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Research

Behavior and Electrophysiological Response of Gravid and Non-Gravid *Lucilia cuprina* (Diptera: Calliphoridae) to Carrion-Associated Compounds

Guanjie Yan,[1,2] Shimin Liu,[1,3] Anthony C. Schlink,[3] Gavin R. Flematti,[4] Bekka S. Brodie,[5] Bjorn Bohman,[4] Johan C. Greeff,[3] Philip E. Vercoe,[1] Jianhong Hu,[2] and Graeme B. Martin[1,6]

[1]UWA Institute of Agriculture, University of Western Australia, Crawley, WA, Australia, [2]Northwest Agriculture and Forestry University, College of Animal Science and Technology, Yangling, China, [3]Department of Primary Industry and Regional Development, Livestock Industries, Agriculture and Food, South Perth, WA, Australia, [4]School of Molecular Sciences, University of Western Australia, Crawley, WA, Australia, [5]Department of Biological Sciences, Ohio University, Athens, OH, and [6]Corresponding author, e-mail:graeme.martin@uwa.edu.au

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Abstract

The Australian blow fly, *Lucilia cuprina* Wiedmann (Diptera: Calliphoridae), is a major cause of myiasis (flystrike) in Merino sheep in Australia and New Zealand and, as a primary colonizer of fresh carrion, also an important species in forensic investigations. Olfaction is considered the most important cue for insects to rapidly locate carrion over long distances, so the first carrion visitors are predicted to be very sensitive to carrion-related volatile compounds. We studied the responses of the Australian blow fly, *Lucilia cuprina*, to the carrion-associated compounds dimethyl trisulfide (DMTS), butyric acid, 1-octen-3-ol and indole. We also tested 2-mercaptoethanol, a compound commonly used in fly traps in Australia. We investigated whether responses of the flies are affected by their ovarian status by comparing responses of gravid and non-gravid *L. cuprina* in electroantennography (EAG) and two-choice laboratory bioassays. All four compounds evoked an EAG response, while only DMTS evoked responses in gas chromatography-mass spectrometry electroantennographic detection (GCMS-EAD) analyses and two-choice bioassays. Gravid flies detected lower doses of the test compounds than non-gravid flies. Our results indicate that DMTS is an important semiochemical for *L. cuprina* to locate carrion resources, and has potential for use in fly traps for flystrike control. Our observations also suggest that the greater sensitivity of gravid *L. cuprina* allows them to find fresh carrion quickly to maximize reproductive success by avoiding unsuitable degraded carrion.

Key words: electroantennography, reproductive state, dimethyl trisulfide, flystrike, blowfly

The blow fly *Lucilia cuprina* Wiedmann (Diptera: Calliphoridae) is a major cause of myiasis (flystrike) in Merino sheep in Australia and New Zealand (Heath and Bishop 2006, Wardhaugh et al. 2007) and causes an economic loss of about US$ 280 million pa in Australia alone (Sackett et al. 2006). It is known that blow fly trapping can be used to reduce fly density and the incidence of strike (Tillyard and Seddon 1933), but success has been limited. Trap efficacy depends on the composition of the attractants used, so veterinary science and livestock industries could benefit from continued research into the olfactory responses of *L. cuprina*.

In addition to being an economically important agricultural pest, *L. cuprina* [as well as its sister species *Lucilia sericata* (Meigen)] are often primary colonizers of carrion (Payne 1965, De Jong and Hoback 2006). Carrion offers a rich supply of nutrients but is ephemeral, so competition for this resource is intense (Janzen 1977, DeVault et al. 2003). For rapid location of carrion over long distances, olfactory compounds are likely to be the most important cues; accordingly, obligate scavengers should be under strong selective pressure to develop keen olfaction (De Bruyne and Baker 2008). Consequently, we would expect the first carrion visitors to be very sensitive to carrion-associated volatile organic compounds (VOCs). VOCs, alone or in combination, have been extensively studied in *L. sericata* and shown to evoke electroantennographic and behavioral responses, with combinations revealing evidence of synergism (Frederickx et al. 2012a, Chaudhury et al. 2015, Brodie et al. 2016, Liu et al. 2016). For *L. cuprina*, on the other hand, most studies investigating the attractiveness of VOCs have been conducted with mixtures of compounds (Emmens and Murray 1983, Morris et al. 1997, Urech et al. 2004, Urech et al. 2009, Scobie and O'Connell 2010, Scott 2014). For example, butyric acid and indole, mixed with

other compounds, are used in non-return insecticide-free traps for *L. cuprina* as a means of prevention of flystrike on sheep (Urech et al. 2004). Several VOCs present in mammalian body odors, such as sulphur-based compounds and 1-octen-3-ol, are also present in decomposing carrion and can attract female *L. cuprina* and elicit neuronal antennal responses from them (Park and Cork 1999). It cannot be determined from these studies whether *L. cuprina* responds to the combination of molecules or to a single compound of the combination. Therefore, as a first step towards a structured series of investigations in female *L. cuprina*, we tested the hypothesis that individual carrion-associated VOCs will evoke responses that can be detected by electroantennography (EAG) or by behavior in laboratory bioassays.

The seeking and exploitation of resources by insects are known to depend on the relationship between their nutritional needs and their physiological status, especially with respect to female reproduction (Stockhoff 1993, Hochuli 2001). In *L. sericata*: 1) non-gravid females respond to both carrion and feces whereas gravid females respond only to carrion (Brodie et al. 2016); 2) gravid females are more attracted to liver odors than non-gravid flies (Ashworth and Wall 1995); and 3) gravid and non-gravid females are attracted by different concentrations of DMDS, phenylacetic acid (PAA) and indole (Liu et al. 2016). There is a very strong likelihood that gravid *Cochliomyia macellaria* and *Chrysomya rufifacies* will be the first to arrive at carcasses but, over the next 2 d, there is a dramatic shift to dominance by non-gravid females (Mohr and Tomberlin 2014). For *L. cuprina*, therefore, we tested whether gravid females are more sensitive to carrion-associated VOCs than non-gravid females.

We tested our hypotheses using four VOCs from the many that could be associated with carrion decomposition (LeBlanc 2008, von Hoermann et al. 2016). Dimethyl trisulfide (DMTS) is from among the many that could be associated with carrion decomposition of cysteine (Jürgens et al. 2013), is present throughout the process of carrion decomposition of piglets (von Hoermann et al. 2016), and attracts necrophagous flies (Wang et al. 2001, Urech et al. 2004, Hu et al. 2010). On the other hand, 1-octen-3-ol appears about 5 d after the death of a piglet (von Hoermann et al. 2016) and is known to attract tsetse flies (Hall et al. 1984) and non-gravid *L. sericata* (Brodie et al. 2016). Butyric acid and indole are linked to the microbial degradation of fat and protein during carrion decomposition (Statheropoulos et al. 2005, Dekeirsschieter et al. 2009, Frederickx et al. 2012b, Chaudhury et al. 2014), and are commonly used in fly traps to reduce flystrike in Australia. We also tested 2-mercaptoethanol because it had been shown to enhance the attractiveness of fleece, and, in a mixture with a combination of indole, pentanoic acid and sodium sulfide, it had been shown to double the number of flies caught, compared to the liver standard (Eisemann 1985, 1995; Urech et al. 2004).

Materials and Methods

Fly Colonies

Flies used for the experiments were reared in the University Field Station (Shenton Park) of The University of Western Australia, after starting a new colony with approximately 100 larvae collected from the breach of fly-struck sheep approximately 3 mo before these experiments commenced. The sheep were hosted on Manurup Research Station (latitude 34° 34′ 39″ S, longitude 117° 31′ 10″ E) near Mount Barker, Western Australia. This area has a Mediterranean environment with an annual rainfall of 650 mm, most of which falls between May and December. The blowflies for the insectary were sourced from the same site. Flies were kept under 12:12 (L:D) h photoperiod at 30–40% relative humidity and 23–25°C.

Clearly, the availability of non-gravid and gravid adult flies is critical for the identification of behavioral differences between the two reproductive conditions. To produce non-gravid flies, ovaries were prevented from developing by providing flies with sugar and water from emergence until they were used in the experiments. To produce gravid flies, full development of the ovaries was ensured by providing water, a mixture of milk powder and sugar (1:1) upon emergence, and liver paste from 4 to 8 d after emergence. However, this approach also raises the possibility of a confounding effect of nutritional history on the behavioral measurements (e.g., Bowdan 1982, Schultzhaus et al. 2017). For this reason, we carried out a pilot experiment to test the effect of nutritional history on attraction behavior in *L. cuprina*: one group was fed with sugar plus water after emergence; the other group was fed with sugar plus milk powder plus water after emergence; both groups were given liver paste when the flies were 4–8 d old. Even after six generations of breeding, nutritional history had no significant effect on the attractiveness of wool or liver.

Ovary status of gravid flies was determined by dissecting 10 randomly selected flies from each feeding cage of about 500 flies—if more than eight were gravid, flies from that cage were used for behavior tests. For the antenna tests, ovary status was determined by dissection of the flies from which the antennae were removed.

Electroantennography

Five chemical compounds were used: DMTS, butyric acid, indole and 1-octen-3-ol, all obtained from Alfa Aesar (Heysham Lancashire, United Kingdom), and 2-mercaptoethanol obtained from Sigma-Aldrich (St. Louis, MO). The compounds were diluted in a series of concentrations (10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1} g/ml) in dichloromethane (DCM), the solvent used in the electrophysiological and behavioral assays, and then applied to the EAG testing system. A piece of filter paper (about 1 × 5 cm) was zig-zag folded and partly inserted into the wide opening of a Pasteur pipette. A 20 µl droplet of each solution (10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} or 10^{-1} g/ml) was applied to each individual filter paper and the DCM was allowed to evaporate completely in a fume hood. In preliminary testing, antennae response for DCM, but 2 min drying was found to be sufficient to eliminate any response in an antenna. The filter paper was then fully inserted into the Pasteur pipette. Each filter paper, therefore, contained 0.02, 0.2, 2, 20, 200 or 2,000 µg of test substance when inserted into the pipette. Except for DMTS, there was no antennal responses at a dose of 0.2 µg. For DMTS, lower doses were tested and a response was no longer detectable at 0.002 µg, so 0.02 µg was chosen as the lowest dose for EAG studies. Both ends of the pipette were sealed with a piece of aluminium foil and were re-sealed for subsequent re-use. After preliminary experiments showed that the pipettes could be re-used at least six times without any change in responses between first and last puff, we used the same pipette for the six replicates, all within 2 h. A Pasteur pipette containing filter paper only was used as control.

Each compound was tested with antennae from six non-gravid and six gravid female flies. The antenna was carefully pulled out from the head of the fly and suspended between two glass capillary electrodes filled with saline solution (Staddon and Everton 1980). The test compound in the pipette was volatilized with a 0.5 s air puff. The control pipette was applied first, followed by the six doses (from lowest to highest) of two compounds, followed by a repeat of the control. The antenna was then replaced, and the two compounds were again tested, but in reverse order, using the same sequence of doses. For each antenna, the operation was completed within 15 min.

Two-Choice Behavioral Bioassays

In the above EAG experiment, the antennae were found to respond to four compounds (DMTS, butyric acid, indole, and 1-octen-3-ol) at specific doses. These four compounds were subsequently used in two-choice behavioral bioassays. The bioassays were carried out in wire mesh cages (45 × 30 × 30 cm) illuminated by fluorescent ceiling lights in a laboratory maintained at 24–26°C. Two plastic funnel bottle traps were placed about 20 cm apart inside each cage (Fig. 1). The traps were made from 0.5-liter transparent plastic bottles (7 cm i.d. × 16 cm height) that were cut 9 cm from the base into two sections, a 9-cm-tall cylinder and a 7-cm-tall funnel. The funnel was inverted and inserted into the cylinder to guide flies into the cylindrical base and discourage them from leaving. To prepare the attractant in the traps, 30 µl of test solution was added dropwise to a piece of filter paper (diameter 3 cm) and allowed to dry for 2 min. The filter paper was placed in the traps. DCM was used as a negative control because, in contrast to the EAG tests, we did not have data from preliminary experiments to verify a lack of effect after 2 min evaporation; pig liver (5 g) on filter paper was used as a positive control.

For each of the 10 replicates, 50 gravid or non-gravid female flies, termed here 'response flies' (RFs), were selected from 200 mixed-sex flies that had been cold-sedated for 5 min and then released into each cage, where they were supplied with sugar and water. A very small number of flies did not recover within 10 min, and were replaced. After overnight adaptation, two traps were placed inside the cage, one for the treatment and one as a negative control. Control and treatment traps were alternated in adjacent cages. The values for the behavioral response of the RFs increase with time, as does the amount of compound volatilized, so the flies in each trap were counted after precisely 1 h. Thereafter, all trapped flies were removed and released into the cage, the treatment trap was replaced with the positive control trap (pig liver) and the RFs were allowed another hour to enter the traps. After each test, the cages were soaked overnight in an aqueous detergent solution and then washed with hot water.

For each compound, the higher doses (300 µg and 3,000 µg) were tested first with gravid flies. These doses were selected on the basis of the outcomes of the above EAG tests, and on findings from studies with *L. sericata* (Brodie et al. 2014). For these two doses, if attractiveness did not differ significantly from the negative control, the compound was not subjected to further testing. If the high doses were attractive, lower doses were tested until there was no significant difference in the negative control.

Gas Chromatography-Mass Spectrometry Electroantennographic Detection

As only DMTS and butyric acid induced responses in both the EAG and behavior bioassays, they were the only two compounds subjected to gas chromatography-mass spectrometry electroantennographic detection (GCMS-EAD) experiments. We tested the ability of this method to detect biologically active compounds at lower concentrations and determined the detection limit by injecting pure compounds into the GC at 20 ng and in 10-fold dilutions until the antenna did not respond.

GCMS-EAD consisted of an HP 5890 GC connected to a 5972 mass selective detector (MSD) equipped with an HP5ms column (30 m × 0.25 mm × 0.25 µm), using helium as the carrier gas at 2 ml/min. The capillary column was split with a Y-glass splitter between the MSD and the EAD as described earlier (Bohman and Peakall 2014, Xu et al. 2017). The gas for EAD was passed through a Syntech effluent conditioner (Syntech, Kirchzarten, Germany) containing a heated (250°C) transfer line, with the outlet placed in a purified and humidified air stream, where the electrodes holding the antenna were positioned.

Two microlitres of a solution containing 20 ng butyric acid and 20 ng DMTS was injected (splitless mode, 1 min) and detected simultaneously with the MSD and EAD. The injector and transfer line temperatures were set at 250°C; the oven temperature was set initially at 40°C and then increased to 160°C at a rate of 15°/min with no holds.

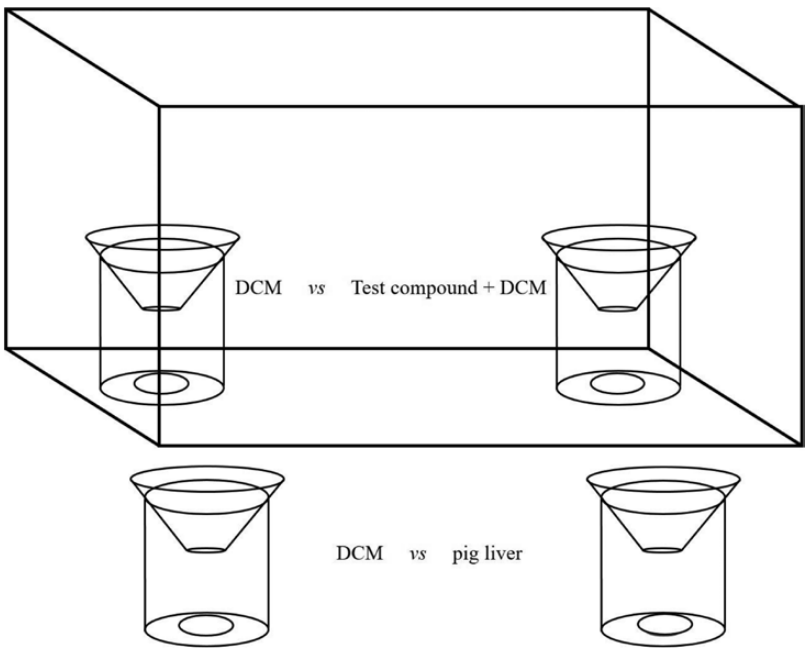

Fig. 1. Design of traps for the two-choice behavioral bioassay. The negative control trap (DCM only) is first compared with a test compound dissolved in DCM. Subsequently, a second negative control (DCM only) is compared with a positive control (pig liver).

Statistical Analyses

As the same antenna was used to examine responses to both the test compound and the control compound (DCM), data were analyzed using paired *t*-test with six replicates. EAG responses were presented as the difference between the treatment and control values, as reported from the Syntech software. To compare these differences in gravid and non-gravid flies for a given compound at all six doses, parametric one-way analyses of variance (ANOVA) were used with dose as a factor.

The difference in the behavioral response of *L. cuprina* to each compound at a given dose was examined using *t*-tests for paired samples with 10 replicates (i.e., cages), as the treatment trap and the negative control trap were inside the cage simultaneously. The differences in behavioral response of *L. cuprina* to negative and positive controls were analyzed by paired *t*-tests. To examine the differences between gravid and non-gravid flies in behavior responses to a compound at each tested dose, the relative behavior response was calculated as the difference between the treatment (%) and the negative control (%). The relative responses were used in a two-way ANOVA, where gravid status and chemical dose were two fixed factors. The interaction between two factors was also included in the model.

Results

EAG

Table 1 shows the responses of the antennae of gravid and non-gravid flies to the control and six doses of test compounds, and the results of paired-sample *t*-tests comparing the control with each dose. For gravid flies, the lowest dose that induced a response was 0.02 µg for DMTS (t = 5.94; df = 5; P = 0.002), 20 µg for butyric acid (t = 3.01; df = 5; P = 0.03), and 200 µg for indole (t = 3.78; df = 5; P = 0.013) and 1-octen-3-ol (t = 4.13; df = 5; P = 0.009). For non-gravid flies, the lowest dose that induced a response was 2 µg for DMTS (t = 3.70; df = 5; P = 0.014), 200 µg for butyric acid (t = 2.88; df = 5; P = 0.035) and 2,000 µg for indole (t = 3.08; df = 5; P = 0.028) and 1-octen-3-ol (t = 5.36; df = 5; P = 0.003). Clearly, the antennae of gravid flies were more sensitive to the test compounds than the antennae of non-gravid flies.

Table 2 shows the differences between control and test compound in EAG response, to the six doses of the test compounds, for gravid and non-gravid flies, and the outcome of the one-way ANOVA. For gravid flies, there were significant dose-responses for DMTS (F = 14.6; df = 5, 30; P < 0.001), butyric acid (F = 22.0; df = 5, 30; P < 0.001), indole (F = 89; df = 5, 30; P < 0.001) and 1-octene-3-ol (F = 67.2; df = 5, 30; P < 0.001). For non-gravid flies, on the other hand, significant differences among doses were only observed for butyric acid (F = 10.6; df = 5, 30; P < 0.001) and 1-octen-3-ol (F = 24.7; df = 5, 30; P < 0.001).

Two-Choice Behavioral Bioassays

Figure 2 shows the proportion of gravid and non-gravid *L. cuprina* that were caught in the negative control traps and the treatment traps for four compounds at various doses. Compared to the negative control traps, DMTS attracted more gravid flies (Fig. 2A) at 0.3 µg (t = 3.35; df = 9; P = 0.008), 3 µg (t = 9.59; df = 9; P < 0.001), 30 µg (t = 7.04; df = 9; P < 0.001), 300 µg (t = 5.48; df = 9; P < 0.001) and 3,000 µg (t = 9.37; df = 9; P < 0.001); more non-gravid flies (Fig. 2B) were attracted at 30 µg (t = 8.41; df = 9; P < 0.001), 300 µg (t = 9.00; df = 9; P < 0.001) and 3,000 µg (t = 7.10; df = 9; P < 0.001). Butyric acid attracted more gravid flies (Fig. 2C) at doses of 30 µg (t = 8.57; df = 9; P < 0.001), 300 µg (t = 7.27; df = 9; P < 0.001) and 3,000 µg (t = 7.67; df = 9; P < 0.001), and more non-gravid flies (Fig. 2D) at doses of 30 µg (t = 9.15; df = 9; P < 0.001) and 300 µg (t = 6.60; df = 9; P < 0.001) when compared to the negative control. For indole (Fig. 2E) and 1-octen-3-ol (Fig. 2F), no significant difference was found between the negative control trap and the traps with doses of 300 and 3,000 µg.

Table 3 shows the relative behavior response of gravid and non-gravid *L. cuprina* to a series of doses of butyric acid and DMTS. There was no significant effect of the interaction between physiological stage and DMTS dose on the behavior response (F = 1.20; df = 3, 90; P = 0.315; Table 3). However, for butyric acid, the interaction was significant (F = 3.29; df = 3, 72; P = 0.025).

The positive control (pig liver) traps had 30.3% (SEM = 0.54) of gravid flies inside the trap, compared with 9.4% (SEM = 0.37) in the negative control traps (t = 33.92; df = 139; P < 0.001). With non-gravid flies, 30.5% (SEM = 0.71) of flies were caught in the positive control traps while only 7.8% (SEM = 0.31) were found in the negative control traps (t = 29.45; df = 79; P < 0.01).

Gas Chromatography-Mass Spectrometry Electroantennographic Detection

As only DMTS and butyric acid induced responses in both the EAG and behavior bioassays, only these two compounds were subjected to GCMS-EAD. Figure 3 shows that DMTS consistently elicited responses from the antennae of both gravid and non-gravid flies at doses of 20 and 2 ng. At 0.2 ng no peak could be detected in the TIC, but all antenna still responded. The antenna of gravid flies

Table 1. The EAG responses (mV) of the antennae of gravid and non-gravid flies to increasing doses of carrion-associated test compounds, as reported by Syntech software

		0 (control)	Dose (µg)					
			0.02	0.2	2	20	200	2,000
DMTS	Gravid	−0.47 ± 0.075	−0.70 ± 0.086†	−0.71 ± 0.087†	−0.74 ± 0.104†	−0.69 ± 0.086†	−0.86 ± 0.111†	−1.25 ± 0.120†
	Non-gravid	−0.69 ± 0.177	−0.77 ± 0.205	−0.79 ± 0.213	−0.89 ± 0.228*	−0.92 ± 0.2197*	−0.87 ± 0.223*	−0.94 ± 0.249*
Butyric Acid	Gravid	−0.46 ± 0.077	−0.57 ± 0.130	−0.53 ± 0.092	−0.55 ± 0.081	−0.60 ± 0.074*	−0.91 ± 0.144†	−1.48 ± 0.177‡
	Non-gravid	−0.79 ± 0.177	−0.85 ± 0.225	−0.94 ± 0.230	−0.80 ± 0.162	−0.87 ± 0.183	−0.94 ± 0.203*	−1.42 ± 0.250†
Indole	Gravid	−0.35 ± 0.056	−0.40 ± 0.067	−0.37 ± 0.064	−0.38 ± 0.077	−0.36 ± 0.058	−0.62 ± 0.098*	−0.84 ± 0.157*
	Non-gravid	−0.35 ± 0.050	−0.31 ± 0.035	−0.36 ± 0.062	−0.32 ± 0.098	−0.37 ± 0.068	−0.41 ± 0.079	−0.50 ± 0.095*
1-octen-3-ol	Gravid	−0.37 ± 0.062	−0.40 ± 0.073	−0.39 ± 0.074	−0.41 ± 0.079	−0.48 ± 0.116	−0.61 ± 0.113†	−1.87 ± 0.209‡
	Non-gravid	−0.20 ± 0.034	−0.25 ± 0.039	−0.23 ± 0.027	−0.24 ± 0.015	−0.23 ± 0.054	−0.28 ± 0.071	−1.31 ± 0.223†
2-mercaptoethanol	Gravid	−0.50 ± 0.068	−0.47 ± 0.090	−0.41 ± 0.063	−0.46 ± 0.083	−0.51 ± 0.121	−0.52 ± 0.089	−0.51 ± 0.105
	Non-gravid	−0.33 ± 0.069	−0.33 ± 0.065	−0.36 ± 0.084	−0.35 ± 0.072	−0.34 ± 0.066	−0.36 ± 0.068	−0.34 ± 0.056

Significant difference between the compound and the control: *P < 0.05, †P < 0.01, and ‡P < 0.001. Values are mean ± SE (n = 6).

Table 2. The EAG responses of the antennae of gravid and non-gravid flies to five compounds over a range of doses, presented as differences in response (mV) between the treatment and control, as reported by Syntech software

	Physiological stage	Dose (μg)						*df*	*F*	*P*
		0.02	0.2	2	20	200	2,000			
DMTS	Gravid	0.23 ± 0.047	0.24 ± 0.030	0.26 ± 0.041	0.22 ± 0.030	0.38 ± 0.054	0.78 ± 0.104	5, 30	14.6	<0.001
	Non-gravid	0.08 ± 0.53	0.10 ± 0.065	0.20 ± 0.055	0.18 ± 0.047	0.18 ± 0.051	0.25 ± 0.085	5, 30	1.1	0.377
Butyric acid	Gravid	0.12 ± 0.085	0.07 ± 0.043	0.09 ± 0.015	0.15 ± 0.049	0.45 ± 0.092	1.02 ± 0.127	5, 30	22.0	<0.001
	Non-gravid	0.06 ± 0.063	0.08 ± 0.049	0.02 ± 0.048	0.09 ± 0.041	0.15 ± 0.052	0.63 ± 0.101	5, 30	10.6	<0.001
Indole	Gravid	0.04 ± 0.039	0.02 ± 0.051	0.02 ± 0.026	0.01 ± 0.019[c]	0.26 ± 0.069	0.48 ± 0.124	5, 30	8.9	<0.001
	Non-gravid	−0.04 ± 0.037	0.01 ± 0.019	−0.03 ± 0.086	0.02 ± 0.042	0.05 ± 0.039	0.14 ± 0.046	5, 30	1.8	0.134
1-octen-3-ol	Gravid	0.03 ± 0.014	0.02 ± 0.013	0.04 ± 0.017	0.11 ± 0.055	0.24 ± 0.057	1.38 ± 0.153	5, 30	67.2	<0.001
	Non-gravid	0.04 ± 0.017	0.03 ± 0.013	0.03 ± 0.028	0.02 ± 0.025	0.07 ± 0.041	1.10 ± 0.206	5, 30	24.7	<0.001
2-mercaptoethanol	Gravid	−0.02 ± 0.053	−0.09 ± 0.021	−0.04 ± 0.058	0.01 ± 0.073	0.02 ± 0.023	0.01 ± 0.043	5, 30	0.7	0.625
	Non-gravid	−0.01 ± 0.016	−0.02 ± 0.030	−0.01 ± 0.024	−0.01 ± 0.017	−0.03 ± 0.022	−0.01 ± 0.039	5, 30	0.2	0.972

Values are mean ± SE (n = 6 for each physiological stage at each dose).

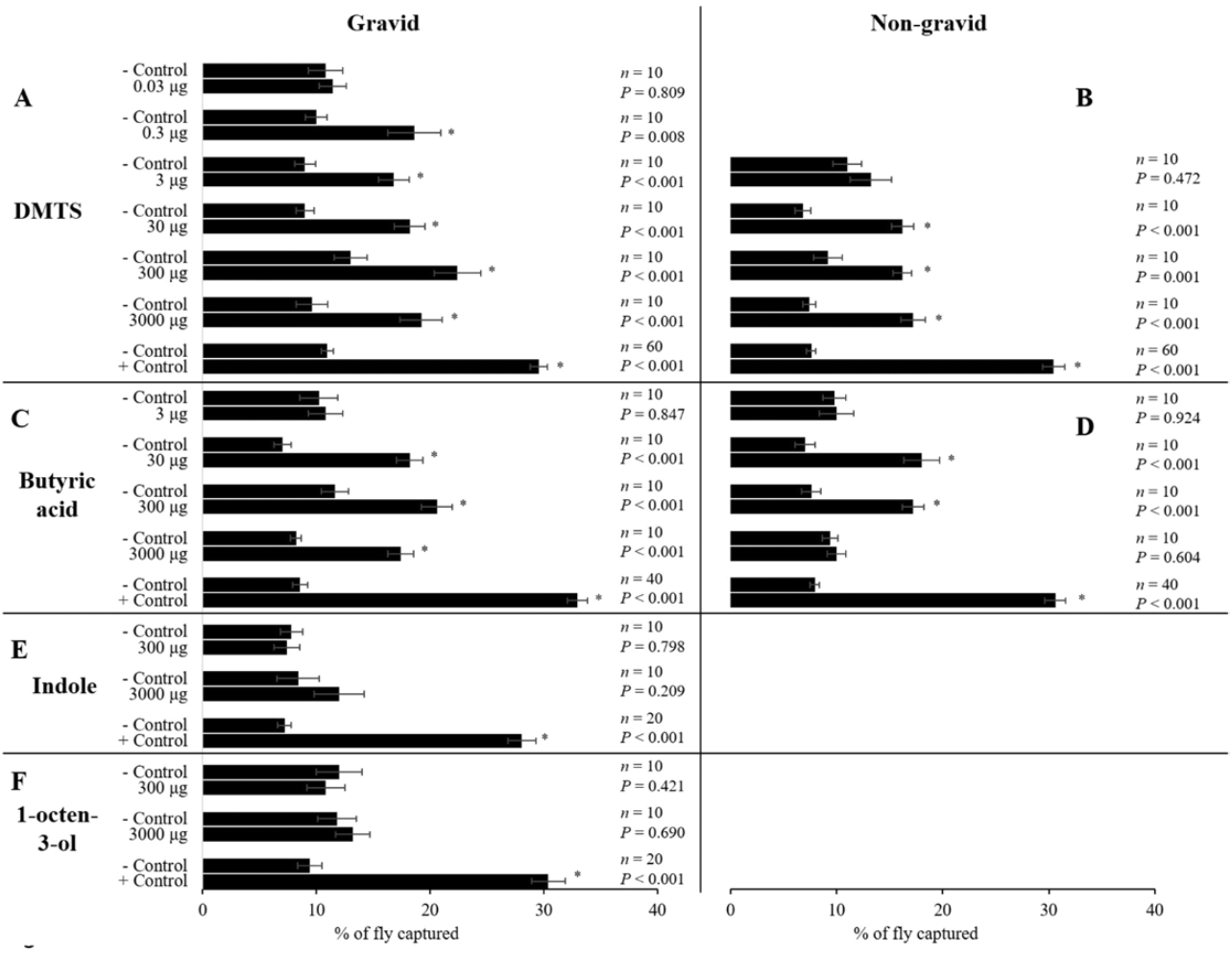

Fig. 2. Percentage of *Lucilia cuprina* captured in two-choice behavior bioassays (each 1 h duration) employing two upside down bottle traps (Fig. 1) baited with a single dose (0.03–3,000 μg) of one of carrion-associated compounds. DCM served as a negative control and pig liver as a positive control. In each experiment, an asterisk (*) denotes a treatment stimulus that captured significantly more flies ($P < 0.05$).

alone responded at the lowest dose (0.02 ng). Further, it is clear that butyric acid is not suitable for GCMS-EAD with this column and instrument configuration. This carboxylic acid strongly interacts with the stationary phase of the column leading to broad peaks, low detection limit, and absence of antennal responses.

Discussion

Two of the carrion-associated VOCs, DMTS and butyric acid, evoked responses that could be detected by both EAG and behavior bioassay in *L. cuprina*, whereas the other two VOCs, 1-octen-3-ol

Table 3. The relative behavior response of gravid and non-gravid flies to butyric acid and DMTS over a range of doses

		Dose (μg)						Factor	*df*	*F*	*P*
		0.03	0.3	3	30	300	3,000				
DMTS	Gravid	0.6 ± 2.40	8.6 ± 2.57	7.8 ± 0.81	9.2 ± 1.31	9.4 ± 1.71	9.6 ± 1.02	Dose	5, 90	5.48	<0.001
	Non-gravid			2.2 ± 2.93	9.4 ± 1.12	7.0 ± 1.44	9.8 ± 1.38	Physiology	1, 90	1.85	0.177
								Dose × physiology	3, 90	1.20	0.315
								Dose	3, 72	16.2	<0.001
Butyric acid	Gravid			0.6 ± 3.01	11.2 ± 1.31	9.0 ± 1.24	9.2 ± 1.20	Physiology	1, 72	3.26	0.075
	Non-gravid			0.2 ± 2.03	11.0 ± 1.20	9.6 ± 1.45	0.6 ± 1.12	Dose × physiology	3, 72	3.29	0.025

Values are mean ± SE (n = 10 for each physiological stage at each dose).

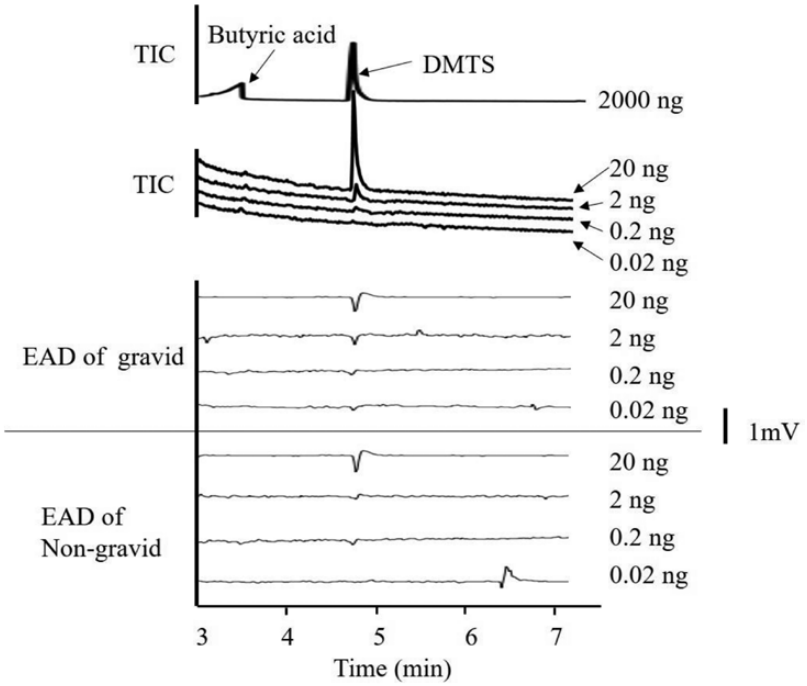

Fig. 3. Combined GCMS-EAD signals of antennae from gravid and non-gravid Lucilia cuprina showing the responses to butyric acid and DMTS. The total ion chromatogram (TIC) for the range 20 to 0.2 ng butyric acid and DMTS are shown in the top traces and the antennal responses of gravid and non-gravid flies are shown in the bottom traces. The TIC (2,000 ng) trace shows the retention time of butyric acid and DMTS.

and indole, only evoked EAG responses. Sulfur-containing VOCs, such as DMTS, are produced by fresh carrion and attract carrion flies that prefer to oviposit on fresh carrion (Archer and Elgar 2003, Kalinova et al. 2009, Frederickx et al. 2012b, Zito et al. 2014, von Hoermann et al. 2016). DMTS evoked an EAG response, as well as responses in laboratory bioassays, suggesting that this compound is important for attracting *L. cuprina* to carrion in the early stages of decomposition. As little as 0.02 ng of DMTS can be detected by the antenna of gravid *L. cuprina*, indicating that there is, in fact, a strong selection pressure on necrophagous insects to fine-tune their olfactory system for the detection of the early stages of carrion decomposition. Interestingly, our findings are also consistent with the role of oligosulfides in floral oviposition site mimics (Jürgens et al. 2013).

It is also clear that gravid females are more sensitive to carrion-associated VOCs than non-gravid females. Only small amounts of DMTS are produced in the early stage of carrion decomposition, and emissions increase as decomposition progresses before declining in the final stages (von Hoermann et al. 2016). Therefore, greater sensitivity to DMTS would help gravid *L. cuprina* to find the carrion rapidly, reducing competition and maximizing opportunities for the growth and survival of their offspring (Archer and Elgar 2003).

By contrast, indole and 1-octen-3-ol, which appear in later stages of carrion decomposition, are known to attract non-gravid *L. sericata* to canine feces whereas, for gravid *L. sericata*, indole helps avoid decomposed carrion (Forbes and Perrault 2014, Brodie et al. 2016, von Hoermann et al. 2016). In *L. cuprina*, the antennae of gravid flies were more sensitive to 1-octen-3-ol and indole than the antennae of non-gravid flies, but our behavioral tests showed that neither of these two carrion-associated VOCs were attractive. These observations are consistent with the view that, just because an insect has a receptor, a specific behavior (e.g., attraction) need not be elicited. This dissociation in between receptor presence and insect behavior could be due to insects having a receptor for the compound but being repelled by it; insects only responding to a specific concentration of the compound; insects being attracted or repelled by combinations of compounds; insects retaining the receptor but having a degenerated behavior response. Alternatively, the function of the receptor might not be known. Compounds which have receptors

but do not attract insects might play a supporting role during the location of resources, or have other roles.

In *L. cuprina*, the responses in EAG and behavioral bioassays were not always linked, supporting observations in studies of other species. For example, in *Musca domestica*, DMTS evokes an EAG response but not a behavioral response (Zito et al. 2014). Both *Tabanus bromius* and *Atylotus quadrifarius* display EAG responses that differ in intensity between 1-octen-3-ol and phenols, but the responses in trap experiments are similar for the two compounds (Baldacchino et al. 2014). This sort of inconsistency between EAG and behavioral outcomes to a single compound has also been demonstrated for flower-visiting insects and floral scents (Jhumur et al. 2007, Xu et al. 2015, Bohman et al. 2016, Bohman et al. 2017). It is important to note that electroantennographic methods only show compounds which can be detected by the insect's antenna, not the function of the compound. That said, in our study, a wide range of doses of DMTS evoked stable EAG and GCMS-EAD responses in *L. cuprina*, suggesting DMTS to be a practical positive control in future EAG or GCMS-EAD analyses with this species. GCMS-EAD could be useful for detecting bioactive compounds in these insects, as the detection limit is much lower than EAG with 'puffing', due to the lower background noise. This increase in sensitivity is twofold: 1) there is no simultaneous mechanical stimulation, and 2) the compounds are injected in solution with the full amount reaching the antenna, rather than in EAG where only the headspace volatiles are delivered to the antenna. By using GCMS-EAD to obtain retention indices of bioactive compounds, the relevant candidate compounds may be identified by GC-MS, or targeted for collection by preparative gas chromatography for further characterization and testing.

Fly traps that contain an aqueous solution of sodium sulfide, butyric acid, 2-mercaptoethanol and indole are selective for *L. cuprina* and are used to reduce populations and thus prevent flystrike (Urech et al. 2009). The inclusion of butyric acid in the mixture significantly increases the attractiveness to *L. cuprina* in laboratory assays (Eisemann 1985). In addition to butyric acid, DMTS attracts gravid *L. cuprina*, as shown in the present study, suggesting that the addition of DMTS will improve the performance of fly traps.

Overall, we have demonstrated that *L. cuprina* is attracted to carrion-associated VOCs, with gravid flies able to detect lower doses than non-gravid flies. The results of our study might be directly applied to flystrike management by modification of population-control traps. Furthermore, we have established reliable EAG and behavioral assays using *L. cuprina* that will assist future studies on the identification of attractive odorous compounds produced by Merino sheep, potentially uncovering the underlying cause of sheep myiasis.

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