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Research

Characterization of the Volatiles' Profiles of the Eggs of Forensically Relevant *Lucilia sericata* and *Phormia regina* (Diptera: Calliphoridae) Blow Flies by SPME-Facilitated GC-MS

Justine E. Giffen-Lemieux,[1] Koji Okuda,[2] Jennifer Y. Rosati,[3] and Rabi A. Musah[1,4]

[1]Department of Chemistry, University at Albany, State University of New York, 1400 Washington Avenue, Albany, NY 12222, [2]JEOL Inc., 11 Dearborn Road, Peabody, MA 01960, [3]John Jay College of Criminal Justice, 524 West 59th Street, New York, 10019, and [4]Corresponding author, e-mail: rmusah@albany.edu

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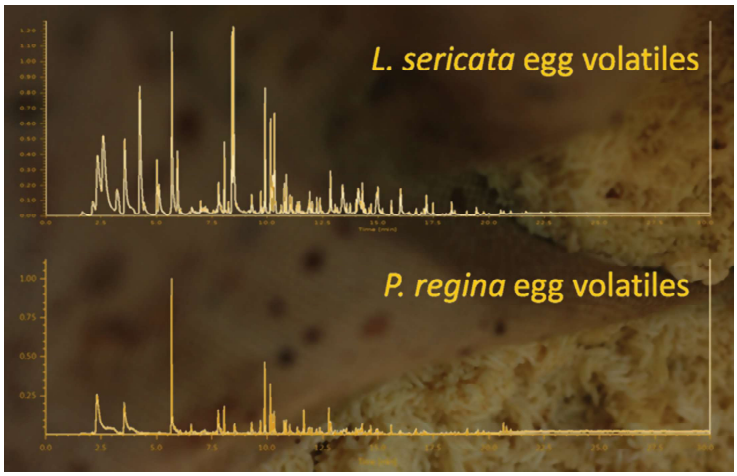
Abstract

The attraction of necrophagous insects, particularly blow flies, to corpses and carrion is of ecological, economic, and agricultural importance, although the mechanisms by which it occurs are not well understood. Much of the published research on blow fly attractants has focused on volatiles emitted from carrion surrogates, but little attention has been given to the possibility that blow fly eggs themselves may emit chemical cues that are responsible for conspecific and heterospecific insect attraction. In this study, the headspace volatiles emitted from eggs representing two aggregated oviposition events that were collected 1 mo apart from two species of the *Calliphoridae* family (Order: Diptera), *Lucilia sericata* (Meigen), and *Phormia regina* (Meigen) were analyzed via solid-phase microextraction-facilitated GC-MS. The volatiles' profiles were found to be consistent between samples representing the same species, but unique between the two species. Over 100 molecules covering a wide range of compound classes that included alcohols, aldehydes, esters, amines, ketones, and organosulfur compounds were identified. The profile of volatiles emitted from the *L. sericata* eggs contained several alkanes and aldehydes, whereas salient features of the *P. regina* headspace included numerous esters and ketones. Between the two species, 42 compounds were shared, several of which were carboxylic acids. Little overlap between the range of compounds detected and those reported to be emitted from decomposing remains was observed.

Graphical Abstract



Key words: necrophagous insect eggs, headspace volatiles, GC-MS, Calliphoridae

Necrophagous insects of the *Calliphoridae* family (Order: Diptera), also known as blow flies, are of immense agricultural, ecological and medical importance. While their larvae fulfill an essential scavenger role by feeding on decomposing remains, thus returning natural resources back to the ecosystem (Greenberg 1991), the larvae are also responsible for economic losses on the order of $20 million annually through myiasis in livestock (Novy 1991). In a forensic context, blow flies are used for the estimation of time since death (i.e., postmortem interval, or PMI), and in medicine, for wound debridement (Benecke 2001, Abela 2017). They are also important pollinators for carrion flowers, which emit the characteristic scent of decaying flesh or feces in order to entice the insects (Hepburn and Nolte 1943, van der Niet et al. 2011). Attraction of blow flies to remains is believed to occur via visual and chemical cues, which signal the presence of a food source for larvae, or a mating or oviposition medium (Aluja et al. 2001, Wall and Fisher 2001, Johansen et al. 2014). The chemical cues in particular serve to communicate information that has a profound influence on fly behavior (i.e., they are semiochemicals). The response of insects to this chemically based language has evolved such that different insects respond to the ever-changing profile of molecules that are characteristic of the different stages of decomposition of decaying flesh (Reed 1958, Matuszewski et al. 2010a). Thus, while the period immediately following death is characterized by colonization by blow flies, later stages of decomposition (such as skeletonization) are known to attract primarily beetles (Order: Coleoptera), and there is a range of insects representative of various other families that appear in between (Reed 1958, Prado e Castro et al. 2013). The highly predictable waves of insect species that appear as a function of the evolving stages of decomposition is known as succession. Since succession is more or less directly correlated with the amount of time that has elapsed since death, it is this phenomenon that facilities PMI determination based on the species of insects found on remains (Tabor et al. 2004, Matuszewski et al. 2010b). However, while succession is a well-known phenomenon, the interplay between individual semiochemicals and/or complex mixtures of them on fly behavior is not well understood. This is due in part to the paucity of information on the identities of the specific molecules involved.

Studies aimed at discovery and characterization of blow fly-specific chemical cues are complicated by a number of factors. First, decomposing remains are known to produce thousands of chemicals, and discovering which compound or subset of compounds is responsible for communicating a given piece of information that influences fly behavior can be tantamount to searching for a needle in the proverbial haystack. Second are the limitations in the technological approaches that are available for real-time determinations of the spatio-temporal relationships that govern the emissions of chemical cues on the one hand, and fly attraction on the other (Caraballo 2014, Rosier et al. 2016). Third, once the structures of emitted volatiles have been determined, identification of semiochemicals requires that the effect on fly behavior of each of the molecular components, as well as combinations of them, be tested (Ashworth and Wall 1994, Frederickx et al. 2012a, Chaudhury et al. 2015). Even if single compounds and up to a combination of only five of them are tested using a single species, 64 fly behavior experiments would be required for just one replicate, which is time- and human resource-intensive, and cost-prohibitive. Fourth, while it is established that decomposing remains produce attraction cues, there is significant evidence that colonizing insects themselves may also produce semiochemicals that, in turn, attract other insects, which adds another dimension to the chemical profiling experiments (Frederickx et al. 2012b, Brodie et al. 2015).

After identification of emitted volatiles has been achieved, olfactory and behavioral studies can then be performed using an electroantennogram or flight tube, respectively, to assess the level of attraction that the insect has for a given compound or cocktail of compounds (Wolbarsht and Hanson 1965, Johansen et al. 2014). These experiments are sometimes performed in the presence of a medium that will elicit the appropriate tactile response, and/or visual cues that have been shown to be important for insect oviposition site selection (Hobson 1935, Harris and Rose 1990, Ashworth and Wall 1994). Regarding chemical cues, there has been an emphasis in the literature on identification of, and behavioral assays associated with pheromones and semiochemicals emitted by adult and larval life stages (Barton Browne et al. 1969, Chaudhury et al. 2014). Little attention has been given to the possibility that eggs (the most immature of the life stages) may themselves release chemicals that have biological importance. Predation by wasps and beetles of blow fly eggs is a common occurrence, as these parasitoids feed on the eggs or lay eggs of their own within the egg mass so that the developing offspring can feed on the host eggs and larvae (Nuorteva 1970, Welch 1993, Joseph et al. 2011). This behavior implies that there may be chemical signals that attract the predators to the eggs. Another case in point is the observation that oviposition by conspecific females has been found to elicit oviposition by heterospecific females within the same egg mass that is produced by the conspecific females (Anderson 2001). This egg aggregation is believed to occur because of the numerous benefits that accrue from simultaneous development including increased moisture and warmth; protection from predators; and faster larval development that is a result of the sharing of digestive fluids (Brodie et al. 2015). However, the common occurrence of heterospecific egg aggregation that appears to be initiated by conspecific oviposition implies the existence of a mechanism by which the presence of the eggs is communicated to other flies, whether they be predators or other gravid females. While it has been proposed that this phenomenon occurs through the action of one or more pheromones secreted by adult females (Barton Browne et al. 1969, Brodie et al. 2015), this hypothesis remains to be proven. Another proposal is that volatiles emitted by the eggs themselves may serve as attraction cues. In support of this premise, it has been reported that when the volatiles emitted from freshly laid eggs of the blackfly, *Simulium damnosum* Theobald (Diptera: *Simuliidae*), were offered to conspecific gravid flies, high levels of attraction and increased oviposition rates were observed (McCall 1995, McCall and Cameron 1995, McCall et al. 1997, McGaha et al. 2015). The volatiles were analyzed by GC-MS, and while two distinctive peaks were observed consistently, their identities have yet to be determined (McCall et al. 1997). Thus, there are a number of fly behaviors the implications of which support the premise that blow fly eggs emit semiochemicals (Omar 1995, Anderson 2000, Jiang et al. 2002).

The detection and recognition abilities of insects regarding chemical cues emitted from other insects have also been examined from an oviposition site selection perspective. For example, gravid *Sphaerophoria rueppellii* Wiedemann (Diptera: *Syrphidae*), or hoverflies, were placed in an olfactometer and given a two-choice test where they were presented with plants (their natural oviposition site) on which were placed: conspecific eggs or larvae; heterospecific eggs or larvae; or no hoverfly eggs or larvae, to serve as a control (Amorós-Jiménez et al. 2015). The plants were placed within the arms of an olfactometer and the behavior of the adult hoverflies was monitored to determine preferences. It was found that adult females strongly preferred the plants that did not contain juvenile life stages of their same species, and it was theorized that this was most likely because laying on these oviposition sites would lead to competition

for resources between offspring. It was also observed that there was no significant change in oviposition site selection when heterospecific larvae were present. These findings imply that discrimination by insects between members of their own and other species is based on variations in the emitted chemical profiles that the females are able to detect.

Important to the goal of discovering and identifying semiochemical volatiles is the development of effective analysis methods. In this regard, Frederickx et al. (2012b) developed an approach for the determination of the compounds emitted by *Calliphora vicina* Robineau-Desvoidy larvae and pupae. This was accomplished through the use of solid phase microextraction (SPME) and GC-MS. They also began cataloging the volatiles that were released by larvae in order to determine which compounds were insect versus decomposition derived, as this difference has often been overlooked in previous reports (Statheropoulos et al. 2007, Vass et al. 2008).

Utilizing a similar SPME- and GC-MS-facilitated approach, characterization of the volatiles associated with blow fly eggs from the *Calliphoridae* family, namely *Lucilia sericata* (Meigen) and *Phormia regina* (Meigen), is reported for the first time. While profiles between batches of egg samples from the same species but laid 1 mo apart were similar, each species exhibited a unique headspace signature. Compounds from the alcohol, aldehyde, alkane, carboxylic acid, and ester classes were observed for both. Only a small subset of these compounds was shared between species.

Materials and Methods

Egg Collection

Original core blow fly colonies were begun in the entomology laboratory of Dr. Jennifer Rosati (John Jay College of Criminal Justice,

Fig. 1. A *P. regina* fly and an egg mound produced by multiple flies. (A) A gravid blow fly with eggs in her abdomen; (B) an egg mass containing several hundred eggs laid by 15–20 *L. sericata* flies on the floor of the cage; and (C) eggs stored in an airtight glass vial just prior to concentration of headspace volatiles.

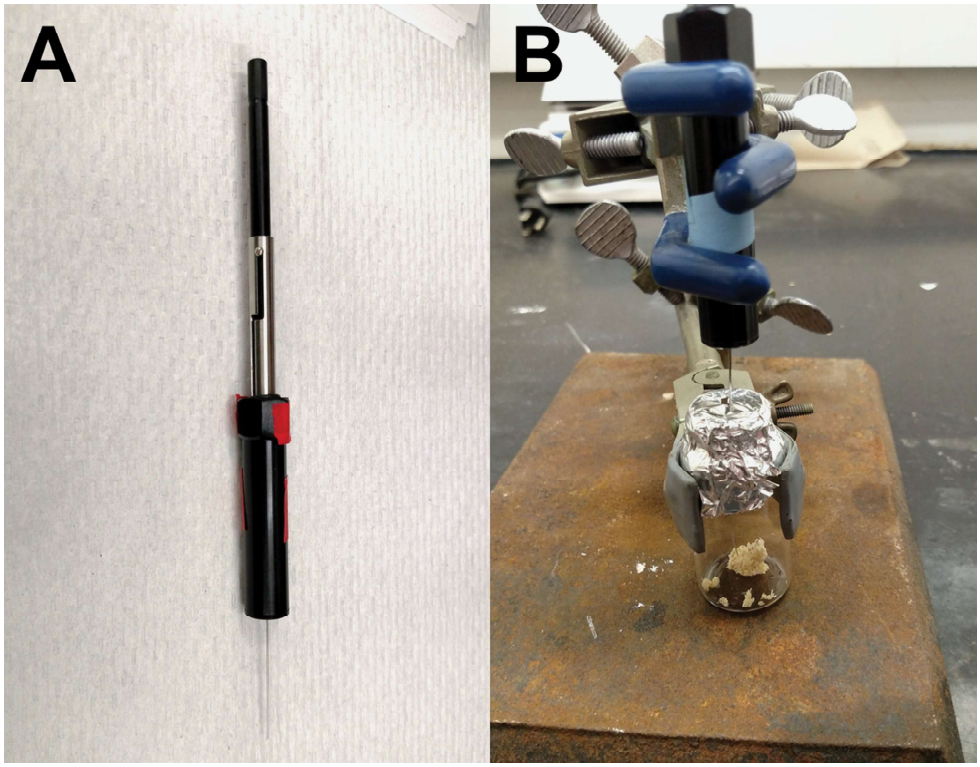


Fig. 2. Headspace sampling of blow fly egg volatiles. (A) SPME fiber holder; (B) glass vial containing ~100 eggs of a single species, covered tightly with aluminum foil, into which was inserted a conditioned DVB/CAR/PDMS SPME fiber through a puncture in the foil. After a 30-min exposure, the fiber was subjected to GC-MS analysis.

Table 1. Compounds detected by GC-MS analysis of the headspace of two subsamples of *Lucilia sericata* eggs that were laid one month apart, using a HP-FFAP column*

Peak no.	Ret. time	Compound	Subsample 1	Subsample 2
1	1.66	Hexane	X	X
2	7.20	Tridecane	X	X
3	8.46	2-Nonanone	X	X
4	8.51	Nonanal	X	X
5	9.12	1-Phenyl-1-butene	X	X
6	10.31	Benzaldehyde	X	X
7	10.37	Propanoic acid	X	X
8	12.37	2-Thiophenecarboxaldehyde	X	X
9	12.84	Methoxy-phenyl-oxime	X	X
10	14.28	Benzyl alcohol	X	X
11	14.99	2-Thiophenemethanol	X	X
12	16.71	9-Tetradecen-1-ol acetate	X	X
13	23.61	Hexadecanoic acid	X	X
α	3.21	Triethylamine	X	
β	4.24	Dimethyl disulfide	X	
γ	5.01	1-Undecene	X	
δ	5.94	2-Methyl-1-butanol	X	
ϵ	8.40	Dimethyl trisulfide	X	
ζ	9.81	2-Decanone	X	
η	10.62	3-Methyl-1*H*-pyrrole	X	
θ	12.49	4-Ethylbenzaldehyde	X	
ι	1.76	Heptane		X
κ	2.04	Octane		X
λ	4.86	3-Methyl-1-butanol acetate		X
μ	5.94	2-Methyl-1-butanol/1-Pentanol**		X
ν	6.32	Ocimene		X
ξ	11.66	4-Hydroxybutanoic acid		X
ο	13.86	Hexanoic acid		X

*Compounds that were detected in one but not both subsamples are labeled with Greek letters.

**Both 2-methyl-1-butanol and 1-pentanol are believed to be present with a shared retention time.

New York). Adult flies were kept in 45 × 45 × 45-cm steel and mesh cages at 21°C with 50% humidity and a 12L: 12D diel cycle (Rosati 2014). Adult flies were given sugar and milk powder ad libitum in 100-mm plastic Petri dishes (Fisher Scientific, Pittsburgh, PA) and had access to water in a 500-ml Erlenmeyer flask plugged with paper towels to prevent drowning. *Lucilia sericata* colonies were maintained since 2014 and *P. regina* colonies were originally obtained from Dr. Christine Picard at Indiana University–Purdue University (Indianapolis) and subsequently established at John Jay College in 2015. All colonies were augmented annually (until 2017) with wild-type females collected from the Manhattan, New York area with traps baited with pork liver. Fresh pork liver was placed in each colony cage for egg collection. After egg deposition, liver was removed and eggs were placed on fresh liver that was placed on a 15 × 15-cm piece of moist paper towel. The paper towel, liver and eggs were then placed in 1-liter Bernardin mason jars filled with 3 cm of kiln dry pine shavings (Lanjay Inc., Quebec, Canada) to absorb excess moisture and to provide a pupation medium. Jars were secured with a metal ring lid with black landscape tarp (The Scotts Company, LLC, Marysville, OH) to provide adequate air flow. Larvae were fed pork liver ad libitum until pupation, after which the liver was removed. Upon emergence, flies were separated based on species and sex with 150 females and 50 males being placed into clean cages. Adult flies were also fed sugar and water ad libitum. These cages were transported and housed at the University at Albany-SUNY (Albany, NY) for the studies reported here. Cages were cleaned twice weekly by wiping all surfaces with deionized water and ethanol.

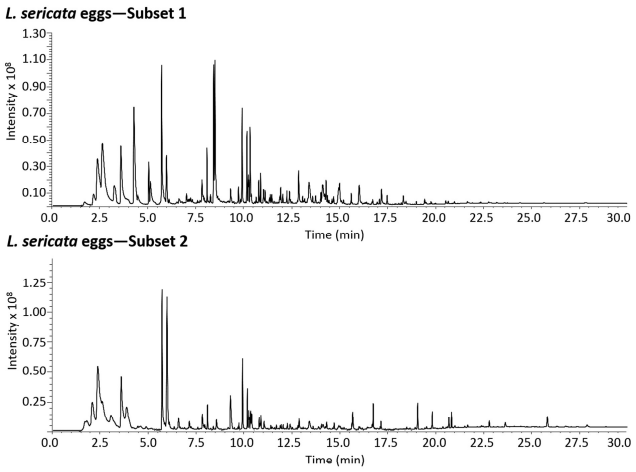


Fig. 3. GC chromatograms of two subsamples of *Lucilia sericata* eggs (collected one month apart), analyzed using a HP-FFAP column. For ease of visualization, the chromatogram corresponding to subsample #1, partitioned into segments representative of 5-min increments, is presented in Supp Fig. 1 (online only), where the peaks are each assigned a number. Peak identities are listed in Table 1.

For the collection of the freshly laid eggs that were used in these experiments, every three days, flies were offered ~5 ml of chicken blood (Perdue, Salisbury, MD) in a plastic Petri dish (Fisher

Scientific, Pittsburgh, PA) in order to provide females with a protein boost to enhance egg production (Fig. 1A). For each species, on two separate occasions, there was an aggregated oviposition event when 15–20 gravid females collectively laid clutches of egg on the metal floor of the cages (Fig. 1 B). These egg masses were not laid in the vicinity of the blood source. Within 10–15 min after egg deposition, the entire egg mass was immediately collected, transferred to glass vials (Kimble Chase, Rockwood, TN), and frozen at −10°C to minimize bacterial contamination until analysis (Fig. 1 C). From these large egg masses, ~100 eggs were subsampled for analysis. The aggregated oviposition and collection events occurred approximately 1 mo apart, after which, egg collection ceased due to adult mortality. Voucher specimens of the eggs of *L. sericata* and *P. regina* are stored in the repository at the John Jay College of Criminal Justice under accession numbers FE-2018-CALLIPH-LS-E1 and FE-2018-CALLIPH-PR-E1, respectively.

Headspace Volatiles Sampling

To collect and concentrate headspace volatiles, SPME fibers were used and the headspace collection set-up was as shown in Fig. 2. The fibers utilized in these experiments were coated with divinylbenzene/carboxen/polydimethysiloxane (DVB/CAR/PDMS; Supelco, Bellefonte, PA). Each fiber was conditioned prior to headspace sampling by insertion into a GC inlet at 250°C for 30 min under a flow of dry helium using a glass GC inlet liner (Supelco, Bellefonte, PA) installed on the instrument to prevent destruction of the fiber. For each egg mass, ~100 blow fly eggs per species were subsampled and placed into a glass vial which was covered tightly with aluminum foil (Fig. 2 B). After conditioning, the fiber was introduced into the vial through a puncture in the foil, via the SPME fiber assembly, and the headspace was sampled for 30 min.

Analytical Standards

The following chemical standards were purchased from Sigma–Aldrich (St. Louis, MO): 1-propanol; 2, 6-dimethylpyrazine; 2,3-butanediol; benzaldehyde; dodecanal; nonanal; and octanal. Pentanoic acid ethyl ester and 2-thiophenecarboxaldehyde were purchased from TCI America (Philadelphia, PA). Acetic acid was purchased from Mallinckrodt (St. Louis, MO), and acetone and ethyl acetate were purchased from Pharmco (Brookfield, CT).

Headspace Volatiles Analysis by GC-MS

After collection and concentration of headspace volatiles, the fibers were analyzed by GC-MS using of a JMS-T200GC AccuTOF GCx mass spectrometer (JEOL USA, Inc., Peabody, MA) coupled to a 7890B Agilent GC (Agilent, Santa Clara, CA). The instrument parameter settings are listed in Supp Table 1 (online only). To detect as broad a range of compounds as possible, the headspaces of eggs deposited during two mass oviposition events from each species (collected 1 mo apart) were analyzed using two columns: one optimized for detection of free fatty acids (HP-FFAP, Agilent J&W GC Columns, Santa Clara, CA), and the other optimized for detection of amines (CP-Sil 8 CB, Agilent J&W GC Columns, Santa Clara, CA). After exposure to egg volatiles, the fibers were inserted into the GC-MS system with a splitless injection and an inlet temperature of 250°C. The mass spectrometer parameters for both columns were as follows: ionization mode was EI; ionization voltage was 70 V; ionization current was 300 µA; mass range was *m/z* 10–800; acquisition time was 0.4 s (2.5 Hz); and sampling time was 0.25 ns (4 GHz). Additional GC-MS parameters for the analyses performed using both columns are listed in Supp Table 1 (online only). Data processing was performed using msAxel software (JEOL Ltd., Akishima, Japan). The fragmentation patterns of the mass spectra of the detected compounds were screened against spectral libraries (NIST 2011) in order to confirm identities.

For a handful of compounds that were detected using the CP-Sil column, attempts at identification using spectral library fragmentation pattern comparisons yielded ambiguous results. In these cases, authentic chemical standards were used to confirm compound assignments. Solutions of the standards were created in acetone to a final concentration of 100 ppm, and GC analysis was performed using a 1-µl injection with a split ratio of 100:1 using the same oven program that was used for the SPME fiber analysis (Supp Table 1 [online only]). The retention times and fragmentation patterns were compared with those of the compounds detected in the egg samples to confirm peak identities.

Results

GC-MS Analysis of Blow Fly Egg Headspace Volatiles

The headspace volatiles profiles of two species of blow fly eggs (*L. sericata* and *P. regina*) were collected and concentrated using SPME fibers, which were subsequently analyzed via GC-MS using both a HP-FFAP and a CP-Sil column. Figure 3 displays the chromatograms obtained using the HP-FFAP column for analysis of the *L. sericata* eggs, and Supp Fig. 1 (online only) shows magnifications of the chromatograms in 5-min increments for clarity. The peaks in the chromatograms are labeled numerically in order of increasing retention time (Supp Fig. 1 [online only]), except for those representing silanes which appeared as a consequence of column bleed. These are indicated with the label 'S'. Additionally, there were a number of compounds that were detected that were not natural products that originated from the blow fly eggs but are instead ubiquitous unnatural products (such as 2,6-bis[1,1-dimethylethyl]-4-[1-methylpropyl] phenol, a known plasticizer), and these are indicated with the label 'U'. In the case of the HP-FFAP column results where two subsamples of eggs were analyzed for each species, peaks common to both chromatograms were assigned the same number.

Lucilia sericata eggs: The compounds identified in the headspace profiles of the two subsamples of *L. sericata* eggs using the HP-FFAP column are listed in Table 1. Supp Fig. 2 (online only) shows comparisons of the EI mass spectra of the compounds corresponding to each of the peaks observed by GC, and those of the compound matches from the NIST mass spectral library, rendered as heat-to-tail plots. In each case, the top spectrum is of the compound derived from the egg sample, and the bottom is that of the library match. The compounds detected covered a range of classes including alcohols, aldehydes,

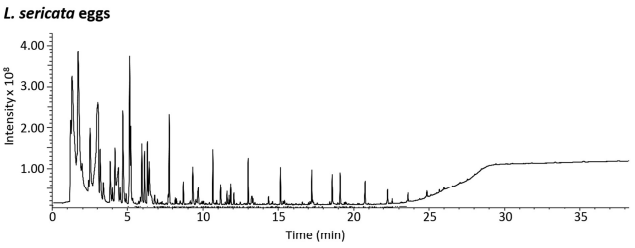


Fig. 4. GC chromatogram of *L. sericata* eggs (subsample #2) analyzed with a CP-Sil column. For ease of visualization, the chromatogram, partitioned into segments representative of 5-min increments, is presented in Supp Fig. 3 (online only), where the peaks are each assigned a number. Peak identities are listed in Table 2.

Table 2. Compounds detected by GC-MS analysis of the headspace of one subsample of *Lucilia sericata* eggs using a CP-Sil column

Peak no.	Ret. time (min)	compound name	Peak no.	Ret. time (min)	Compound name
1	1.60	Unknown	33	8.34	Heptanal
2	2.38	Carbon disulfide	34	8.50	Methional
3‡	2.47	1-Propanol	35‡	8.59	2,6-Dimethylpyrazine
4‡	2.62	Acetic acid	36	9.00	1,4-Butanediol
5	2.80	Acetic acid	37	9.35	Hexanoic acid
6	3.16	Ethyl acetate	38‡	9.66	Benzaldehyde
7	3.50	Tetrahydrofuran	39	9.70	Unknown
8	3.74	2-Butenal	40	9.79	3-(Methylthio)-1-propanol
9	3.84	3-Methylbutanal	41	9.99	Hexanoic acid ethyl ester
10	4.00	2-Methylbutanal	42	10.18	Octanal
11	4.41	Propanoic acid	43‡	10.38	2-Thiophenecarboxaldehyde
12	4.46	2,3-Pentanedione	44	11.07	Benzeneacetaldehyde
13	4.52	Heptane	45	11.65	2-Nonanone
14	4.70	Unknown	46	11.77	Undecane
15	5.16	3-Methyl-1-butanol	47	11.85	2,9-Undecadiene
16	5.24	2-Methyl-1-butanol	48‡	11.91	Nonanal
17	5.36	2-Methyl-2-butenal	49	12.23	Phenylethyl alcohol
18	5.60	2-Methylpropanoic acid ethyl ester	50	12.67	Unknown
19	5.78	Butanoic acid	51	13.53	Decanal
20	5.89	Toluene	52	14.22	Nonanoic acid
21‡	6.00	2,3-Butanediol	53	14.86	Tridecane
22	6.18	Unknown	54	16.27	Tetradecane
23	6.37	Octane	55‡	16.49	Dodecanal
24	6.87	3-Methylbutanoic acid	56	16.73	5-Formyl-2,4-dimethyl-pyrrole-3-carbonitrile
25	7.05	2-Methylbutanoic acid	57	17.41	5-Hexyldihydro-2(3*H*)-furanone
26	7.30	2-Methylbutanoic acid ethyl ester	58	18.29	Unknown
27	7.36	3-Methylbutanoic acid ethyl ester	59	18.75	Dodecanoic acid ethyl ester
28	7.60	3-Methyl-1-pentanol	60	20.00	Heptadecane
29	7.79	3-Methyl-1-butanol acetate	61	20.96	Ethyl 9-tetradecenoate
30	7.86	Methoxy-phenyl-oxime	62	22.97	Unknown
31	8.10	2-Heptanone	63	25.60	Docosahexaenoic acid
32	8.26	Nonane			

‡Compound identity was confirmed by matching retention time with that of an analytical standard.

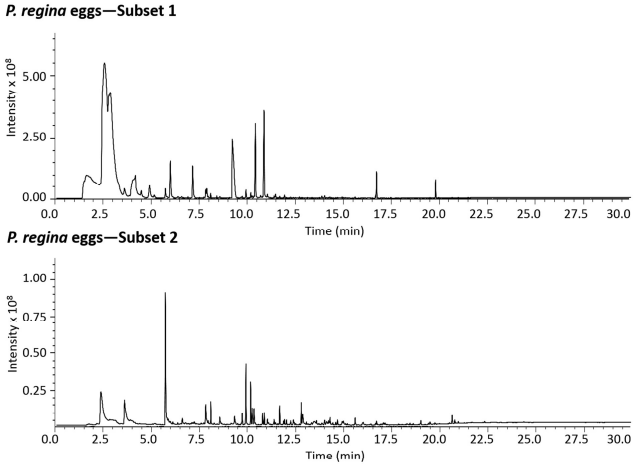


Fig. 5. GC chromatograms of two subsamples of *Phormia regina* eggs (collected one month apart), analyzed using the HP-FFAP column. For ease of visualization, the chromatogram corresponding to subsample #2, partitioned into segments representative of 5-min increments, is presented in Supp Fig. 5 (online only), where the peaks are each assigned a number, the identities of which are listed in Table 3.

alkanes, and ketones, among others. Overall, the chromatograms for both subsamples were quite similar. Excluding the compounds that were attributed to column bleed-derived silanes and other minor synthetic contaminants (e.g., 2-ethyl-1-hexanol at 9.70 min, 2-[2-ethoxyethoxy]-ethanol at 11.37 min, 1,1'-oxybisoctane at 12.78 min and diethyl phthalate at 19.00 min), there were 21 and 20 peaks in the Sample 1 and Sample 2 chromatograms, respectively. Of these, compounds represented by chromatogram peaks 1 through 13 listed in Table 1 were common to both samples. However, there were some notable variations between the two samples. For example, in Sample 1, triethylamine, dimethyl disulfide, 1-undecene, 2-methyl-1-butanol, dimethyl trisulfide, 2-decanone, 3-methyl-1*H*-pyrrole, and 4-ethylbenzaldehyde were detected at 3.21, 4.24, 5.01, 5.94, 8.40, 9.81, 10.62, and 12.49 min, respectively. However, these compounds were not observed in the Sample 2 eggs. Conversely, heptane (1.76 min), octane (2.04 min), 3-methyl-1-butanol acetate (4.86), 2-methyl-1-butanol/1-pentanol (5.94 min), ocimene (6.32 min), 4-hydroxybutanoic acid (11.66 min), and hexanoic acid (13.86 min) were detected in Sample 2 but not Sample 1.

Figure 4 illustrates the chromatogram resulting from GC-MS analysis of *L. sericata* eggs using the CP-Sil column, and Supp Fig. 3 (online only) shows magnifications of the chromatogram displayed in 5-min increments for clarity. In total, 63 compounds were detected, 56 of which were identified including several aldehydes, alkanes, and carboxylic acids, as well as esters and ketones. Peak retention times and the corresponding compound assignments based on mass spectral fragmentation patterns are listed in Table 2. Supp Fig. 4 (online only) shows the mass spectral fragmentation patterns corresponding to the indicated peaks in the chromatogram, compared

Table 3. Compounds detected by GC-MS analysis of the headspace of two subsamples of *Phormia regina* eggs that were laid one month apart, using a HP-FFAP column*

Peak no.	Ret. time	Compound	Subsample 1	Subsample 2
1	8.53	Nonanal	X	X
2	9.29	Acetic acid	X	X
3	10.31	Benzaldehyde	X	X
4	11.44	Butanoic acid	X	X
5	11.65	4-Hydroxybutanoic acid	X	X
6	12.21	4-Butoxy-1-butanol	X	X
7	12.37	3-Thiophenecarboxaldehyde	X	X
8	12.70	Pentanoic acid	X	X
9	12.85	Methoxy-phenyl-oxime	X	X
10	13.87	Hexanoic acid	X	X
11	14.29	Benzyl alcohol	X	X
12	14.99	2-Thiophenemethanol	X	X
13	15.60	Phenol	X	X
14	16.72	9-Tetradecen-1-ol acetate	X	X
15	18.91	1-Hexadecanol	X	X
16	19.53	Benzoic acid	X	X
17	19.83	Docosahexaenoic acid	X	X
α	4.77	3-Methyl-1-butanol acetate	X	
β	5.10	1-Butanol	X	
γ	6.60	3-Octanone	X	
δ	7.46	2-Heptanol	X	
ϵ	6.57	Ocimene		X
ζ	7.12	Octanal		X
η	7.20	Tridecane		X
θ	8.23	2,6-Dimethyl-2,4,6-octatriene		X
ι	9.13	1-Phenyl-1-butene		X

*Compounds that were detected in one but not both subsamples are labeled with Greek letters.

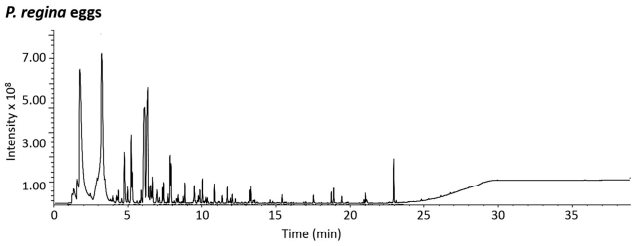


Fig. 6. GC chromatogram of *Phormia regina* eggs (subsample #1) analyzed with a CP-Sil column. For ease of visualization, the chromatogram, partitioned into segments representative of 5-min increments, is presented in Supp Fig. 7 (online only), where the peaks are each assigned a number. Peak identities are listed in Table 4.

with NIST library spectra or spectra acquired using authentic standards, rendered as head-to-tail plots in which the top spectrum represents the compound detected in the eggs, and the bottom, the standard or NIST spectrum. A subset of these compounds was also detected using the HP-FFAP column, including 2-methyl-1-butanol, 2-nonanone, 2-thiophenecarboxaldehyde, 3-methyl-1-butanol acetate, benzaldehyde, heptane, hexanoic acid, methoxyphenyl-oxime, nonanal, octane, propanoic acid, and tridecane.

Phormia regina eggs: The chromatograms observed in the analysis of two subsamples of *P. regina* eggs laid 1 mo apart, and using the HP-FFAP column, are shown in Fig. 5. Magnifications of the chromatograms in 5-min increments for clarity are shown in Supp Fig. 5 (online only). The retention times and corresponding compound identities that were determined by spectral library comparisons or comparisons with the mass spectra of authentic standards are listed in Table 3. Eliminating chromatographic peaks that were the result of column bleed, plasticizers, or other synthetic molecules, 26 compounds were detected overall. Comparison of the results from Sample 1 and Sample 2 revealed that most of the compounds (17) were shared. These included several carboxylic acids such as benzoic, butanoic, pentanoic and hexanoic acids, and compounds featuring alcohol, aldehyde and ester functional groups. Supp Fig. 6 (online only) shows the mass spectral fragmentation patterns corresponding to the indicated peaks in the chromatogram, compared with NIST library spectra or spectra acquired using authentic standards, rendered as head-to-tail plots in which the top spectrum represents the compound detected in the eggs, and the bottom, the standard or NIST spectrum. Compounds detected in Sample 1 but not Sample 2 were 3-methyl-1-butanol acetate, 1-butanol, 3-octanone, and 2-heptanol (at retention times (RT) of 4.77, 5.10, 6.60, and 7.46 min, respectively), and those detected in Sample 2 but not Sample 1 were ocimene (RT: 6.57 min), octanal (RT: 7.12 min), tridecane (RT:7.20 min), 2,6-dimethyl-2,4,6-octatriene (RT: 8.23 min), and 1-phenyl-1-butene (RT: 9.13 min).

Figure 6 displays the chromatogram observed from analysis of *P. regina* egg headspace using the CP-Sil column, and Supp Fig. 7 (online only) shows magnifications of the chromatogram in 5-min increments for clarity. Excluding peaks representing synthetic compounds and silanes derived from column bleed, a total of 78 peaks were detected, 71 of which were identified. The corresponding compound identities that were determined through mass spectral library comparisons are listed in Table 4. Supp Fig. 8 (online only) shows comparisons of the mass spectra of the detected peaks with those from the assigned match in the spectral library analyses or to the authentic standards analyzed using the CP-Sil column for the *P. regina*

Table 4. Compounds detected by GC-MS analysis of the headspace of one subsample of *Phormia regina* eggs using a CP-Sil column

Peak no.	Ret. time (min)	Compound name	Peak no.	Ret. time (min)	Compound name
1	2.41	Carbon disulfide	40	10.23	Acetic acid hexyl ester
2	2.86	Acetic acid	41	10.37	4-Ethylcyclohexanone
3‡	3.17	Ethyl acetate	42	10.58	2-Methyloctanoic acid ethyl ester
4	3.37	Ethyl acetate	43	11.08	9-Octadecenoic acid phenylmethyl ester
5	3.75	2-Butenal	44	11.65	2-Nonanone
6	3.85	3-Methylbutanal	45	11.78	5-Ethyl-2-heptanol
7	3.92	1-Butanol	46‡	11.92	Nonanal
8	3.98	Unknown	47	12.24	Phenylethyl alcohol
9	4.46	3-Pentanone	48	12.32	*N*-(3-Methylbutyl) acetamide
10	4.53	Carbonic acid butyl 2-pentyl ester	49	12.41	Octanoic acid ethyl ester
11	4.71	Acetoin	50	12.82	Dihydro-5-propyl-2(3*H*)-furanone
12	4.95	Unknown	51	13.28	Octanoic acid ethyl ester
13	5.17	3-Methyl-1-butanol	52	13.36	Dodecane
14	5.24	2-Methyl-1-butanol	53	13.54	Decanal
15	5.57	2-Methylpropanoic acid ethyl ester	54	14.12	4-Methyloctanoic acid ethyl ester
16	5.74	1-Pentanol	55	14.18	Benzeneacetic acid ethyl ester
17	6.09	2,3-Butanediol	56	14.38	2-Acetic acid phenylethyl ester
18	6.31	2,3-Butanediol	57	14.72	α-Ethylidene-benzeneacetaldehyde
19	6.37	Octane	58	14.77	Nonanoic acid ethyl ester
20	7.08	2-Methylbutanoic acid	59	14.81	2-Undecanone
21	7.29	2-Methylbutanoic acid ethyl ester	60	14.86	Dodecane
22	7.36	3-Methylbutanoic acid ethyl ester	61	15.96	5-Hexyldihydro-2(3*H*)-furanone
23	7.59	Pentanoic acid	62	16.09	4-Decenoic acid ethyl ester
24	7.66	1-Hexanol	63	16.17	Decanoic acid ethyl ester
25	7.79	3-Methyl-1-butanol acetate	64	16.27	Tetradecane
26	7.86	Methoxy-phenyl-oxime	65‡	16.48	Dodecanal
27	8.08	2-Heptanone	66	17.02	6,10-Dimethyl-5,9-undecadien-2-one
28‡	8.26	Pentanoic acid ethyl ester	67	17.34	8-Heptadecene
29	8.46	Acetic acid pentyl ester	68	18.28	Unknown
30	8.59	2,6-Dimethylpyrazine	69	18.50	Unknown
31	8.97	1,4-Butanediol	70	18.75	Dodecanoic acid ethyl ester
32	9.38	Hexanoic acid	71	20.80	Unknown
33	9.54	3,5-Dimethyl-2-hexene	72	20.85	Unknown
34	9.66	Benzaldehyde	73	20.96	Ethyl 9-tetradecenoate
35	9.72	1-Octen-3-ol	74	21.42	Hexadecanal
36	9.79	3-(Methylthio)-1-propanol	75	22.07	Unknown
37	9.93	2-Octanone	76	22.99	Palmitelaidic acid ethyl ester
38	10.00	Hexanoic acid ethyl ester	77	23.15	Hexadecanoic acid ethyl ester
39‡	10.20	Octanal	78	25.62	Docosahexaenoic acid

‡Compound identity was confirmed by matching retention time with that of an analytical standard.

eggs. These images are presented as head-to-tail plots, in which the top spectrum represents the compound as it was detected in the eggs, and the bottom panel is that of the NIST spectrum or standard. A broad range of functional groups were represented, including oxygen-containing compounds (alcohols, aldehydes, carboxylic acids, esters, and ketones) and hydrocarbons (alkanes and alkenes) among others. A subset of these compounds was also detected using the HP-FFAP column, including 1-butanol, 2-methyl-1-butanol acetate, acetic acid, benzaldehyde, docosahexaenoic acid, hexanoic acid, methoxyphenyl-oxime, nonanal, octanal, and pentanoic acid.

Discussion

One hypothesis that may explain why conspecific egg laying elicits heterospecific egg oviposition within the conspecific egg mound is that the initially laid eggs emit a chemical cue that promotes oviposition in other females. A first step in assessing the validity of this hypothesis is to identify the headspace volatiles of blow fly eggs so that identified compounds can be used in controlled studies of fly behavior. In this first report on the volatile organic compounds associated with *L. sericata* and *P. regina* eggs, SPME fiber-facilitated GC-MS analysis using two different types of columns was used to begin cataloguing the compounds present. The utilization of two types of columns and the analysis of egg samples collected from 10 to 15 females permitted assessment of the consistency of the constituent volatiles profiles of the eggs, and enabled detection of a broad range of compounds. While the results are preliminary and a broader range of studies representing many more species and rearing conditions will need to be examined to determine whether the trends hold, it was noted that the detected volatiles were quite similar for a given species, but very distinct between species, despite the presence of shared compounds. This marked difference in volatiles profiles between species aligns with the results of analyse of the small molecule profiles of ethanol suspensions of eggs, which have recently been shown to be distinct enough to serve as the basis of the ability to identify them based on their unique chemical signatures (Giffen et al. 2017).

Among the classes of molecules represented were alcohols, aldehydes, alkanes, alkenes, amides, amines, arenes, carboxylic acids, esters, and ethers. Table 5 lists the molecules detected, disaggregated by functional group class, and indicates which of them were common

Table 5. Molecules detected in the headspace of the eggs of *Lucilia sericata* and *Phormia regina*, disaggregated by functional group*

	Lucilia sericata	*Phormia regina*
Alcohol		
1,4-Butanediol	X	X
1-Pentanol	X	X
2,3-Butanediol	X	X
2-Methyl-1-butanol	X	X
3-Methyl-1-butanol	X	X
Benzyl alcohol	X	X
1-Butanol		X
1-Hexadecanol		X
1-Hexanol		X
1-Octen-3-ol		X
2-Heptanol		X
4-Butoxy-1-butanol		X
5-Ethyl-2-heptanol		X
Phenol		X
1-Propanol	X	
3-Methyl-1-pentanol	X	
Aldehydes		
2-Butenal	X	X
3-Methylbutanal	X	X
Benzaldehyde	X	X
Decanal	X	X
Dodecanal	X	X
Nonanal	X	X
Octanal	X	X
Hexadecanal		X
α-Ethylidene-benzeneacetaldehyde		X
2-Methyl-2-butenal	X	
2-Methylbutanal	X	
4-Ethylbenzaldehyde	X	
Benzeneacetaldehyde	X	
Heptanal	X	
Alkanes		
Octane	X	X
Tetradecane	X	X
Tridecane	X	X
Dodecane		X
Heptadecane	X	
Heptane	X	
Hexane	X	
Nonane	X	
Undecane	X	
Alkenes		
Ocimene	X	X
2,6-Dimethyl-2,4,6-octatriene		X
3,5-Dimethyl-2-hexene		X
8-Heptadecene		X
1-Undecene	X	
2,9-Undecadiene	X	
Amides		
N-(3-Methylbutyl) acetamide		X
Amines		
2,6-Dimethylpyrazine	X	X
3-Methyl-1*H*-pyrrole	X	
5-Formyl-2,4-dimethyl-pyrrole-3-carbonitrile	X	
Triethylamine	X	
Arenes		
1-Phenyl-1-butene	X	X
Methoxy-phenyl-oxime	X	X
Phenylethyl alcohol	X	X
Toluene	X	
Carboxylic acids		
2-Methylbutanoic acid	X	X
4-Hydroxybutanoic acid	X	X
Acetic acid	X	X
Butanoic acid	X	X
Docosahexaenoic acid	X	X
Hexanoic acid	X	X
Benzoic acid		X
Pentanoic acid		X
3-Methylbutanoic acid	X	
Hexadecanoic acid	X	
Nonanoic acid	X	
Propanoic acid	X	
Esters		
2-Methylbutanoic acid ethyl ester	X	X
2-Methylpropanoic acid ethyl ester	X	X
3-Methyl-1-butanol acetate	X	X
3-Methylbutanoic acid ethyl ester	X	X
9-Tetradecen-1-ol acetate	X	X
Dodecanoic acid ethyl ester	X	X
Ethyl 9-tetradecenoate	X	X
Ethyl acetate	X	X
Hexanoic acid ethyl ester	X	X
2-Acetic acid phenylethyl ester		X
2-Methyloctanoic acid ethyl ester		X
4-Decenoic acid ethyl ester		X
4-Methyloctanoic acid ethyl ester		X
9-Octadecenoic acid phenylmethyl ester		X
Acetic acid hexyl ester		X
Acetic acid pentyl ester		X
Benzeneacetic acid ethyl ester		X
Carbonic acid butyl 2-pentyl ester		X
Decanoic acid ethyl ester		X
Hexadecanoic acid ethyl ester		X
Nonanoic acid ethyl ester		X
Octanoic acid ethyl ester		X
Palmitelaidic acid ethyl ester		X
Pentanoic acid ethyl ester		X
Ether		
Tetrahydrofuran	X	
Ketones		
2-Heptanone	X	X
2-Nonanone	X	X
5-Hexyldihydro-2(3*H*)-furanone	X	X
2-Octanone		X
2-Undecanone		X
3-Octanone		X
3-Pentanone		X
4-Ethylcyclohexanone		X
6,10-Dimethyl-5,9-undecadien-2-one		X
Acetoin		X
Dihydro-5-propyl-2(3*H*)-furanone		X
2,3-Pentanedione	X	
2-Decanone	X	
Organosulfur compounds		
2-Thiophenemethanol	X	X
3-(Methylthio)-1-propanol	X	X
Carbon disulfide	X	X
3-Thiophenecarboxaldehyde		X
Dimethyl disulfide	X	
Dimethyl trisulfide	X	
2-Thiophenecarboxaldehyde	X	
Methional	X	

*Gray shading indicates compounds that were common to both species. Nonshaded areas indicate compounds that were detected in one but not both species.

to both species. Out of a total of 112 identified compounds, 42 (i.e., 37.5%) were shared. These included alcohols (1,4-butanediol, 1-pentanol, 2,3-butanediol, 2-methyl-1-butanol, 3-methyl-1-butanol and benzyl alcohol); aldehydes (2-butenal, 3-methylbutanal, benzaldehyde, octanal, nonanal, decanal and dodecanal); alkanes (octane, tridecane and tetradecane); an alkene (ocimene); arenes (1-phenyl-1-butene, methoxyphenyl-oxime and phenylethyl alcohol); carboxylic acids (acetic acid, butanoic acid, 4-hydroxybutanoic acid, 2-methylbutanoic acid, hexanoic acid and docosahexaenoic acid); esters (ethyl acetate, 2-methylbutanoic acid ethyl ester, 3-methylbutanoic acid ethyl ester, 3-methyl-1-butanol acetate, hexanoic acid ethyl ester, 2-methylpropanoic acid ethyl ester, dodecanoic acid ethyl ester, ethyl 9-tetradecenoate and 9-tetradecen-1-ol acetate); ketones (2-heptanone, 2-nonanone and 5-hexyldihydro-2[3*H*]-furanone); organosulfur compounds (carbon disulfide, 3-[methylthio]-1-propanol and 2-thiophenemethanol); and a pyrazine (2,6-dimethylpyrazine).

There were 29 compounds that were identified that were unique to *L. sericata* eggs. The subset that were aldehydes included: 2-methyl-2-butenal, 2-methylbutanal, 2-thiophenecarboxaldehyde, benzeneacetaldehyde, 4-ethylbenzaldehyde, heptanal, and methional. Other compounds unique to *L. sericata* were 1-propanol, 2,3-pentanedione and tetrahydrofuran. In total, 41 compounds were identified only in the *P. regina* eggs. A number of these were esters, including ethyl esters (pentanoic acid, benzeneacetic acid, octanoic acid, 2-methyloctanoic acid, 4-methyloctanoic acid, nonanoic acid, decanoic acid, 4-decenoic acid, hexadecanoic acid, and palmitelaidic acid ethyl esters); pentyl esters (acetic acid and carbonic acid butyl 2-pentyl esters); 9-octadecenoic acid phenyl methyl ester, acetic acid hexyl ester, and 2-acetic acid phenylethyl ester. There were also several alcohols including 1-butanol, 1-octen-3-ol, 1-hexanol, phenol, 2-heptanol, 5-ethyl-2-heptanol, 1-hexadecanol, and 4-butoxy-1-butanol.

From these observations, the following trends were noted: 1) While low molecular weight amines have been shown to be prominently featured as components of decomposition volatiles, only two aliphatic amines were detected (i.e., triethylamine and 5-formyl-2,4-dimethylpyrrol-3-carbonitrile in *L. sericata*). In addition, the aromatic amine 2,6-dimethylpyrazine was detected in both species and 3-methyl-1*H*-pyrrole was observed in *L. sericata*; 2) Amides and alkenes were also poorly represented, with one amide and four alkenes being observed in *P. regina* (i.e., *N*-[3-methylbutyl] acetamide, 3,5-dimethyl-2-hexene, ocimene, 8-heptadecene, 2,6-dimethyl-2,4,6-octratriene, respectively), and three alkenes (ocimene, 2,9-undecadiene and 1-undecene) and no amides being detected in *L. sericata*. The paucity of unsaturated hydrocarbons and amides also contrasts with their representation as components of decomposition volatiles. Previous studies have shown that *N,N*-dimethylformamide, *N*-butylformamide, butanamide, benzene, methylbenzene, xylene, and α-pinene have been detected in the volatiles emitted from decomposing human, dog, and pig remains (Vass et al. 2004, Statheropoulos et al. 2005, Paczkowski and Schutz 2011, Dekeirsschieter et al. 2012, Vass 2012); 3) Alkanes were more prominently featured in *L. sericata* than in *P. regina*. Nine alkanes ranging from 8 to 17 carbons were detected in *L. sericata*, only three of which were shared with *P. regina* (i.e., octane, tetradecane and tridecane). In addition to these three latter alkanes, one additional alkane (dodecane) was detected in *P. regina*; 4) There are several compound classes for which both species exhibit similar numbers of detected molecules, some of which were shared. Examples include carboxylic acids (10 detected in *L. sericata* and 8 detected in *P. regina*, with 6 of these being shared between the two species) and 4 arenes detected in *L. sericata* and 3 arenes detected in *P. regina*, respectively; and 5) For some classes of compounds, *P. regina* was observed to have a much broader representation of compounds than *L. sericata*. This difference was most dramatic in the ester class. While 9 esters were detected in *L. sericata*, all of which were shared with *P. regina*, 15 additional esters were detected in *P. regina*. In another example, 11 ketones were detected in *P. regina* and 5 were detected in *L. sericata*, with 3 ketones being shared between the two species.

A salient feature of the observed results is that there was little overlap between the identified volatiles associated with the eggs on the one hand, and the molecules commonly detected in carrion and/or corpse decomposition headspace on the other. Thus, only a small subset of the total number of compounds detected (i.e., 14 molecules or 12.5%) have been reported to be emitted by decomposing remains. These include 2-methylbutanal, carbon disulfide, decanal, dimethyl disulfide, dimethyl trisulfide, heptanal, heptane, hexane, nonanal, nonane, octanal, octane, toluene, and undecane (Vass et al. 2008, Vass 2012). Interestingly, some of the compounds detected have also been reported to be present in the volatiles emissions of *C. vicina* larvae and pupae, including: 2-methylbutanal, 3-methylbutanal, acetic acid, benzaldehyde, dimethyl disulfide, dimethyl trisulfide, ethyl acetate, heptanal, nonanal, octane, and phenol (Frederickx et al. 2012b).

Since blow fly attraction to decomposing remains is believed to be mediated by chemical cues, it was notable that there were a small subset of compounds that are known to be emitted by decaying tissue, and which were also detected in the egg headspace of one or the other species, but not in both. For example, phenol and 1-butanol, both of which are well-known decay compounds, (Dekeirsschieter et al. 2009) were detected in *P. regina* headspace but not in that of *L. sericata*. Another decay compound, dimethyl disulfide, was detected in *L. sericata* headspace but not in the headspace of *P. regina*. These molecules have been shown to cause species-specific responses (Frederickx et al. 2012a). Thus, the presence of a compound in one species but not the other could indicate that the compound is a source of attraction for the latter species. For example, phenol has been found to elicit a strong response in *L. sericata* (Frederickx et al. 2012a), so its detection in *P. regina* but not in *L. sericata* could attract *L. sericata* to *P. regina* eggs.

The possibility that compounds associated with the eggs of one species might act as chemical cues that promote heterospecific egg laying or serve as attractants to predators, makes the subset of detected compounds that are not shared between species particularly interesting. It was observed, for example, that while there were 9 esters that were shared between *L. sericata* and *P. regina*, 15 additional esters were observed in *P. regina*. In principle, a compound or combination of these unique compounds could serve as chemical cues for other species in which these molecules do not appear. Exploration of this hypothesis will require detailed systematic studies of oviposition behavior in *L. sericata* and *P. regina* flies in order to first determine which species, if either, promotes egg laying in the other. The influence of the unique compounds that were detected in the eggs of the species that elicits egg laying in the other can be used in systematic tests of fly behavior and is a subject of on-going studies in our laboratories.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

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