitor over time changes in odor profile of aging mice carrion							
Objective 6: Test the effect of key semiochemical(s) on the acceptance or rejection of fresh mouse carrion							
23	Laboratory	**T_1:** Mouse carrion (24-h old); **T_1** + **T_2**: Canine feces (fresh; 20 g)			10		1 h
24	Field[c]	**T_1:** Mouse carrion (fresh); **T_2**: **T_1** + canine feces (fresh; 20 g)					3 d
25	Field[c]	**T_1:** Mouse carrion (fresh) + ether; **T_2**: **T_1** + indole (100 μg in ether)					3 d

[a] Headspace volatile (HSV) extract and the synthetic blend (SB) were tested at 10 fecal g-hr-equivalents (10 g of fresh canine feces during 1 h)

[b] SB: acids [propanoic (30 ng), 2-methylpropanoic (30 ng), butanoic (300 ng), 2-methylbutanoic (300 ng), 3-methybutanoic (300 ng)]; aldehydes [phenylacetaldehyde (3 ng), (*E*)-2-octenal (3 ng), nonanal (9 ng), decanal (9 ng)]; alcohols [phenol (1260 ng), 1-octen-3-ol (15 ng), *m-/p*-cresol (12 ng each)]; ketones [sulcatone (9 ng), geranylacetone (6 ng)]; indole (660 ng), dimethyl trisulfide (DMTS, 15 ng)

[c] 10 & 11 replicates

filter paper (55 mm diam; Springfield Mill, UK). For all tests, one randomly assigned treatment and control cup were placed 10 cm apart in the center of the cage. Immediately thereafter, the number of flies that alighted on the cheesecloth of each cup was recorded for 5 min. and averaged across all replicates.

Objective 1: Compare the Attractiveness of Fresh Canine Feces and Fresh Rat Carrion to Young and Gravid Females Experiments 1–4 (Table 1) offered young females (Exps. 1, 2) and gravid females (Exps. 3, 4) a choice between two Ziploc® plastic containers (828 ml; SC Johnson, Racine, WI, USA), covered in 2-ply cheese cloth to standardize visual cues, containing, in one set, canine feces (10 g; see above) or nothing (control) and, in another set, rat carrion or nothing. Ziploc® plastic containers were used instead of Solo cups in experiments 1–4 to house the larger rats (♂ mean=463 g; ♀ mean=224 g). Each rat was CO_2-euthanized 5 h prior to testing and incised (Brodie *et al.* 2014) to simulate the death of an injured rat.

Objective 2: Obtain Head Space Volatile (HSV) Extracts from Fresh Canine Feces and Bioassay its Attractiveness to Flies After placing an homogenized 10-g sample of fresh canine feces in a Pyrex® glass chamber (34 cm high × 12.5 cm

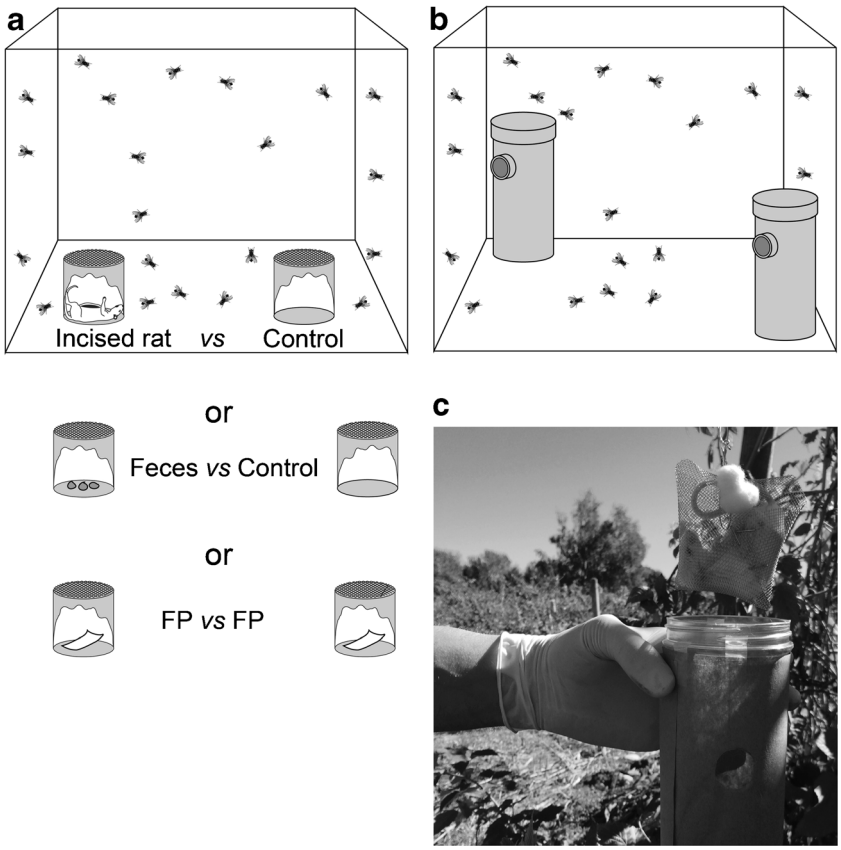

Fig. 1 **a** Design of laboratory experiments recording alighting of flies on paired Ziploc storage containers (experiments 1–4) and on paired Solo cups (experiments 5–22) containing (*i*) canine feces (10 g) or nothing (empty control), (*ii*) rat carrion or nothing, and (*iii*) filter paper impregnated with (synthetic) semiochemicals or a solvent control; **b** design of laboratory experiment 23, which recorded captures of flies in paired Oak Stump traps covered in brown construction paper and baited with a mouse carcass (see **c**) or canine feces (20 g); **c** Oak Stump trap baited for field experiments 24 and 25 with a mouse carcass in a mesh bag; the white cotton ball was impregnated with either indole (100 μg) dissolved in ether or ether alone (control)

wide), a pump drew charcoal-filtered air, at 0.5 $l.min^{-1}$, for 5 h through the chamber and a glass column (6 mm o.d. × 150 mm) containing 200 mg of Porapak-Q™ (50–80 mesh) adsorbent (Byrne *et al.* 1975). Feces-derived headspace volatiles captured on Porapak-Q were desorbed in sequence using pentane (1 ml) and ether (1 ml). Pentane and ether extracts were concentrated and combined, such that 30-μl aliquots of HSV extract contained 10 g-hr equivalents of feces volatiles (i.e., the amount of volatiles given off by 10 g of feces over 1 h).

In experiment 5 (Table 1), aliquots of the HSV extract at 10 g-hr equivalents were then tested for attractiveness to flies, following the general experimental design described above. Because only young flies responded well to fresh canine feces (see Results), only they were tested for response to HSV extract of canine feces.

Objective 3: Identify Candidate Semiochemicals in Bioactive HSV Extracts Aliquots of Porapak-Q HSV extract of canine feces were analyzed by gas chromatographic-electroantennographic detection (GC-EAD) and GC/mass spectrometry (MS), with procedures and equipment previously described in detail (Arn *et al.* 1975; Gries *et al.* 2002). For GC-EAD recordings ($N=10$), an antenna was carefully pulled from the head of a 3–5-d-old female fly and suspended between two glass capillary electrodes (1.0×0.58×100 mm; A-M Systems, Carlsborg, WA, USA) filled with saline solution (Staddon and Everton 1980). Volatile components that elicited responses from at least seven out of 10 antennae were considered candidate semiochemicals. The GC-EAD apparatus utilized a Hewlett-Packard 5890 gas chromatograph (GC) fitted with a DB-5 GC column (30 m×0.32 mm i.d.; J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas (35 $cm.s^{-1}$) with the following column oven temperature program used: 50 °C for 5 min., 20 $°C.min^{-1}$ to 280 °C. The injector port and flame ionization detector (FID) were set at 250 °C. Candidate semiochemicals were analyzed on a Saturn 2000 Ion Trap GC/MS operated in full-scan electron impact mode and fitted with a DB-5 column (50 m×0.25 mm i.d.). Helium was the carrier gas (35 $cm.s^{-1}$). The column oven temperature program was 50 °C for 1 min, 10 $°C.min^{-1}$ to 280 °C (held for 10 min). The injector port and ion trap were set at 250 °C and 260 °C, respectively. Components that elicited responses from fly antennae were identified by comparing retention indices (Van den Dool and Kratz 1963) and mass spectra with those reported in the literature (Adams 1989; Jennings and Shibamoto 1980) and with those of authentic standards.

To confirm the structural assignment of candidate semiochemicals and to prepare synthetic blends (see objective 4), the following compounds were purchased: propanoic acid (>99.5 % chemically pure), 2-methylpropanoic acid (99 %), butanoic acid (>99 %), 2-methylbutanoic acid (>98 %), 3-

methylbutanoic acid (99 %), phenylacetaldehyde (90 %), nonanal (95 %), decanal (>95 %), phenol (>95 %), 1-octen-3-ol (>98 %), *m*- and *p*-cresol (99 %), sulcatone (>98 %), geranylacetone (>97 %), indole (>99 %), dimethyl trisulfide (>95 %) (all Sigma Aldrich, St. Louis, MO, USA), and (*E*)-2-octenal (Bedoukian, Danbury, CT, USA).

Objective 4: Determine the Key Semiochemical(s) in Bioactive HSV Extracts Experiment 6 (Table 1) tested a synthetic blend (SB) of all candidate semiochemicals in HSV extracts of feces that had elicited responses from fly antennae in GC-EAD recordings (see Results). The SB included propanoic acid, 2-methylpropanoic acid, butanoic acid, 2-methylbutanoic acid, 3-methybutanoic acid, phenylacetaldehyde, (*E*)-2-octenal, nonanal, decanal, phenol, 1-octen-3-ol, *m*- and *p*-cresol, sulcatone, geranylacetone, indole, and dimethyl trisulfide (DMTS). As *m*- and *p*-cresol could not be separated, they were both included in the SB. All components were prepared in pentane, in a ratio equivalent to that found in GC/MS analyses of HSV extracts, and were tested at 10 g-hr equivalents, following the general bioassay procedure described above.

With evidence that HSV extract of fresh canine feces and the SB were both effective in attracting flies (see 'Results'), the next set of experiments was designed to determine the essential semiochemical(s) that mediate the response of flies to the SB. Accordingly, parallel-run experiments 7–12 (Table 1) tested the SB (experiment 7), and SBs that lacked groups of chemicals, including alcohols (experiments 8), aldehydes (experiment 9), ketones (experiment 10), acids (experiment 11), and N- or S-containing compounds (experiment 12). All SBs were tested following the general bioassay procedure described above.

With evidence that an alcohol and a N- or S-containing compound (DMTS or indole) were key semiochemicals in feces (see Results), experiments 13–18 (Table 1) tested the SB (experiment 13) and SBs lacking a specific alcohol, namely phenol (experiment 14), 1-octen-3-ol (experiment 15) or *m/p*-cresol (experiment 16), or lacking DMTS (experiment 17) or indole (experiment 18). All SBs were tested following the general bioassay procedure described above.

With evidence that indole, but not DMTS or any one specific alcohol, were key semiochemicals of feces (see Results), experiments 19–22 (Table 1) investigated potential interactive effects between components, by testing the SB (experiment 19), the three alcohols combined (phenol, 1-octen-3-ol, *m-/p*-cresol) (experiment 20), indole alone (experiment 21), and a mixture of the alcohols and indole (experiment 22). All synthetic stimuli were tested following the general bioassay procedure described above.

Objective 5: Monitor Changes over Time in Odor Profile of Aging Mouse Carrion To quantify the amount of indole and DMTS in the headspace of mouse carrion, 10 CO_2-euthanized mice (3 replicates) were placed in a Pyrex® glass aeration chamber, capturing the headspace on Porapak-Q (see Objective 2 and Table 1 for detailed methods). After the first 16 h of volatile capture, the Porapak-Q volatile traps were replaced every 12 h, up to 76 h. Aliquots of the Porapak-Q extracts were analyzed by GC/MS, using hexyl acetate as an internal standard.

Objective 6: Test the Effect of Key Semiochemical(s) on the Acceptance or Rejection of Mouse Carrion With evidence that young and gravid flies differed in their resource preferences (see Results), and that indole was absent from fresh carrion, but essential for attraction of young flies to canine feces (see Results), experiments 23–25 tested the effect of fresh canine feces, or of indole specifically, on the response of flies to fresh mouse carrion. Laboratory experiment 23 and field experiment 24 (Table 1) tested the response of aged (13-d-old) flies and wild-type flies, respectively, to one mouse carcass at the bloat stage (24 h *post* CO_2-euthanizition at room temperature) and the same type of mouse carrion in combination with fresh canine feces (20 g) applied to the outside of a mesh bag (50×63 mm; Fig. 1c) enclosing the carrion. The mesh bag was suspended from a wire above a soap water moat inside an Oak Stump trap covered with brown paper to occlude test stimuli. For each experimental replicate, 50 cold-sedated female flies were allowed to acclimate for 1 h before the two traps were placed 0.45 cm apart in opposite corners of the bioassay cage. After 1 h, the traps were removed, captured flies counted, and their reproductive status (non-gravid, gravid) determined by dissection of the ovaries (Adams and Reinecke 1979).

Field experiment 24 (13–15 September 2014; Table 1) was conducted on a berry farm (49°01′31.55″N, 122°18′43.61″W) next to a poultry farm in Abbotsford, BC, Canada. Testing the same two stimuli as in experiment 23 (mouse carrion with or without canine feces), Oak Stump traps were suspended 0.5 m above ground from a fence in randomized complete blocks with 10 m between traps within and among blocks. Near the end of the photophase of each of the 2 days, the number, sex, and physiological status (see above, experiment 23) of captured specimens of each fly species were determined. Combined data for days 1–2 are reported.

Field experiment 25 (21–23 September 2014; Table 1) had the same design as experiment 24, but the freshly deceased mouse carrion in each trap was combined with a cotton ball (Fig. 1c), impregnated with ether (control) or indole (100 μg) dissolved in ether, and attached to the mesh bag enclosing the carrion.

Statistical Analyses All data were analyzed with JMP 11® (SAS Institute Inc., Cary, NC, USA). In experiments 1–4, 7–12, 13–18, and 19–22, the mean proportions of fly landings on

paired Solo cups were analyzed by one-tailed *t*-tests, expecting a preferred stimulus to induce >65 % of the fly responses. Data (mean cumulative number of fly landings on treatment stimuli) in experiments 7–12 and 19–22 were log-transformed and evaluated for normality using a Q-Q plot. Within experiments 7–12, 13–18, and 19–22, respectively, the alighting responses of flies to treatments were analyzed by ANOVA followed by Tukey's HSD test. The alighting responses of flies to treatments in experiments 5 and 6 were compared by a Wilcoxon test. Data in each of experiments 23–25 were analyzed using a non-parametric Wilcoxon signed rank test.

Results

Objective 1: Compare the Attractiveness of Fresh Canine Feces and Fresh Rat Carrion to Young and Gravid Females Fresh canine feces received more (>65 %) alighting responses from young females than did the control (Fig. 2, Exp. 1: df=9, t=30.84, P<0.001), whereas there was no difference (Fig. 2; Exp. 3: df=9, t=−1.394, P=0.896) in alighting responses of gravid females on fresh canine feces over the control. This indicates that fresh canine feces was attractive only to young flies. In contrast, fresh rat carrion received >65 % of the alighting responses from both young (Fig. 2; Exp. 2: df=9, t=33.99, P<0.001) and gravid females (Fig. 2; Exp. 4: df=9, t=47.12, P<0.001), indicating that fresh rat carrion was attractive to both young and gravid flies.

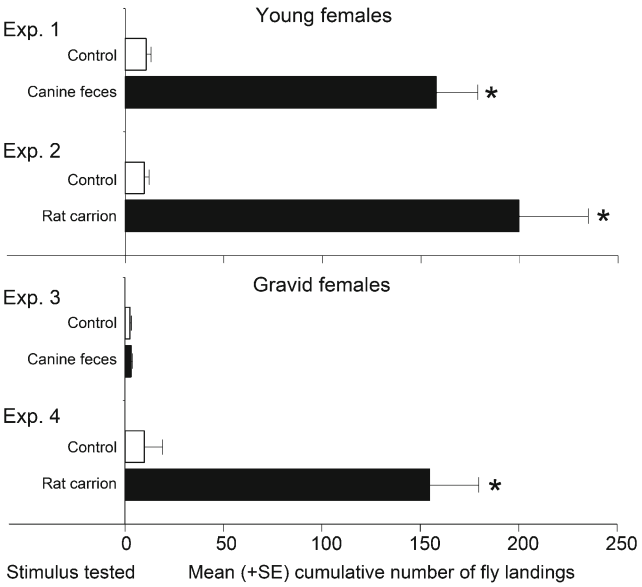

Fig. 2 Mean (+SE) cumulative alighting responses by young and gravid female *Lucilia sericata* in experiments 1–4 (N=10 each) on paired, cheesecloth-covered Ziploc plastic containers that were left empty (control) or baited with fresh canine feces or fresh rat carrion. In experiments 1, 2, and 4, an asterisk denotes that the treatment received more than 65 % of the alighting responses (one-tailed *t*-test; Exp. 1: t=30.84, df=9, P<0.001; Exp. 2: t=33.99, df=9, P<0.001; Exp. 4: t=47.12, P<0.001)

Objective 2: Obtain Head Space Volatile (HSV) Extract from Fresh Feces and Bioassay its Attractiveness to Flies Aliquots of Porapak Q HSV extract of fresh canine feces received >65 % of the landing responses of flies, different from that of the control (Fig. 3; Exp. 5: t=7.83, df=13, P<0.001), indicating that the essential semiochemical(s) associated with fresh canine feces were present in HSV extract.

Objective 3: Identify Candidate Semiochemicals in Bioactive HSV Extracts In GC-EAD analyses of HSV extract of fresh canine feces, 12 compounds consistently elicited responses from fly antennae (Fig. 4): phenylacetaldehyde, (*E*)-2-octenal, nonanal, decanal, phenol, 1-octen-3-ol, *m*- and *p*-cresol, sulcatone, geranylacetone, indole, and DMTS. Antennal responses to propanoic acid, 2-methylpropanoic acid, butanoic acid, 2-methylbutanoic acid, and 3-methybutanoic acid varied greatly between runs, likely due to poor chromatography of these acids on the GC column.

Objective 4: Determine the Key Semiochemical(s) in Bioactive HSV *Extracts* The SB of candidate semiochemicals from fresh canine feces had more (Fig. 3; Exp. 6: t=6.69, df=9, P<0.001) landing than the solvent control, indicating that the SB contained key semiochemicals associated with canine feces. Moreover, the mean response of flies to the SB (Exp. 6: 70.0±7.3 landings) and to HSV extract of feces (Exp. 5: 62.6±5.1 landings) did not differ (Z=140.5, df=1, P=0.380), indicating further that the SB contained all the key volatile semiochemical(s) of canine feces.

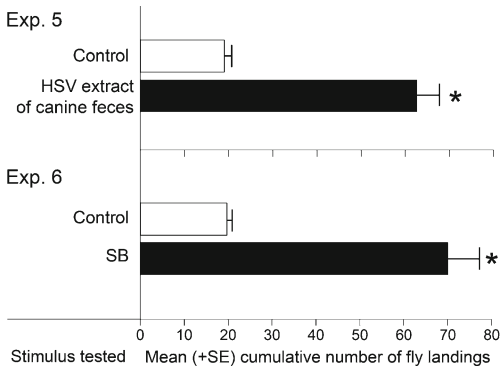

Fig. 3 Mean (+SE) cumulative alighting responses by young female *Lucilia sericata* in experiment 5 (N=14) and 6 (N=10) on paired cheesecloth-covered Solo cups containing filter paper impregnated with (*i*) headspace volatile (HSV) extract of fresh canine feces in pentane/ether at 10-g-hr equivalents (amount of volatiles given off by 10 g of feces over 1 h) (Exp. 5), and (*ii*) a synthetic blend (SB) of candidate semiochemicals (see Table 1) in pentane/ether vs. pentane/ether (control; Exp. 6). In experiments 5 and 6, an asterisk denotes that the treatment received more than 65 % of the alighting responses (one-tailed *t*-test; Exp. 5: t=7.83, df=13, P<0.001; Exp. 6: t=6.69, df=9, P<0.001)

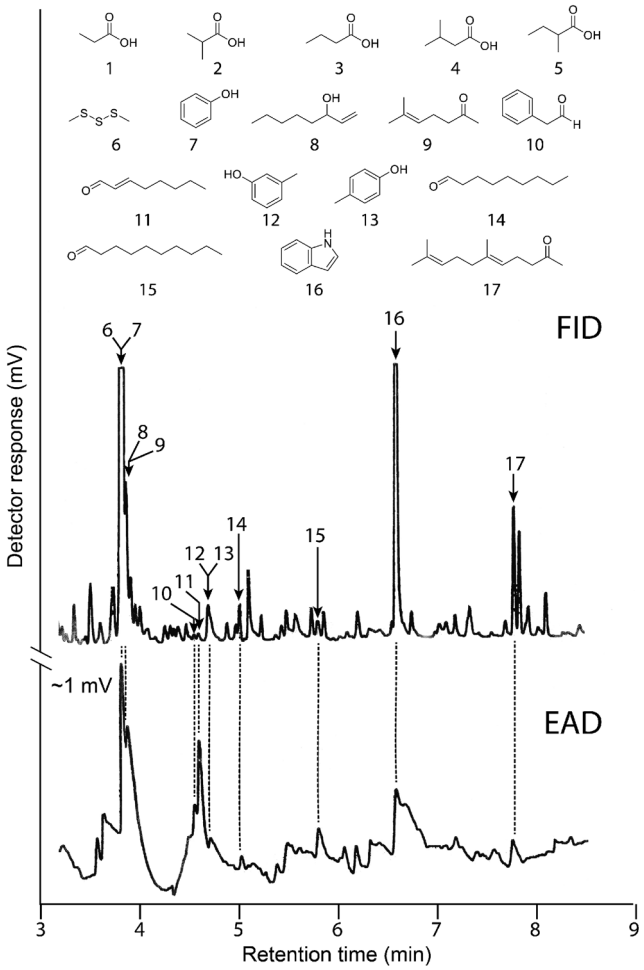

Fig. 4 Representative recordings from a gas chromatographic flame ionization detector (FID) and an electroantennographic detector (EAD; of female *Lucilia sericata* antenna) to an aliquot of Porapak Q headspace extract of fresh canine feces. Antennal responses to propanoic acid (**1**), 2-methylpropanoic acid (**2**), butanoic acid (**3**), 3-methybutanoic acid (**4**), and 2-methylbutanoic acid (**5**) varied greatly between runs due to poor chromatography of these acids and are not depicted here. Other EAD-active odorants: dimethyl trisulfide (**6**), phenol (**7**), 1-octen-3-ol (**8**), sulcatone (**9**), phenylacetaldehyde (**10**), (*E*)-2-octenal (**11**), *meta*- and/or *para*-cresol (**12**, **13**), nonanal (**14**), decanal (**15**), indole (**16**), and geranylacetone (**17**)

The response of young females to the complete SB or to SBs lacking specific components differed (Exps. 7-12; $F_{(5,54)}$=4.45, P=0.002; Fig. 5). In comparison to the control, the complete SB and SBs lacking acids, aldehydes, or ketones all received >65 % of landing responses from flies (df=9 each; Exp. 7: t=7.45, P<0.001; Exp. 8: t=6.46, P<0.001; Exp. 9: t=7.09, P<0.001; Exp. 11: t=5.79, P<0.001; Fig. 5). A preferred response was not evident for SBs lacking alcohols (Exp. 10: t=1.29; P<0.115) or indole and DMTS (Exp. 12: t=−1.46; P<0.911), indicating that at least one alcohol, as well as indole and/or DMTS, mediate attraction of young females to fresh canine feces.

The response of young females to the complete SB or to SBs lacking a specific component differed (Exps. 13–18; $F_{(5,54)}$=10.5, P<0.001; Fig. 5). In comparison to the control, the complete SB and SBs lacking phenol, 1-octen-3-ol, *p*/*m*-cresol, or DMTS all received >65 % of the landing responses of flies (df=9 each; Exp. 13: t=4.73, P=0.001; Exp. 14: t=5.96, P<0.001; Exp. 15: t=2.73, P=0.023; Exp. 16: t=3.82, P=0.004; Exp. 17: t=7.30, P<0.001; Fig. 5), indicating that neither of these odorants alone mediates attraction of young females to fresh canine feces. In contrast, the SB lacking indole failed to receive >65 % of the landing responses of flies (Exp. 18: t=0.70, P=0.22; Fig. 5), indicating that the presence of indole is important for attracting young females to fresh canine feces.

The response of young females to test stimuli consisting of the complete SB, three alcohols (phenol, 1-octen-3-ol, *m*- and *p*-cresol), indole, or a mixture of the alcohols and indole differed (Exps. 19–22; $F_{(3,36)}$=18.03, P<0.001; Fig. 5). In comparison to the solvent control, the complete SB and the mixture of alcohols and indole both received >65 % of the landing responses of flies (df=9 each; Exp. 19: t=11.81, P<0.001; Exp. 22: t=9.62, P<0.001; Fig. 5). The same type of preferred response was not evident when we tested the alcohols (Exp. 20: df=9, t=0.44, P=0.439; Fig. 5) or indole (Exp. 21: df=9, t=0.04, P=0.489; Fig. 5), which received 12.9±3.3 fly landings and 12.6±4.6 fly landings, respectively, five times fewer than that received by the mixture of alcohols and indole, indicating synergistic attractiveness between indole and one or more of the alcohols.

Objective 5: Monitor Changes over Time in Odor Profiles of Aging Mouse Carrion During 16 hr *post mortem*, indole was detectable in only one of three replicates (10 mice carcasses per replicate) and in only a small amount (mean of <0.01 μg; Fig. 6), whereas DMTS was detectable in all three replicates, averaging 1.33 μg (Fig. 6). During the subsequent 12-h periods, the amounts of DMTS and indole released from mice carcasses increased substantially. At each sampling interval, the amount of DMTS exceeded the amount of indole by 13–3 times (Fig. 6).

Objective 6: Test the Effect of Key Semiochemical(s) on the Acceptance or Rejection of Mouse Carrion In laboratory experiment 23 with (>90 %) gravid females, traps baited with both mouse carrion and canine feces captured fewer females (Z=−3.745, df=1, P<0.001; Fig. 7) and fewer gravid females (Z=−3.75, df=1, P<0.001; Fig. 7) than traps baited with mouse carrion alone, revealing a repellent effect of canine feces on the responses of oviposition-site-seeking flies. In contrast, non-gravid females responded equally to both baits (Z=−1.793, df=1, P=0.073; Fig. 7), consistent with prior results (Fig. 2, exps. 1, 2) that canine feces and recently deceased rat carrion are equally attractive to protein-hungry flies with immature oocytes.

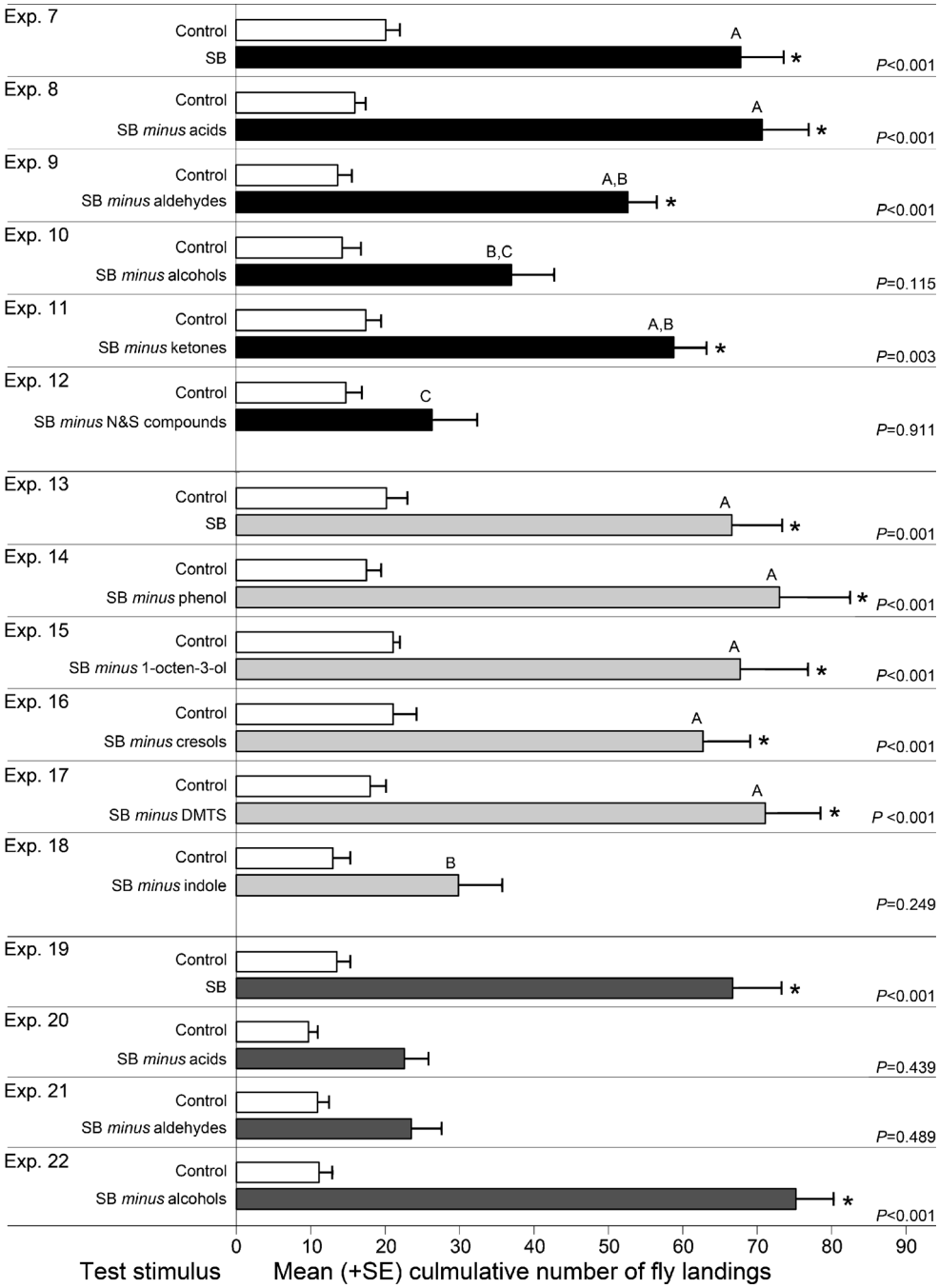

Fig. 5 Mean (+SE) cumulative number of alighting responses by young females of *Lucilia sericata* in experiments 7–22 (N=10 each) on paired cheesecloth-covered Solo cups containing filter paper impregnated with (*i*) a synthetic blend (SB; see Table 1) of the 17 components that elicited antennal responses in headspace volatile extracts of canine feces (see Fig. 4), (*ii*) partial blends of these components, or (*iii*) 1–3 select key components. Filter paper impregnated with the corresponding amount of pentane was the control. In each experiment, an asterisk denotes a treatment that received more than 65 % of alighting responses (one-tailed t-test: $P<0.001$). Within experiments 7–12, 13–18, and 19–22, bars with different letters indicate differences in the number of alighting responses by young female flies (Tukey's HSD: $P<0.05$)

The type of trap bait had an effect on captures of wild female flies (Z= −2.293, df=1; P=0.022, Fig. 7, exp. 24). Traps baited with both mouse carrion and canine feces attracted more non-gravid flies (Z=−2.973, df=1, P=0.003) than traps baited with mouse carrion alone, indicating a preference of these flies to the proteinaceous feces. However, there was no preference for either bait by gravid flies (Z=−0.873, df=1, P=0.383).

In field experiment 25, the type of trap bait had no effect on the overall number of wild flies captured (Z=0.325, df=1, P=0.985, Fig. 7). However, traps baited with both mouse carrion and indole (an indicator semiochemical of feces) captured fewer gravid female flies than traps baited with mouse carrion alone (Z=−2.89, df=1, P=0.004). In contrast, captures of non-gravid females in the same paired traps were not affected by the presence of indole (Z=−0.38, df=1, P=0.704).

Discussion

Our data demonstrate that: (1) the physiological state of *L. sericata* females affects their resource preference, with

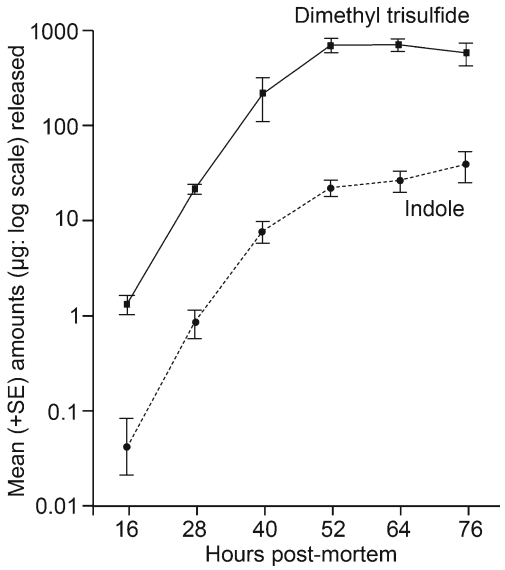

Fig. 6 Mean (±SE) amount of dimethyl trisulfide and indole released from 10 euthanized mice (N=3) over six time intervals *post mortem*

fresh canine feces attracting young (protein-hungry) flies but not gravid flies, and fresh rat carrion attracting both young and gravid flies; (2) attraction of young flies to canine feces is mediated by semiochemicals, of which indole and one or more of phenol, *m-/p*-cresol, and 1-octen-3-ol play key roles; (3) at an advanced, but not early, stage of decay, mouse carrion produces indole; and (4) fresh canine feces, or indole, repels gravid females seeking oviposition sites, indicating that indole

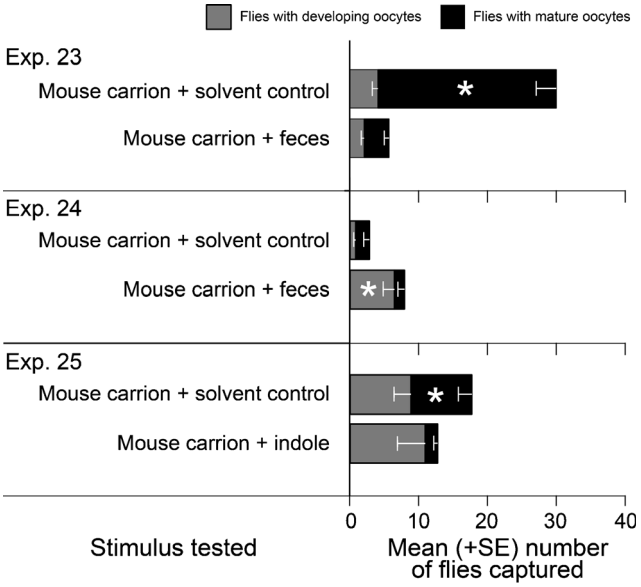

Fig. 7 Mean (±SE) number of *Lucilia sericata* females captured in paired Oak Stump traps baited with (*i*) one mouse carcass alone or in combination with fresh canine feces (20 g) (laboratory experiment 23, 24-h-old mouse carcass; field experiment 24, fresh mouse carcass), or (*ii*) one fresh mouse carcass alone or with indole (100 µg) dissolved in ether and applied to a cotton ball (field experiment 25). In each experiment, an asterisk denotes the stimulus that attracted more flies of a particular reproductive stage (Wilcoxon signed rank test; P<0.05).

signifies the presence of animal feces (protein resources) rather than an oviposition site, or that carrion is at an advanced stage of decay and thus unsuitable for oviposition.

Indole was the second most abundant (18 %) odorant in feces that elicited responses from *L. sericata* antennae. It has a strong fecal odor (Jensen *et al.* 1995) and is produced during the degradation of tryptophan, a major building block of proteins (Whitley and Thornton 2012). by intestinal bacteria, such as *Escherichia coli* (Dawes 1948; Schulz and Dickschat 2007). *Enterobacter* spp., *Klebsiella* spp. (Schulz and Dickschat 2007). *Lactobaccillus* spp., and *Clostridium* spp. (Jensen *et al.* 1995) that are present in most animal feces. Both its relative abundance and the fly's inherent sensitivity could make indole a reliable foraging cue for *L. sericata* females seeking feces for protein.

While indole is essential for attracting *L. sericata* females, its attractiveness hinges on the presence of one or more of the alcohols phenol, *m-/p*-cresol, or 1-octen-3-ol. Unlike indole, which has moderate volatility and thus likely serves as a long-range attractant, the more volatile alcohols could function as close-range attractants to help blowflies pinpoint the location of feces. An analogous system was reported for the aggregation pheromone of the bark beetle *Ips typographus*, in which the less volatile pheromone component (4*S*)-*cis*-verbenol attracts beetles over long distances, whereas the more volatile pheromone component 2-methyl-3-buten-2-ol attracts beetles at short range (Bakke *et al.* 1977; Schlyter *et al.* 1987).

The three fecal alcohols exhibit some degree of redundancy, because omitting any one from our SB did not reduce blend attractiveness (Fig. 5, Exps. 14–16). Intense fecal odor typically is associated with recently deposited, and thus moist, feces, and less so with dry feces. As the alcohols may dissipate more quickly than other components such as indole, their decreasing contribution to the odor bouquet might reflect the decreasing moisture content of feces. This would be important to foraging blowflies that have difficulty feeding on dry feces (Hanski 1987).

There are contrasting reports on the response of calliphorid flies to indole or feces. Indole previously was not known to attract calliphorid flies to animal feces, but was known to be part of a semiochemical blend that induced attraction to suboptimal oviposition sites, such as larval waste from artificial rearing material (Chaudhury *et al.* 2014) and fleece from an oviposition host (Eisemann 1995). Indole also had no effect on the attraction of blowflies in studies that investigated the attractiveness of oviposition sites rather than food sources, and that exclusively bioassayed the response of protein-fed flies (Easton and Feir 1991; Frederickx *et al.* 2011). The results of these previous studies and our own data indicate that age, physiological need, and reproductive status of blowflies affect their propensity to respond to semiochemicals from specific resources.

Our conclusion that fecal semiochemicals, in general, and indole, in particular, are repellent to gravid, oviposition site-seeking *L. sericata* females is supported by both laboratory and field data. Gravid *L. sericata* females were not more attracted to canine feces than they were to unattractive control stimuli, but strongly preferred mouse carrion over canine feces, and discriminated against mouse carrion when presented in combination with indole. In contrast, young flies were strongly attracted to canine feces, headspace extract of canine feces, and to various blends of synthetic fecal semiochemicals. Avoiding aging carrion, through perception of indole, as oviposition sites may help gravid *L. serricata* females minimize adverse fitness effects for their progeny. If females were to oviposit on animal carrion during later stages of decay, they might subject their offspring to predation by scavenging vertebrates or to resource competition by detritus-consuming insects, fungi, or microbes (DeVault *et al.* 2003). These might explain why gravid female flies typically do not oviposit on carrion at advanced stages of decomposition (Huntington *et al.* 2008). That gravid females and other flies still respond to these resources might be explained by their searching for food (which liquefied carrion provides) or for mates (which often gather on or near food resources) (Archer and Elgar 2003).

In summary, the resource preference of *L. sericata* females depends on their physiological state. Young, protein-seeking *L. sericata* females are strongly attracted to feces and to carrion. We demonstrated that they respond as readily to fresh canine feces as they do to a semiochemical blend of indole and one or more of the alcohols phenol, *m-/p*-cresol, and 1-octen-3-ol. In contrast, gravid females, which have fed already on protein, need to locate carrion for oviposition and to distinguish between fresh and aging carrion, with recently deceased carcasses (oviposition resources) likely providing less resource competition for offspring. Female *L. sericata* appear to accomplish this, in part, by responding to trace amounts of DMTS that emanate from fresh carrion and by discriminating against carrion as soon it begins to produce appreciable amounts of indole, which is the second most abundant semiochemical in fresh canine feces and apparently serves as an indicator of food rather than oviposition resources. Our results emphasize the importance of studying foraging choices by flies, and possibly insects, in accordance with physiological stage. Being cognizant of the physiological state of bioassay insects should facilitate better interpretation of experimental data, more accurate conclusions about foraging choices, and help facilitate development of Earth-friendly management of insect pests in urban, agricultural, or forest settings.

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