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PAPER

PATHOLOGY/BIOLOGY

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Decaying Mouse Volatiles Perceived by *Calliphora vicina* Rob.-Desv.*,†

ABSTRACT: Volatiles emitted by decaying human remains are in the focus of recent research. The identification of core volatiles in this field is of high importance, because cadaveric volatiles generally show high variation. In this study, the volatile profiles of five mice (*Myodes glareolus*) were sampled with charcoal filter tubes from their time of death until advanced decay. Eleven compounds were quantitated by means of gas chromatography–mass spectrometry. Electroantennographic experiments with female *Calliphora vicina* antennae led to the identification of dimethyl trisulfide, dimethyl disulfide, nonanal, hexan-1-ol, 1-octen-3-ol, 3-methylbutan-1-ol, and heptanal as electrophysiologically active compounds. When these were compared, dimethyl trisulfide (17 ng/μL) and dimethyl disulfide (11 ng/μL) were found to be emitted in higher concentrations. The roles of these compounds and nonanal as core volatiles for cadaver detection or postmortem time determination and their correlation to the stages of decay and the accumulated degree days are discussed.

KEYWORDS: forensic science, forensic chemoecology, forensic entomology, *Calliphora vicina*, electroantennogram, decomposition process, volatile emission pattern, stages of decay, odor analysis, postmortem time, core compounds, accumulated degree days

Vertebrates are degraded by autolytic and microbial processes after their death (1–11). The chemical reactions catalyzed by the tissue's own enzymes, as well as microbial enzymes, yield volatile organic compounds as byproducts or end products (9,12–20). These volatile compounds are currently being researched and considered as possible applications in forensic investigations. In 2004, Dent et al. (19) published a review of human decomposition processes in soil. In the same year, Vass et al. (14) introduced a postmortem volatile database for the purpose of developing a portable sensor capable of detecting buried remains. Dent et al. (19) discuss the likely pathways of protein, fat, carbohydrate, and bone decomposition, while Vass et al. (14) provide a first analysis on the volatile profile of human decomposition in graves. Both groups showed the existence of a dynamic in the volatile pattern.

Statheropoulos et al. (16) were the first to present experimental data on the volatiles emitted by human remains on the soil surface at a specific time point in the decaying process. In 2007, Statheropoulos et al. (15) published the quantitative dynamic of the volatile pattern in the early stages of human decomposition. This discusses the practical use of the volatile pattern of human decay for canine training or the detection of human remains. In 2009, Dekeirsschieter et al. (12) sampled the volatile profile of pigs in three separate habitats. They discuss the influence of factors affecting the decompositional process and proposed a common core of volatiles, which were identified in all habitats. A study by Hoffman et al. (17) shows similarities across various regions and different tissues of decaying remains. All of these studies found a high variability of the volatile pattern of decaying vertebrates and discuss constant dynamics in the volatile emissions.

The general problem seems to be the identification of core volatiles by means of common trace analytical methods. They should have three characteristics: First, they should be removed from as much variation as possible, enabling cadaver dogs to find human remains despite disparities in climate, habitat, or condition of the cadaver. A portable sensor system for tracing cadavers or postmortem interval (PMI) estimation would also rely on these characteristics. Second, the volatiles should be emitted in high quantities, as cadaver dogs are then able to trace them over larger distances, and gas sensor applications have a relatively high detection threshold. Third, the emitted quantities of the core volatiles should show a constant dynamic to correlate them to the process of decay. Such a correlation could include several volatiles, some typical for the early stages of decay, and some typical for the later stages.

To identify the core volatiles in question, it was decided that mice (*Myodes glareolus*) would be used in the experiments. Core volatiles should form from basic chemical compounds such as those found in muscle tissue or neurons; therefore, a constant dynamic should be detectable in mice as well as in larger vertebrates. A small cadaver also emits less volatiles; hence, the selection of volatiles with the listed characteristics should be easier. However, any realistic experimental design would most probably not allow validating core volatiles. Therefore, it was decided that the female blowfly *Calliphora vicina* would be used as an information filter for the scent of decay. The fly feeds and oviposits on deceased vertebrates (21–23). Ovipositing begins

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shortly after death and continues until advanced decay (22,23); therefore, this fly is a generalist among carrion visitors. Its olfaction might be adapted to perceive core volatiles, which would allow tracking of dead vertebrates for ovipositing.

The biochemistry of insect olfaction is different from the olfaction in mammals and not yet completely understood. The most accepted hypothesis is that the odor molecules enter specialized olfactory sensillae on the surface of an insect antenna. Here, they are bound to odorant binding proteins. This binding complex is able to activate olfactory receptors on the surface of axons inside the sensillae, which can lead to an electrophysiological reaction (24). In two separate studies, Kaib (25) and Huotari and Mela (26) identified several compounds, which were electrophysiologically active on the antenna of *C. vicina*. In both studies, the compounds were not identified as products of vertebrate decay, but chosen randomly. Compounds such as dimethyl disulfide or dimethyl trisulfide, which occur frequently in recent literature as byproducts of vertebrate decomposition, were not tested during these earlier studies. The first study, combining trace analysis of dead vertebrates with electrophysiology on *C. vicina,* was performed by Stensmyr et al. (20). They identified dimethyl sulfide, dimethyl disulfide, and dimethyl trisulfide as active on the antenna of the blowflies *C. vicina* and *Lucilia caesar*. However, they sampled the decaying vertebrate only once and did not provide information on its stage of decay. To fill this gap, the volatile patterns of five decaying mice were analyzed. Thereby, the antenna of *C. vicina* was used as a phylogenetically adapted information filter to select core volatiles of vertebrate decay.

Materials and Methods

Collection of Volatiles

During the late summer of 2008, five mice (*M. glareolus*) were placed in a deciduous forest near Göttingen, Lower Saxony, Germany. The mice were caught in the forest in live traps to avoid the killing of preserved mouse species. The traps were controlled twice a day, and mice of the species *M. glareolus* were captured and killed using CO_2. Approval from the university's Animal Ethics Committee was obtained prior to trapping the mice. Wild animals were used so as to allow a natural variation of the chemical composition of the mouse tissues. The mouse cadavers were placed inside casings made from wire and fixed to the ground with tent pegs to protect them against scavengers. To collect the volatiles, the dead mice were placed in 1.5-L polyethylene bags (Confresco Frischhalteprodukte, Minden, Germany) connected to membrane pumps (Thomas Division, Sheboygan, WI). The pumps withdrew air from the environment (70 L/h) through a 3Å molecular sieve filter (Carl Roth GmbH & Co KG, Karlsruhe, Germany), the bag, and an adsorbent trap. The volatiles were trapped on charcoal (CLSA; Gränicher & Quartero, Daumazan, France) for 60 min and TENAX® (TDS; Gerstel, Mühlheim, Germany) for 30 min. In 2008, between August 29 and September 24, 29 samples were taken. During sample collection, the temperature was recorded with a HOBO© data logger (Onset Computer Corporation, Bourne, MA). The accumulative degree days (ADDs) were calculated by dividing the sum of the minimum and maximum temperature (°C) per day by 2 and subtracting five (27). Table 1 shows the sampling regime, the start of sampling, and the average temperature between the sampling. Controls with both TENAX® and charcoal traps attached to empty plastic bags with filters were placed 20 m away from the decaying mice to collect the volatiles of the sampling site.

Trace Analysis

TDS Gas Chromatography–Mass Spectrometry (GC-MS)—The TDS tubes were thermally desorbed by a TDS thermodesorption system (Gerstel). The volatiles were focused in a KAS cryotrap (Gerstel) and separated on a 30 m × 0.25 mm HP-5MS (95% dimethyl–5% diphenyl-polysiloxane) nonpolar column (0.25 μL film thickness) in a 6890N Network GC System; both systems were from Agilent Technologies (Santa Clara, CA). The carrier gas was helium with 99,999% purity and a gas flow of 1 mL/L. The oven program was set to an initial temperature of 40°C for 1.5 min, which was then increased at a rate of 7.5°C per min to 200°C with a final hold of 5 min. The 5973 quadrupole mass spectrometer (Agilent Technologies) was operated in the scan mode in the m/z detection range of 20–354 per 0.5 sec. The resulting chromatograms were further evaluated using the Enhanced Chemstation version D00.00.38 software (Agilent Technologies), Mass Spectral Search Library of the National Institute of Standards and Technology (NIST, Gaithersburg, MD), and MS Interpreter version 0.9f (NIST). Authentic standards were employed to confirm the identity of the compounds found in the samples (Table 2). Straight-chain hydrocarbons (C8–C24) were used to calculate the linear retention index (28).

CLSA GC-MS—The CLSA tubes were eluted with 75 μL of a dichloromethane/methanol (2:1) mixture. One microliter of the solution was injected through the injection port of a 6890N Network GC System onto a 30 m × 0.25 mm HP INNOWAX (poly(ethylenglycol)) polar column (0.25 μm film thickness); both systems were from Agilent Technologies. The carrier gas was helium with 99,999% purity and a gas flow of 1 mL/L. The oven program was set to an initial temperature of 50°C for 1.5 min, which was then increased at a rate of 7.5°C per min to 250°C with a final hold of 5 min. A 5973 quadrupole mass spectrometer (Agilent Technologies) was operated in the scan mode in the m/z detection range of 20–354 per 0.5 sec. The resulting chromatograms were further evaluated using the Enhanced Chemstation version D00.00.38 software, Mass Spectral Search Library of the National Institute of Standards and Technology, and MS Interpreter version 0.9f. Authentic standards were used to confirm the identity of the compounds in the samples (Table 2). Straight-chain hydrocarbons (C8–C24) were used to calculate the linear retention index (28). The identified compounds were quantitated from the peak areas of selected single ions in the CLSA eluate. Dimethyl disulfide was quantitated on the basis of m/z = 94; undecane, m/z = 71; heptanal, m/z = 70; 3-methylbutan-1-ol, m/z = 55; octan-3-one, m/z = 99; hexan-1-ol,
m/z = 56; dimethyl trisulfide, m/z = 126; nonanal, m/z = 57; 1-octen-3-ol, m/z = 57; phenol, m/z = 94; and indole, m/z = 117. The median, minimum, and maximum of the compound concentrations are listed in Table 3. The samples were grouped into the following stages based on the optical impression of the stages of decay of the mice: fresh, bloated, active decay, and advanced decay (Table 4). The characteristics of each stage were presented by Dekeirsschieter et al. (12) and reviewed by Carter et al. (29). In few cases, the decaying mice showed characteristics of two stages, for instance, of the active stage and the advanced stage. The same effect was described by Matuszewski et al. (30), in relation to decaying pig carcasses. In these cases, the predominant stage was selected to evaluate the

TABLE 1—*Sampling regime of the 29 samples of the five mice, the average daily temperature, the minimum and maximum temperature, the stage of decay according to Table 4 and the accumulated degree days (ADDs).*

Date	Average daily temperature (°C)	Minimum daily temperature (°C)	Maximum daily temperature (°C)	Mice (stage of decay;ADD)
29 August 2008	12	6	19	1 (fresh;0)
30 August 2008	11	5	19	1 (fresh;40)
31 August 2008	11	5	17	1 (bloated;53)
1 September 2008	11	4	16	
2 September 2008	10	4	16	1 (active;76),2 (fresh;0)
3 September 2008	11	3	16	1 (active;85), 2 (bloated;20)
4 September 2008	10	2	16	
5 September 2008	10	3	16	1 (advanced;103),2 (active;38)
6 September 2008	10	3	16	
7 September 2008	12	6	17	1 (advanced;124), 2 (advanced;60)
8 September 2008	15	10	27	
9 September 2008	16	10	30	3 (fresh;0)
10 September 2008	16	10	19	3 (fresh;22)
11 September 2008	16	10	20	3 (bloated;35)
12 September 2008	16	11	22	3 (active;47), 4 (fresh;0)
13 September 2008	16	11	21	3 (advanced;55), 4 (fresh;20)
14 September 2008	16	11	21	
15 September 2008	15	11	20	4 (fresh;30), 5 (fresh;0)
16 September 2008	14	11	20	
17 September 2008	13	10	19	4 (fresh;48), 5 (fresh;22)
18 September 2008	13	9	19	4 (bloated;57), 5 (fresh;31)
19 September 2008	13	8	19	
20 September 2008	12	8	19	
21 September 2008	12	7	19	4 (active;84), 5 (bloated;59)
22 September 2008	13	9	18	4 (advanced;90), 5 (active;65)
23 September 2008	10	2	15	
24 September 2008	18	11	30	5 (active;77)

correlation between the optical impression, ADDs, and selected compounds in the volatile profile (Fig. 1).

Flies

In the autumn of 2008, female *C. vicina* were caught from fresh ground meat (half pork, half beef) in the city of Göttingen. The species *C. vicina* was chosen as it is one of the first to arrive on a dead vertebrate and is present during all stages of decay, excluding the dry stage. This species is considered one of the most important in forensic entomology (22,23) and is a model organism for the olfaction of blowflies (20,25,26). Although *Calliphora vomitoria* is reported to be more abundant in forests of central Europe (31), it was decided nevertheless that *C. vicina* would be used. After removing the antennae for electrophysiological experiments, the development of the ovaries was assessed (32). This was used as an indicator of the predilection of the flies to oviposit and therefore exhibits high electrophysiological performance. Only flies with developed ovaries were selected for the electrophysiological measurements.

Electroantennogram

All compounds identified in the CLSA samples were tested in paraffin oil (Uvasol quality; Merck/VWR, Darmstadt, Germany) dilutions from 10^{-9} to 10^{-2} mg/mg. For each dilution step, a 2 cm^2 filter paper was drenched with 200 µL of the dilution. The filter paper was inserted into a 10-mL glass syringe, and 2.5 mL of the air was blown into a stream of humidified air (500 mL/min, 23°C, 80% relative humidity), which was passed over the excised antenna of female *C. vicina*. For each dilution, three antennae were used, and every antenna was blown three times. The depolarization of the antennae was monitored by an electroantennographic (EAG) setup (Professor Koch, Kaiserslautern, Germany) consisting of a preamplifier, main amplifier, frequency filter, and adjustment amplifier The signals were analyzed by the GC Chemstation software. (for more details, see Weissbecker et al. [33]). Significant differences in responses to the negative paraffin oil control on filter paper and the compound dilution were calculated by the *U*-test or *t*-test.

Results

Decaying Process

Table 4 shows the four different stages of decay according to the description given by Dekeirsschieter et al. (12). This approach was used to group the volatile samples. *C. vicina* is present during all of these stages (22,23). In Table 1, the sampling regime, the average daily temperature, the stage of decay, and the ADDs are shown. During the experiment, the average day temperature varied between 10°C and 18°C. While the first three mice entered the bloated (Fig. 3) or active stage (Fig. 4) after one or 2 days, mouse four entered the bloated stage after 6 days and mouse five after 4 days.

The ADD was calculated to compare the temperature dependent processes of decay. However, the values of the ADDs were not correlated to the stages of decay in this experiment. The fresh stage (Fig. 2) shows a maximum ADD of 48, while the bloated stage shows a minimum ADD of 35. The ADD range of the active stage of decay (ADD_{min} = 20; ADD_{max} = 85) overlaps with all other stages, whereas only the advanced stage (Fig. 5) (ADD_{min} = 55; ADD_{max} = 124) has a constantly higher ADD value than the fresh stage.

In this experiment, insects were present during all four stages of decay, most of them being members of the order Diptera or Coleoptera. The predominant fly families were Calliphoridae (*C. vicina*, *C. vomitoria*) and Dryomyzidae. Calliphoridae were present throughout the decaying process and oviposited occasionally, whereas Dryomycidae preferred the fresh and bloated stages for feeding. A greater number of flies visited the remains during the decay of mouse one and two

TABLE 2—*Compounds identified by both analytical methods, in the order of their linear retention index on the polar column.*

No.	Compound	CLSA (LRI) INNOWAX	TDS (LRI) HP-5MS	Chemical Classes	Authentic Standards (Company, Purity, CAS No.)	Literature
1	Dimethyl disulfide	1083	755	Sulfur compound	Merck, 98%, 624-92-0	(9,12–20)
2	Undecane	1100	1100	Alkane	Acros, 99%, 1120-21-4	(13,14)
3	Heptanal	1181	906	Aldehyde	Acros, 95%, 111-71-7	(9,12,13,17,18,42)
4	3-Methylbutan-1-ol	1195	759	Alcohol	Aldrich, 99,5%, 123-51-3	(1,8,12,47)
5	Octan-3-one	1251	992	Ketone	VWR, 96%, 106-68-3	(8,58)
6	Hexan-1-ol	1341	884	Alcohol	Aldrich, 98%, 111-27-3	(9,16,17,25,42)
7	Dimethyl trisulfide	1386	976	Sulfur compound	SAFC, 98%, 3658-80-8	(9,12–16,19,20)
8	Nonanal	1392	1113	Aldehyde	Merck, 98%, 124-19-6	(8,13,14,17,42)
9	1-Octen-3-ol	1440	985	Alcohol	Merck, 98%, 3391-86-4	(8,17,48,57)
10	Phenol	1980	1021	Aromatic	Aldrich, 99%, 108-95-2	(12,13,15,17,68)
11	Indole	2442	1331	Aromatic heterocycle	Fluka, 99%, 120-72-9	(1,12,16–19,34)

LRI, linear retention index; CAS, Chemical Abstracts Service.

than during the decay of mouse three, four, and five. Oviposition occurred only on mouse one and two. These discrepancies can possibly be explained by shorter daylight hours, which decreased from 14 h during the decay of mouse one and two, to 12 h during the decay of the other mice. The prevalent beetle species observed were *Geotrupes spp.*, Geotrupidae and *Nicrophorus spp*., Silphidae (*Nicrophorus vespilloides*, *Nicrophorus vespillo*).

Trace Analysis

Eleven compounds were identified by both methods adopted in the experiment. The compounds are listed in the order of their retention index on the polar column (CLSA) and are assigned to the following chemical classes: alcohols, ketones, alkanes, aldehydes, sulfur compounds, aromatic compounds, and aromatic heterocycles (Table 2). The quantitative data are presented in Table 3. Both sulfur compounds were predominantly found in the active stage of decay (dimethyl trisulfide, <1–104 ng/μL; dimethyl disulfide, <1–60 ng/μL), as well as 3-methylbutan-1-ol (1–20 ng/μl) and phenol (<1–7 ng/μL). Nonanal was found in high concentrations during the fresh stage of decay (<1–52 ng/μL) and in lower concentrations during the other stages (<1–12 ng/μL). Hexan-1-ol, 1-octen-3-ol, heptanal, and indole were found in very low quantities throughout the decaying process (<1–1 ng/μL). Undecane was present in higher concentrations in samples throughout the entire experiment (2–5 ng/μL), with the exception of one sample in the fresh stage of decay with low concentration of undecane.

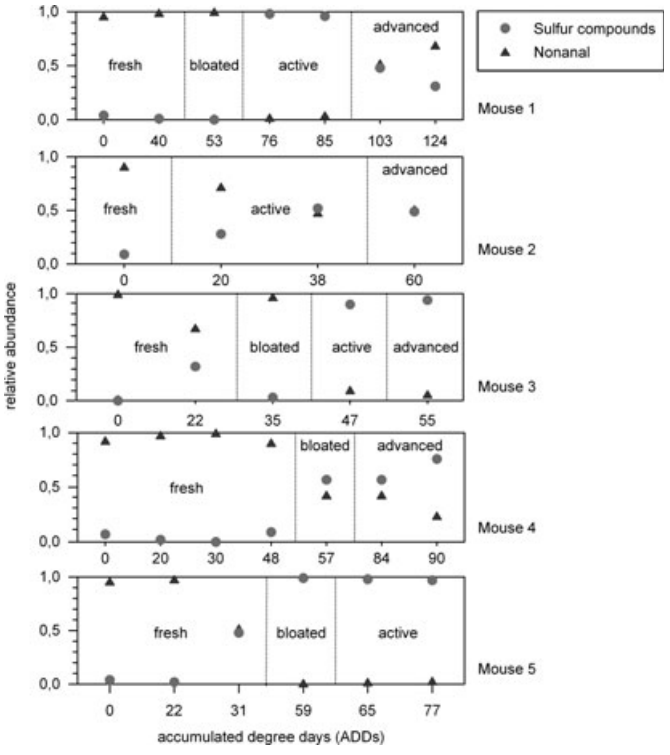

FIG. 1—*Relative emission patterns of nonanal and the sulfur compounds dimethyl disulfide and dimethyl trisulfide across the four stages of decay in the five mice. On the* x*-axis the ADD value of each sample is given and the* y*-axis shows the relative abundance of nonanal and the sulfur compounds.*

Electrophysiology

The results of the EAG experiments are presented in Table 5. Significant differences between the paraffin control and the stimulus were observed with dimethyl trisulfide at a dilution of 10^{-9} and dimethyl disulfide at a dilution of 10^{-8}. Nonanal, hexan-1-ol, 1-octen-3-ol, 3-methyl-butan-1-ol, and heptanal were detected at dilutions of 10^{-5} or 10^{-4}. Octan-3-one, indole, and phenol stimulated the antennae at higher concentrations, and undecane did not stimulate the antennae at all.

Discussion

The idea of distinct stages of vertebrate decay is used by several authors (for review, see [29]). Clearly, this approach is always subjective (29), as the process of decay is continuous. The stage model ignores the fact that a carcass can show the characteristics of two stages of decay at the same time (30). Matuszewski et al. (30) discuss processes-oriented approach and describe the decaying process using the onset, duration, and rate of the decay characteristics. It is most likely that the mosaic effect, discussed by Matuszewski et al. (30), influences the volatile profile, particularly of larger vertebrates. This could be problematic for volatile-based PMI estimation, as the quantity of the emitted volatiles would depend on several overlapping processes. The volatile profile of a small cadaver should not show the influence of the mosaic effect, because the decaying process is much faster. Therefore, an inhomogeneous decay would not affect the volatile quantities to the same extent as in larger vertebrates.

FIG. 2—*Fresh stage of decay of mouse one (29 August 2011).*

FIG. 4—*Active stage of decay of mouse one (2 September 2011).*

FIG. 3—*Bloated stage of decay of mouse two (3 September 2011).*

FIG. 5—*Advanced stage of decay of mouse one (5 September 2011).*

Temperature is another important aspect to be considered. The process of decay is mainly driven by microbial metabolism and insect feeding, and both factors are strongly influenced by the temperature regime. Calculating the ADDs for PMI determination was inspired by entomological studies and evaluated for forensic science (27,34) to compare decaying processes. A linear model seems to be an inappropriate approach, because the temperature has a nonlinear influence on enzymatic activity (5), as well as on the vapor pressure of volatile compounds (35). Vass et al. (34) attempted to solve this problem by calculating the cumulative degree hours for every 12 h, thereby separating day and night temperatures. However, they state that their results still lack any strong correlation between temperature and rate of decay in graves, indicating the influence of other factors like humidity, oxygen availability, or soil composition (34). Michaud and Moreau calculated a nonlinear regression model based on four ADDs, which explained the decay of the 12 exposed pig carcasses in their study (27).

In the experiment conducted in Göttingen, only the fresh and advanced stages of the decaying mice showed no overlapping ADD values. This confirms the limitations of the stages approach and the linear model for rapid decaying processes. In general, describing the decomposition in distinct stages is problematic; however, insect succession on a cadaver is partly correlated to the process of decomposition (22,23,31). This correlation might occur, because certain insect species detect core volatiles, which are always present at a certain stage of the decaying process. The electrophysiological studies in the experiment show dimethyl disulfide and dimethyl trisulfide were detected at very low concentrations of 10^{-9} and 10^{-8} by female blowfly antennae (Table 5). Figure 6 shows the dose–response curve for female *C. vicina* antennae with a calculated ED_{10} of 6×10^{-10} (36). The curve shows biphasic development, which is typical for two olfactory receptor neuron populations of different sensitivities. Furthermore, the formation of dimethyl disulfide and dimethyl trisulfide has been reported in most experimental literature on the decomposition of vertebrates (9,12–17,20). The cited literature, as well as the trace analysis and electrophysiology presented in this study, lead to the conclusion that both compounds are important cues for the process of decay and potential core volatiles. In general, sulfur compounds are formed by the microbial degradation of methionine and cysteine, which are two sulfur-containing amino acids in the body (1,19,37,38). Among the sulfur compounds formed, methanethiol is emitted by the following bacteria: *Hafnia alvei*, *Enterobacter agglomeran*, *Serratia liquefaciens*, *Alteromonas putrefaciens*, and *Aeromonas hydrophila*, which grow on vertebrate tissue (39,40). The oxidative dimerization of methanethiol contributes to the formation of dimethyl disulfide and dimethyl trisulfide both *in vivo* and *in vitro* (41). Furthermore, the bacteria belonging to the Thiobacillus group can transform sulfur compounds under aerobic conditions and might therefore catalyze this reaction (19).

The high sensitivity of female *C. vicina* toward sulfur compounds is important for tracing cadavers, which was demonstrated in behavioral tests (21). However, it is unlikely that sulfur compounds play a role in initial cadaver detection, because the biochemical processes described here do not yield

TABLE 3—*Emission rates of the volatile compounds quantitated in the CLSA samples in ng/µl. The median displays the average concentration in all samples per stage. The* n *value shows the number of samples analyzed in each stage of decay according to Table 4. Min and Max show the minimum and maximum concentration of the volatile compounds in the samples. If no value is given for the minimum, the compound was absent in at least one sample. Less than one indicates the presence of the compound below the threshold for quantitation in all samples.*

	Fresh, $n = 12$			Bloated, $n = 3$			Active, $n = 8$			Advanced, $n = 6$		
Compound	Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max
Dimethyl trisulfide	0	–	<1	<1	0	1	17	<1	104	<1	–	2
Dimethyl disulfide	<1	–	1	1	<1	2	11	<1	60	2	<1	10
Nonanal	2	<1	52	2	2	2	2	<1	12	1	<1	3
Hexan-1-ol	<1	–	1	<1	<1	1	<1	–	1	<1	<1	1
1-Octen-3-ol	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
3-Methylbutan-1-ol	<1	<1	1	1	<1	1	5	1	20	2	<1	3
Heptanal	<1	–	1	<1	<1	<1	<1	–	<1	<1	<1	<1
Octan-3-one	<1	–	2	<1	–	<1	<1	–	1	<1	0	<1
Indole	<1	–	<1	<1	–	<1	<1	–	1	<1	–	<1
Phenol	<1	–	<1	<1	<1	<1	2	<1	7	<1	<1	<1
Undecane	3	–	4	3	2	3	2	2	4	2	2	5

TABLE 4—*Four stages of the decomposition process of the mice during the sampling period.*

Stage of Mice Decay	Length of Time (Days)	Characteristics	Pictures
Fresh	1–4	Rigor mortis; lowered body temperature	Figure 2 shows the fresh stage of decay of mouse one (29 August 2011)
Bloated	1	Putrefaction inside the mice causes bloating; loss of hair begins	Figure 3 shows the bloated stage of decay of mouse two (3 September 2011)
Active decay	1–3	Cadaver inflates and microbial degradation of inner tissues increases	Figure 4 shows the active stage of decay of mouse one (2 September 2011)
Advanced decay	1–3	Most of the soft tissues are assimilated by microbial activity; only hair, bones, cartilage, and skin are left	Figure 5 shows the advanced stage of decay of mouse one (5 September 2011)

sulfur compounds immediately after death. Different volatiles or a combination of factors could be involved in initial cadaver detection. The aldehydes nonanal and heptanal are electrophysiologically active at dilutions of 10^{-5} or 10^{-4}. Among the volatiles in Table 3, nonanal is the only compound present in high concentrations at the onset of decay. Therefore, nonanal could be a cue for the detection of dead vertebrates shortly after death. In general, aldehydes are important in the decay process of vertebrates. Nonanal and heptanal have been identified by several authors (8,9,12–14,17,18,42). Degradation of fat on the skin and fur of the mice might lead to the formation of nonanal in the beginning of the decaying process. It is possible that the same processes that yield nonanal on the living skin (42) also contribute to its formation in the fresh stage of decay. As *C. vicina* is reported to be abundant on cadavers within minutes after death, flies may follow additional indications, for example, the immobility of the cadaver or carbon dioxide concentration. After the onset of decay, the degradation of depot fat or intracellular fat might contribute to the formation of nonanal. Dekeirsschieter et al. (12) mentioned an increase in aldehyde formation during the later stages of decay, and Vass et al. (13) identified aldehydes in high relative concentrations in bones. As fat is a component of marrow that persists even in ancient bones (43–46), the increasing amount of aldehydes in the volatile pattern of decaying vertebrates could be derived from lipid oxidation in bones (47). Unfortunately, there is a lack of detailed studies on biogenic aldehyde formation arising from fatty acid degradation (48,49). Although the beta-oxidation enzymes in prokaryotes, fungi, and plants differ from each other (49,50), their catabolic activity is comparable and yields acetyl-CoA or propionyl-CoA (51). This indicates that aldehydes are not typically emitted from cadavers, but from any source in which lipids are oxidized, and that the emission of nonanal alone is too unspecific for tracing a cadaver in any stage of decay. Therefore, volatile-based PMI estimation or cadaver dog training should include more than one volatile.

Trace analytical and electrophysiological results from the experiment indicate that nonanal and the sulfur compounds are core volatile candidates. The first and second requirements for core volatiles are fulfilled, because both nonanal or the sulfur compounds are present during the whole process of decay and are emitted in high quantities (Table 3). To evaluate whether the volatiles show a constant quantitative emission pattern, the relative quantities of the compounds were displayed. Temperature variation, variation in the adsorption performance of the charcoal tubes, and slightly different suction rates of the pumps can influence the quantity of the volatiles in the samples. Therefore, within one sample, the sum of nonanal, dimethyl disulfide, and dimethyl trisulfide was regarded as 100%. The absolute quantities of both nonanal and the sulfur compounds were expressed as the percentage (0.0–1.0) of this sum. As an example, if 10 ng/µl nonanal, 20 ng/µL dimethyl disulfide, and 20 ng/µL dimethyl trisulfide are found in one sample, the sum is 50 ng/µL. In this case, the relative amount of nonanal in this sample is 10 ng/µL/50 ng/µL = 0.2, and the relative amount of the sulfur compounds in this sample is (20 ng/µL + 20 ng/µL)/50 ng/µL = 0.8. Assuming that temperature and suction rate influence the emissions of the three compounds to the same extent, the relative quantities of all samples are compared in Fig. 1. There is a constant relation between nonanal and the sulfur compounds during the process of decay. In the early stages of decomposition, nonanal dominates, whereas in the later stages, this relation changes and the sulfur compounds are predominant.

TABLE 5—*Dose–response relationship of female* Calliphora vicina *antennae. All compounds were tested. Shown are medians and the upper and lower quartiles of the antennal response in mV;* n = *3 female* C. vicina *for each dilution and the paraffin oil control. The* t*-test or* U*-test showed significant differences between the dilution and the paraffin oil control.*

	Paraffin Oil	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}
Dimethyl trisulfide	21 (17,28)	31 (27,43)***	41 (34,51)***	59 (53,79)***	86 (72,89)***	100 (94,104)***	111 (102,120)***	132 (121,136)***	146 (130,146)***
Dimethyl disulfide	24 (16,65)	29 (25,58)	26 (26,75)*	31 (25,101)**	26 (21,75)*	56 (29,104)***	79 (26,93)***	83 (34,151)***	96 (28,148)***
Nonanal	4 (2,28)	4 (3,30)	5 (3,31)	4 (3,34)	3 (3,34)	8 (6,52)***	24 (12,68)***	22 (12,76)***	36 (22,116)***
Hexan-1-ol	6 (4,35)	4 (4,29)	6 (4,38)	7 (4,37)	6 (4,51)	7 (6,64)**	19 (14,75)***	43 (36,160)***	87 (74,249)***
1-Octen-3-ol	8 (5,18)	6 (6,25)	5 (5,39)	6 (5,33)	7 (5,49)	8 (6,54)*	11 (10,67)***	17 (16,113)***	50 (46,227)***
3-Methylbutan-1-ol	20 (14,23)	23 (13,26)	18 (16,27)	22 (12,25)	29 (13,31)	29 (12,35)	32 (15,36)**	30 (23,51)**	52 (48,88)***
Heptanal	30 (20,65)	23 (18,81)	25 (13,100)	28 (21,86)	31 (25,82)	35 (24,108)	50 (32,120)*	79 /36,184)***	164 (38,257)***
Octan-3-one	20 (14,23)	20 (17,21)	19 (13,24)	21 (11,25)	20 (9,21)	25 (10,30)	28 (10,31)	23 (21,30)**	40 (37,59)***
Indole	30 (20,65)	28 (26,58)	38 (14,68)	44 (13,96)	36 (14,84)	36 (20,74)	40 (20,97)	44 (23,107)*	40 (25,122)*
Phenol	20 (14,23)	22 (20,24)	22 (15,25)	18 (8,33)	25 (8,35)	24 (9,31)	21 (11,28)	28 (15,38)	32 (25,51)***
Undecane	20 (14,23)	20 (17,20)	14 (13,17)	17 (10,17)	17 (8,29)	18 (10,23)	21 (10,30)	19 (12,26)	26 (17,49)

$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

During the decay of mouse one and mouse three, this relation changed abruptly, whereas mouse two, four, and five show a slower change in the relation. Abrupt temperature changes did not occur (Table 4); therefore, different factors influenced the relation between nonanal and the sulfur compounds. One important factor could be the condition of the intestine during the early stages of decay. An intact intestine will lead to an accumulation of volatiles inside the cadaver. In this case, the cadaver would bloat until the skin breaks and the volatiles leave the body, changing the volatile profile abruptly. If there are holes in the intestine because of insect feeding or physical injury, the release of decompositional volatiles will occur earlier and in slowly increasing quantities. The mosaic effect might also influence the volatile emission in the early stages of decay. Most probably, the process of bloating cannot be regarded as the bloating of the intestine as a whole. While gases accumulate in intact regions of the intestine, they might leave through injuries in other parts. This could be important, especially where larger vertebrates are concerned.

The emission pattern presented in Fig. 1 shows a constant development of the relation between nonanal and the sulfur compounds for each mouse. However, the changes in the volatile pattern cannot be correlated accurately to the stages of decay. In the fresh stage of the decay (Fig. 2), nonanal clearly dominates the volatile profile. The only exception is the third sample of mouse five, where nonanal and the sulfur compounds are emitted in equal concentration. The volatile composition in the bloated stage (Fig. 3) does not show such a consistency. Mouse one and three show a high concentration of nonanal, whereas mouse five shows a high concentration of the sulfur compounds. Mouse four shows an almost equal concentration of both. In the active stage, the sulfur compounds are predominant in the volatile profile. Exceptions are the first sample of mouse two (ADD 20), where the concentration of nonanal is high, and the second sample in the active stage (Fig. 4) of mouse two where the concentrations of nonanal and the sulfur compounds are equal. In the advanced stage (Fig. 5), the compounds are either emitted in the same relative quantities (mouse one, ADD 103; mouse two and mouse four, ADD 84), or the sulfur compounds are emitted in relatively higher quantities (mouse three and mouse four, ADD 90). In the sample with the highest ADD, nonanal dominates (mouse one, ADD 124). An increasing amount of aldehydes in the later stages of decay has already been reported by Dekeirsschieter et al. (12) and might be connected to lipid oxidation in bones (13,43–46).

The calculated ADD values do not sufficiently explain the volatile emission. A nonlinear model might show a stronger correlation. In this study, the dominance of the nonanal emission lasts to an ADD range between ADD 20 and ADD 53. The ADD range in which the relation between nonanal and the sulfur compounds changes begins at ADD 31 and ends at ADD 84, while the ADD values of the sulfur compound dominance range between ADD 47 and ADD 90.

Neither the stages approach nor the linear temperature model can explain the volatile emission in this experiment. New models have to be developed to describe the process of decay to evaluate the hypothesis that the PMI can be estimated by means of volatile analysis. Temperature-based models might lack availability of data, as it has been found that temperature data provided by weather stations are not always sufficient for PMI estimation (52). More quantitative data on volatile emissions must be collected. Additional core volatiles emitted by larger vertebrates could increase the correlation of the volatile

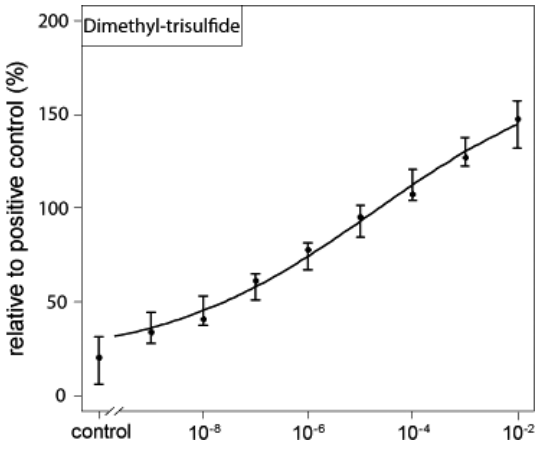

FIG. 6—*Biphasic development of the dose–response reaction of female* Calliphora vicina *antennae toward dimethyl trisulfide.*

emission during the decaying process. Investigation into the olfaction of other carrion visitors would be interesting, especially those species that appear during the early stages of decay, and may detect important core volatiles (31).

The higher fusel alcohol 3-methylbutan-1-ol is a candidate for an additional core volatile in the model presented in Fig. 1. Female blowflies can detect this compound at a dilution of 10^{-4} (Table 5), but in this experiment, the compound showed a high quantitative variation between the samples. 3-Methylbutan-1-ol is a byproduct of L-leucine degradation by the Ehrlich pathway (53). It is also a byproduct of the anabolic biosynthetic pathways in microbes (47,54,55). Dekeirsschieter et al. (12) identified the compound in the headspace of decaying pig, and Ercolini et al. (8) detected the compound in the headspace of tainted meat. *Moraxella phenylpyruvica*, *Staphylococcus xylosus*, *S. starnosus transforme*, *Saccharomyces cerevisiae,* and *Candida utilis* degrade leucine to 3-methylbutan-1-ol (53,54,56). The experiments performed in Göttingen showed that female blowflies could detect hexan-1-ol and 1-octen-3-ol at dilutions of 10^{-5} (Table 5). Both compounds showed very low absolute and relative emission in this experiment (Table 3), which discriminates them as core volatiles. However, human remains might emit the compounds in higher quantities. The eight-carbon volatile compound 1-octen-3-ol is formed during lipid oxidation of compounds such as linoleic acid (48) and arachidonic acid (57). The compound is predominantly emitted by fungal activity (58–63) and is used as the main fungal aroma compound in the food industry (64). 1-Octen-3-ol forms during the degradation of muscle tissue (8,17) human blood, bone, and fat (17); and the skin of fish (57). Haze et al. (42) identified hexan-1-ol in the headspace of living human bodies, while Hoffman et al. (17) identified this compound in the headspace of decaying human blood, testicle, muscle, fat, and bone. The compound is also emitted by tainted meat (9) and human cadavers (16). Fungal metabolism (58–63) and lipid oxidation in plants (65) also yield hexan-1-ol. The detection of 1-octen-3-ol and hexan-1-ol by *C. vicina* antennae (Table 5) could be useful for tracking the uptake of nutrition from flowers (25) and fungi (58), but is not necessarily important when tracing a cadaver.

Octan-3-one, indole, phenol, and undecane are, essentially, insignificant compounds to female blowflies, and among these volatiles, only undecane shows slightly higher concentrations in the volatile samples of decaying mice (Table 3). This discriminates them as core volatiles, although this might be different for larger vertebrates. Octan-3-one is the second eight-carbon volatile identified in this experiment. It has been identified in the headspace of tainted meat (8) and is prominent in the headspace of fungi (58). Lipid oxidation in fungi yields octan-3-one (48). Indole, a degradation product of the amino acid tryptophan (66), is found to be emitted from decaying pig (12); human blood, testicles, and adipoceres (17); and decaying humans (16,34). The volatile compound phenol was identified in the headspace of decaying pig (12) and decaying human remains (13,15) and is derived from the bacterial metabolism of tyrosine in organisms such as the human intestine (67,68). Undecane was detected in the headspace of human bones and at human burial sites (13,14). Further information on postmortem volatiles of vertebrate tissue can be found in Paczkowski and Schütz (69).

Conclusions

The pattern of volatiles emitted from decaying vertebrates is complex. The olfactory perception of *C. vicina* and other carrion visiting invertebrates can help in the selection of core volatiles. Among the detected substances, sulfur compounds are the most important cues for tracing deceased vertebrates. Examination of the relative amounts of sulfur compounds and nonanal facilitates differentiation within the decaying process showed that the relative amounts are correlated neither to the stages of decay nor to the calculated ADD. This information might be a step toward establishing a volatile-based postmortem estimation method with potential forensic application.

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