DOCTOR OF VETERINARY SCIENCES

The smell of death

Development of detection methods and applications for « cadaver dogs » training

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Award date:
2023

Awarding institution:
Université de Liège
University of Namur

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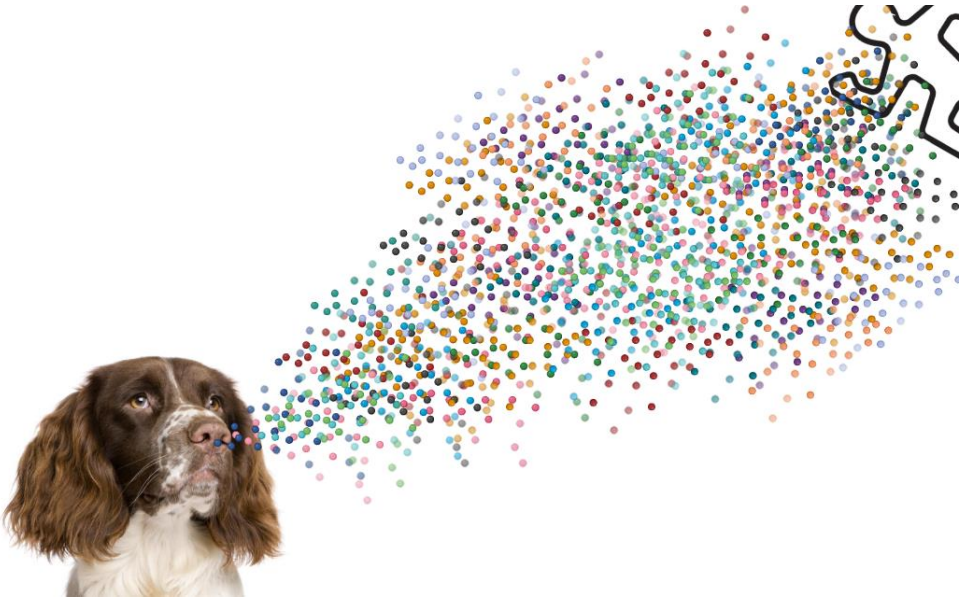

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THE SMELL OF DEATH

Development of detection methods and applications for « cadaver dogs » training

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In fulfilment of the requirements of the PhD Degree in agricultural sciences and biological engineering (GxABT – "docteur en Sciences Agronomiques et Ingénierie Biologique") and in veterinary sciences (UNamur – "docteur en Sciences Vétérinaires)

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Academic Year: 2022-2023

“Fear of a name increases fear of a thing itself”

Albus Dumbledor

À ma fille Abigaël

À ma famille

Abstract

The decomposition of a human corpse is a complex process in which the body's building blocks (e.g., carbohydrates, proteins, lipids) are broken down into by-products thank to chemical reactions mediated by bacterial activity. Volatile organic compounds (VOCs) are quickly released, but their diversity and abundance are changing over the course of the decomposition. Although several studies have attempted to characterize its composition, the methodologies used would greatly benefit from being optimized. The characterization of the so-called "the smell of death" can lead to several applications, including the training of police dogs specialized in the location of human remains. Unfortunately, this research topic is still largely understudied.

During the present PhD thesis, we have performed two reviews of the scientific literature: (i) on the odor profile of human cadavers and (ii) on the selection and training procedures of "cadaver dogs". Then, we have set three specific -but complementary- objectives: (i) Optimizing methods for collecting cadaveric compounds, especially in complex environments, and at the same time deciphering the impact of the environment a corpse decays in on the emission of VOCs; (ii) Developing a gas chromatography method to characterize cadaveric VOCs of freshly deceased people; and (iii) Optimizing training conditions for police dogs in order to improve their performance when searching for human bodies.

In **the first experimental chapter** of this thesis, we have evaluated the impact of three environmental conditions on the decomposition of vertebrate cadavers, using rats as surrogate human models: open-air decomposition, underground decomposition, and underwater decomposition. We have developed original methodologies to collect the cadaveric VOCs. In all scenarios, sulfur and nitrogen containing compounds were highlighted. During the open-air decaying process, we found necrophagous insects not only hasten the decaying process, but also impact the diversity of volatiles released at the beginning of the decomposition. When studying underground decomposition, we found the texture of the soil to influence the diffusion of VOCs in surrounding soil layers, where most decomposition VOC could be identified. Finally, while studying immersed vertebrate remains, we collected less cadaveric compounds than in other scenarios, suggesting that many could be dissolved in the water. No influence of the water salinity has been observed on the odor profile released at the water surface.

Investigating human cadaveric profile remains a major issue, which justifies the use of animal models (*i.e.*, pig and rats) Limitations in the existing literature dealing with

human cadaveric volatilome include the small sample size and the high variability of investigated decomposition stages. In **the second experimental chapter**, we developed a target ion gas chromatography method to characterize the VOCs profile released by freshly deceased people, a decomposition stage barely studied. We succeeded in identifying 30 cadaveric compounds among which those containing sulfur were the most abundant.

The use of dogs to locate decaying remains is common. However, we have very limited information to understand what make these dogs efficient or how they could be efficiently trained to perceive specific cadaveric odors. In **the third experimental chapter**, we explored the post-training abilities to locate cadaveric volatiles in human remains detection dogs. For the first time, a definition of an efficient detection dog is suggested as well as a method to assess dogs' performance. Behavioral assays performed on police dogs also highlighted that sulfur containing compounds drive the target recognition, and that they could either belonged to cadaveric compounds or not.

To conclude, the results of this thesis provide new and complementary information on the cadaveric volatile profile, as well as on the behavior of human remains detection dogs.

Résumé

La décomposition d'un corps humain est un processus complexe au cours duquel les éléments constitutifs du corps (*e.g.*, les glucides, les protéines, les lipides) sont dégradés en sous-produits par le biais de réactions chimiques et via l'activité bactérienne. Des composés organiques volatils (COVs) sont rapidement libérés. Cependant leur nature ainsi que leur abondance varient au cours de la décomposition. Même si plusieurs études se sont efforcées de caractériser sa composition, les méthodologies utilisées gagneraient grandement à être optimisées. La caractérisation de ces composés peut déboucher sur plusieurs applications, dont celles sur le dressage des chiens policiers spécialisés dans la recherche de restes humains. Malheureusement, ce domaine d'application reste largement sous-étudié.

Lors de cette thèse, nous nous sommes fixés trois objectifs distincts mais néanmoins complémentaires : (i) Décrypter l'impact de l'environnement dans lequel un cadavre de rat se décompose sur l'émission de COVs ; (ii) Développer une méthode de chromatographie en phase gazeuse pour caractériser les COVs émis par des cadavres humains frais ; et (iii) Explorer les bases de l'entraînement sur la capacité des chiens détecteurs à localiser ces composés volatils.

Dans le premier chapitre expérimental de cette thèse, nous avons évalué l'impact de trois conditions environnementales sur la décomposition de cadavres de vertébrés : la décomposition à l'air libre, la décomposition sous terre et la décomposition aquatique. Parmi ces scénarios, certains COVs communs ont été mis en évidence (*e.i.*, les composés contenant du soufre et de l'azote). Néanmoins, certains sont impactés par l'environnement dans lequel la décomposition a lieu. D'abord, les insectes nécrophages, non seulement, accélèrent le processus de décomposition, mais ont également un impact sur les volatils libérés au début de la décomposition à l'air libre. Ensuite, la texture du sol dans lequel les restes se décomposent, influence la diffusion des COVs au sein des couches de sol environnantes. Enfin, les restes de vertébrés immergés libèrent moins de COVs que dans d'autres scénarios, laissant penser que certains pourraient rester dissous dans l'eau. Aucune influence de la salinité de l'eau n'a été observée sur le profil en COVs émis à la surface de l'eau.

L'utilisation d'animaux (*e.g.*, porcs ou rats) pour localiser les restes en décomposition est courante. Les données scientifiques permettant de mieux comprendre leur efficacité ou leurs capacités à percevoir des odeurs cadavériques sont cependant peu nombreuses. **Dans le deuxième chapitre expérimental**, nous avons développé une méthode de chromatographie en phase gazeuse ciblant certains fragments ioniques pour caractériser le profil des COVs émis par des personnes

récemment décédées. Malgré que la chromatographie en phase gazeuse bidimensionnelle soit recommandée pour l'étude des COVs cadavériques, notre méthode a permis de mettre en évidence 30 composés cadavériques, parmi lesquels les composés soufrés constituent la classe chimique la plus représentée.

Malgré que les chiens soient recommandés pour la détection de cadavres, les données scientifiques permettant de mieux comprendre leur efficacité ou leurs capacités à percevoir des odeurs cadavériques sont rares. **Dans le troisième chapitre expérimental**, nous avons cherché à explorer les capacités post-formation des chiens de détection de restes humains. Pour la première fois, une définition d'un chien de détection efficace a été proposée ainsi qu'une méthode d'évaluation de ses performances. Des tests comportementaux effectués sur des chiens de détection de restes humains de la police ont mis en évidence que les composés soufrés entraînent la reconnaissance de la cible, qu'ils soient d'origine cadavérique ou non.

En conclusion, ces résultats contribuent à mieux comprendre l'émission de COVs cadavériques, ainsi que le comportement des chiens de recherche de restes humains.

Acknowledgments

« *L'éducation est une chose admirable, mais il est bien de se rappeler de temps en temps que rien de ce qui vaut la peine d'être appris ne peut être enseigné.* »

Oscar WILDE

Je suis très fier d'avoir réalisé cette thèse de doctorat qui m'a permis d'acquérir beaucoup de compétences. J'ai toutefois tout autant appris en compagnie des gens que j'ai côtoyés pendant cette période que lors de la réalisation de ce travail. C'est pourquoi, je souhaite remercier toutes les personnes, qui ont participées ou non à ce travail mais qui, chacun à leur manière, l'ont rendu possible !

Tout d'abord, je souhaite remercier l'ensemble des membres de mon jury de thèse pour leur bienveillance et leurs conseils avisés. La plupart d'entre vous ont commencé l'aventure bien avant le stade du jury final puisque vous m'avez accompagné tout au long de mon parcours, pour certains depuis ma première année de bachelier pour d'autres depuis le début de ma thèse en tant que membres de mon comité. Malgré le sujet qui, je dois bien avouer, est particulier, vous avez été enthousiastes et positifs. Vous m'avez donné suffisamment de latitude pour que je puisse m'épanouir dans ce travail. Je suis très heureux de passer ce cap avec vous. Je vous adresse donc un grand merci !

Ma première pensée va à ceux sans qui je ne me serais jamais lancé dans l'aventure. **Antoine** et **Solène** mes compères des 400 coups. Vous avez toujours été là et cela depuis mon travail de fin d'études, pendant lequel, je vous amenais mes mouches une par une pour commencer un élevage … Pas très « fute-fute » le garçon ! Vous avez bien sûr été présents pour m'accompagner dans mes recherches mais on n'est pas ici pour parler sciences ! Quand je pense à vous, je me rappelle nos soirées téquila, nos soirées premier train à Bruxelles et bien évidement nos sorties en Ardennes (là où l'Orval coule à flot). Toutes ces soirées ont d'ailleurs laissé quelques séquelles (#Antoine) … Mais nous ne sommes pas uniquement des compagnons de beuverie ! Non seulement on a beaucoup ri mais vous m'avez aussi permis de grandir. Vous m'avez aidé à dompter mon tempérament électrique. Vous m'avez aussi appris que toutes les batailles ne valent pas la peine d'être menées. Ce qui est sûr c'est que sans vous, cette thèse n'aurait jamais commencé. On a certes eu quelques bas mais au fond, il n'y a que les gens qu'on aime qui nous font sortir de nos gons, les autres on les ignore ! Un grand merci et une grosse lèche à vous deux !

Maryse, comment te dissocier de Solène et Antoine lorsque je pense au

commencement de l'aventure ! On s'est aussi rencontrés pendant mon travail de fin d'études. Tu as d'ailleurs accompli une mission impossible : me faire aimer les statistiques. Tu m'as aussi transmis ta rigueur. Tu es une vraie source d'inspiration. Tu m'as appris qu'il faut toujours se relever après un échec. Quand une porte se ferme, d'autres s'ouvrent ! De professeur, tu es vite devenue une amie et une confidente ! Tu n'es pas pour rien dans le début de ce travail (toi-même tu sais !). Je tenais à te dire que j'admire ton courage et ta persévérance. D'ami à ami, un immense merci.

Bérénice, alias Batman ou plutôt Batgirl ! Une rencontre exceptionnelle. Je suis très fier d'avoir gagné ton amitié. Tu es une très belle personne. Je sais que compter parmi tes proches est un privilège ce qui rend cette amitié encore plus belle. Je peux compter sur toi et l'inverse est vrai aussi. J'ai trouvé en toi un alias. Ça m'a permis de moins me sentir à part. Les gens excessifs ne courent pas les rues. Certains diront que c'est un défaut mais on s'en fou n'est-ce pas ? Un grand merci pour m'avoir compris et supporté. Le bar à cocktail est prêt pour fêter ça !

François quelle aventure ! Déjà 6 ans que l'on travaille ensemble. On ne peut pas dire que le chemin n'a pas été semé d'embuches. Cependant, je suis très content du travail que nous avons fait ensemble. Il faut dire qu'on a traversé quelques tempêtes (l'arrivé à terra, le covid, ton année sabbatique). Malgré tout, on a gardé une très chouette relation professionnelle de confiance. Tu sais te rendre disponible et j'admire ta capacité à te mettre à la place de l'autre pour mieux comprendre ses positions. C'est une qualité rare. Je ne peux que te remercier car ce qui est sûr c'est que ce travail tu en es à l'origine. Tu as cru en moi et en mon projet là où beaucoup auraient lâché prise à la sortie du FRIA. Je te remercie pour tout ce temps que tu m'as accordé et pour avoir cru à mes idées un peu loufoques, je te l'accorde. On a eu nos hauts et nos bas, mais finalement c'est ça une relation d'amitié !

Claire, tu t'es impliquée dans ce travail dès le début. Tu as toujours été présente pour moi. De membre du comité, tu es très rapidement passée dans les acteurs principaux de ce travail. Tu auras été une très belle rencontre. Tu m'as appris à me faire confiance. Tu m'as intégré dans des projets très intéressants. Je te remercie pour ton encadrement et ces moments de complicité qu'on a pu avoir.

Je tenais aussi à te remercier **Frédéric.** Tu m'as fait confiance en m'offrant la possibilité d'être assistant au sein de ton équipe. Je t'en suis très reconnaissant d'autant plus que ce poste est passionnant de par sa diversité de tâches. J'espère sincèrement avoir rempli la mission. Je voulais aussi te remercier pour les opportunités de collaboration que tu m'as données. Cela m'a permis de rencontrer plein de personnes avec lesquelles j'ai appris énormément de choses. J'ai aussi toujours pu te parler franchement, sans fioritures. C'est une de tes qualités que j'apprécie beaucoup. Pour tout cela, je ne peux que te remercier sincèrement.

C'est naturellement que je remercie mon Poulet**,** probablement la plus belle rencontre depuis le début de ma thèse. Je t'accord qu'on a souvent eu des problèmes de communication. Enfin, j'ai souvent eu du mal à te comprendre. Je ne sais pas de qui venait le problème, même si j'ai appris récemment que Laetitia avait aussi beaucoup de mal à comprendre tes blagues. Mon confident, ma soupape de sécurité, à l'occasion mon secrétaire, je parle évidement de toi, **Nicolas**. Ton départ à changer la donne ! J'ai dû faire peur aux autres parce que ta place est restée vide très longtemps… Bon je dois avouer que j'ai plutôt été clair à ce sujet ^^. Une période de deuil s'imposait. Pour tout ce que tu m'as apporté, je ne pourrai jamais assez te remercier.

Comment écrire des remerciements sans parler de ceux que j'aime appeler mes conseillers, mes balises, mes gardes fous ! **Emilie Bosqué** et **Greg.** Emilie, au final, on n'a fait que se croiser. Quelques mois après mon arrivé, tu partais vers de nouvelles aventures. Il était donc peu probable que l'on garde de si bons contacts. Néanmoins, le peu de temps passé ensemble à suffit pour créer une belle complicité. Nos rencontres annuelles pour discuter de nos vies sont toujours un réel plaisir. Greg à l'inverse on a en quelques sortes commencer l'aventure ensemble, et quelle aventure ! On a partagé nos victoires et nos défaites, nos moments de doutes et nos certitudes. Les meilleurs moments restent nos anecdotes sur les étudiants (sans jamais se moquer bien sûr …). Pour toutes ces raisons et chacun à votre manière vous avez épicé cette thèse. Un immense merci à vous deux !

Je tenais aussi à remercier ceux que j'ai souvent qualifié de piliers : **Jeannine**, **Didier**, **Catherine** et **Sandra**. On a passé de très bons moments ensemble. Vous m'avez appris à m'épanouir tant professionnellement que personnellement. Votre oreille attentive était toujours là pour m'écouter dans mes hauts comme dans mes bas. Je ne peux pas parler des piliers, sans consacrer une partie plus particulière à toi Jeannine, ma coach de travaux pratiques. Tu m'as formé et m'a permis de m'épanouir dans mes enseignements. J'ai passé de très beaux travaux pratiques. Cela n'a jamais été une corvée. J'ai beaucoup de plaisir à partager ces moments avec toi. On aura quand même beaucoup rigolé. Je ne peux que te remercier pour tous ces moments passés ensemble. Tu es sans aucun doute un modèle d'engagement. Les étudiants et l'ensemble des assistants ont beaucoup de chance de t'avoir. Du fond du cœur, Jeannine, je te dis merci pour tout.

Je remercie également tous mes collègues de début de thèse : **Laurent**, **Gwendoline**, **Bertrand**, **Diana**, **Landry** et **Julien**. Ils m'ont tous accompagnés et soutenu pendant les débuts de cette thèse. Un big up spécial à Diana pour sa bienveillance et son intérêt tant pour mes projets de recherches que pour mes accomplissements personnels. J'adresse également un grand merci aux collègues

arrivé plus tardivement : **Chloé**, **Joachim, Ibtissem, Arnaud, Lallie**, **Fanny**, **Solène**, **Julie**, **Andréa**, **Régis**, **Mathieu, Sofia, Asma** et **Marta**. J'ai eu le plaisir de vous rencontrer et vous voir vous épanouir au sein de l'équipe. Vous êtes tous de très belles personnes. J'ai beaucoup aimé discuter avec vous surtout lors de nos midis thématiques. Le vendredi cul reste l'incontournable. Le boulot d'assistant n'est pas facile car on voit partir des amis qui se font remplacer par d'autres. Les transitions sont compliquées. Et pourtant ! Vous avez été tous géniaux. Vous m'avez permis de passer les caps difficiles. Merci à tous pour ça !

Je remercie également l'ensemble des étudiants qui m'ont aidé à réaliser ce travail : **Damien**, **Noémie**, **Sorenza**, **Vincent**, **Margot** et **Audrey**. J'ai aussi une pensé pour tous les étudiants avec lesquels j'ai pu collaborer : **Florence**, **Quentin**, **Jessica**, **Anne**, **Rhôxane**, **Nicolas**, **Pauline**, **Thibaut**, **Brice**, **Remy**, **Gian Carlo**…

Je remercie chaleureusement l'ensemble des membres du DVI ainsi que les maitres-chiens. Vous avez toujours été là et cela depuis mon stage de master. Je vous remercie pour votre enthousiasme et votre intérêt. Sans vous ce projet n'aurait surement pas été possible. J'ai une pensée particulière pour **Claudia** et **Patricia** du DVI avec qui j'ai toujours d'excellent contacts et qui ont toujours cru en mon projet. Je ne peux pas parler des Maitres-chiens sans parler de toi, **Kris** ! Meri pour ton implication et ton intérêt pour le projet. Tu as répondu présent dès que cela était nécessaire. Tu n'hésitais pas à m'impliquer dans vos activités. Enfin, je remercie tous les chiens cadavres (**Bones**, **Turbo, Wallace** et **Davon**) de la police belge qui ont été extraordinaire tout au long des expériences.

A vous mes colloques et amies, **Clothilde** et **Morgane**. Je ne peux pas parler de cette thèse sans évoquer nos moments de partage. On a commencé l'aventure ensemble. Je repense à tous ces moments qui n'ont fait que renforcer nos liens ! Nos sorties, nos discussions interminables, nos pétages de câbles. Qui aurait cru que ce trio fonctionnerait si bien : Clothilde la perfectionniste, Morgane la tempérée et Clément l'excessif ! Et pourtant … on s'est complétés. On a traversé les épreuves ensemble en se soutenant mutuellement. Pour tout ce que vous êtes et ce que vous m'apportez, je vous fais une énorme lèche. Je nous souhaite le meilleur. On a commencé le chemin de la thèse ensemble et on a trouvé notre chemin dans notre vie personnelle ensemble ! Tout ça m'amène forcément aux pièces rapportées : **Franck**, **Davie**. Vous avez été tous deux de très belles rencontres. Je suis très heureux que vous ayez rencontré ces deux folles. A vous quatre MERCI !

Il y a aussi **Emilie** et **Ariane** mes deux consœurs de masters. On a suivi des chemins différents mais on est toujours restés proches. On s'est soutenus et cela bien avant que je débute ma thèse, que ce soit nos travaux de groupe qui ont laissé des séquelles a beaucoup de personnes ^^, que nos soirée synthèse. Pour avoir toujours été présentes,

je vous remercie.

Je tenais aussi à remercier tous les membres de l'équipe du MIPI qui nous ont accueilli les bras ouverts lors de notre arrivée à TERRA. Spéciale dédicace à **Antoine**, **Barbara**, **Jelena, Grégory** et **Barbara**. Vous avez permis que cette transition mouvementée soit agréable et joviale. Merci pour tous ces moments passés ensemble (Cologne, les 6 heures, les soirées Medzo et le médiéval run).

Sophie, **Marjorie**, notre première rencontre date des travaux pratiques durant mes études. Puis, en début de thèse, on a très vite beaucoup discuté. Vous m'avez toutes deux données de bons conseils. Votre soutient a été d'une grande aide surtout pendant certaines périodes un peu compliquées que j'ai pu traverser pendant cette thèse. Pour tout ça, un immense merci!

I cannot write acknowledgment without mentioning my favorite English colleagues: **Estelle** and **Fiona**. You both know how it was a pleasure to talk with you during our monthly lunch meetings or during the different faculty events. Beside all your support to correct some of my papers or my speaking, you were always present to talk about my researches, the funny situation met during courses and even about our private life. Our relation was a prerequisite (# 6h 2023) to go through this thesis. From teacher to colleagues, you quickly became amazing friends. I thank both of you and I wish you the best!

En outre les personnes que j'ai rencontrées pendant ma thèse, il y a toutes celles qui m'accompagnent depuis bien plus longtemps. Je ne peux pas écrire de remerciements honnêtes sans leur consacrer une partie.

On va commencer avec les trois drôles de dames : **Lulu**, **Suzon** et **Françoise**. Vous avez toujours cru en moi. Vous m'avez soutenu dans mon choix d'études là où d'autres n'y croyaient pas du tout. Depuis ce voyages rhéto, inoubliable en Croatie ou encore la retraite à Saint-Hubert, il y en a des choses qui se sont passées. Nos rencontres annuelles font toujours énormément de bien. Vous êtes toutes une source d'inspiration et en particulier de par votre force de caractère face aux épreuves. Je suis très heureux que le feeling soit si bien passé. Un grand merci pour tout !

A mes meilleures amies et leur pièces rapportées : **Anaïs** et **Christophe**, **Iris** et **Gauthier**, **Cyrielle** et **Maxime**, **Juliane** et **Rémy**. Trop de choses à dire, il me faudrait une thèse pour expliquer à quel point vous êtes des personnes particulières à mes yeux. Vous n'êtes pas que des amis, vous faites partie de la famille. Vous savez déjà tous ce que j'ai à vous dire. Je me contenterai de vous dire que je vous aime mes P****.

A ma famille ! **Maman**, **Papa**, qui l'aurait cru ? Je vous aurais fait suer avec ces études à rallonge. Je me rappelle encore mes paquets de feuilles d'exercices que je

devais faire en primaire avant mes examens. Au final, des blocus j'en aurai fait toute ma vie. Ce qui est certain, c'est que sans votre persévérance et surtout votre patience, je n'en serais pas là aujourd'hui. J'espère pouvoir un jour être un aussi bon parent avec Abigaël que vous l'avez été avec moi. Pour tout ce que vous avez fait, pour ce que vous faites et pour ce que vous allez faire dans le future : MERCI. **Baptiste** que je suis fier de t'avoir pour frère. Je te remercie d'être toi. J'ai de la chance de t'avoir et j'en connais une qui a encore plus de chance de t'avoir pour parrain ! Restes comme tu es, excessif, borné mais surtout sincère ! On en a encore des choses à vivre ensemble ! J'ai évidemment aussi une pensée particulière à ma belle-sœur préférée, **Manon**. Le compliment est facile, tu es la seule ^^ ! Honnêtement, je suis très heureux que tu sois rentrée dans le cercle familiale. **Tante Anne**, bien évidemment tu as ta place aussi. Tu as toujours été là, tu as cru en moi et pour ça je ne peux que te remercier. Une citation dit qu'on ne choisis pas sa famille… Ce que je peux vous dire c'est que je vous aurais tous choisis ! Un grand merci à vous tous, d'avoir fait de moi ce que je suis aujourd'hui. Je remercie aussi **ma bonne étoile** qui me suit de loin depuis longtemps maintenant… Merci aussi à mes beaux-parents : **Andrée** et **Jean-Louis**. Andrée merci pour ta disponibilité et pour l'aide que tu nous apportes au quotidien. Tu prends une place importante dans la finalisation de ce travail.

"The last but not least", **Mélanie**, tu es sans aucun doute la meilleure chose qui m'es arrivée pendant cette thèse. J'ai rencontré la personne avec qui je veux passer ma vie. Certains diront que ça a été vite, mais finalement pourquoi attendre quand l'évidence se présente à nous. Je te remercie pour tout ce que tu fais pour moi, pour ton soutient sans faille. Tu me forces à me remettre en question, tu me pousses dans mes retranchements. Cela me permet de grandir. Tu as toujours cru en moi. Tu m'as aussi offert ma plus grande fierté, Abigaël. Merci pour tout lou, Je t'aime.

Abigaël, on ne peut pas dire que tu as toujours facilité cette fin de parcours. Je te remercie pour tout ce que tu m'apportes au quotidien. Tu me permets de relativiser les choses. J'espère que tu garderas ce caractère fort que tu tiens de tes deux parents. Cultive ta différence, ce sera ta force. Soit telle que tu es, tu auras toujours mon soutient.

Je finirai ces remerciements par une citation qui compte beaucoup pour moi :

« *L'imagination est plus importante que la connaissance, car la connaissance est limitée, tandis que l'imagination englobe le monde entier.* »

Albert Einstein

Table of contents

Abstract **7**

Résumé **8**

Acknowledgments **15**

Table of contents **23**

Figure list **27**

Table list **31**

General introduction **35**

Introduction **37**

Odor profile of human corpses: a review **39**

Abstract 39

Introduction 40

Thanatochemistry and volatile organic compounds 41

Protein degradation 41

Lipid degradation 42

Human subjects for VOC analysis 43

Sampling of human cadaveric VOCs 45

Separating and identifying human cadaveric VOCs 48

Conclusion and perspectives 55

Cadaver Dogs and the Deathly Hallows— A Survey and Literature Review on Selection and Training Procedure **56**

Abstract 56

Introduction 57

Methods 58

Selection of human remains detection dogs 61

Training of human remains detection dogs 73

Conclusions 78

Objectives 81

Environmental impact on the cadaveric volatilome 87

Introduction 89

Impact of necrophagous insects on the emission of volatile organic compounds released during the decaying process 91

Abstract 91

Introduction 92

Material & Methods 93

Results 95

Discussion 104

Forensic taphonomy: characterization of the gravesoil chemistry using a multivariate approach combining chemical and volatile analyses . 106

Abstract 106

Introduction 107

Material and methods 108

Results 112

Discussion 122

Conclusions 125

On the Styx bank - Characterization of headspace cadaveric VOCs profile released by submerged decaying rats 126

Abstract 126

Introduction 127

Material and methods 128

Results 130

Discussion 136

Conclusions 137

Potential of direct immersion solid-phase microextraction to characterize dissolved volatile organic compounds released by submerged decaying rat cadavers 138

Abstract 138

Introduction 139
Materials & methods 140
Results & discussion 142
Conclusions and recommandations 144
Human smell of death 147
Introduction 149
All equal in the face of death! – Volatile cadaveric compounds of human corpses in fresh stage. 150
Abstract 150
Introduction 151
Material and methods 152
Results and discussion 154
Conclusions 157
Human remains detection dogs: from performance to perception 159
Introduction 161
What a good boy! Deciphering the efficiency of detection dogs 162
Abstract 162
Introduction 163
Material and methods 164
Results 169
Discussion 173
Conclusions 175
Copycatting the smell of death – Deciphering the role of cadaveric scent components used by detection dogs to locate human remains 176
Abstract 176
Introduction 177
Material and methods 178
Results 182

Discussion..... 183
Conclusions 185
General discussion 187
References 197

Figure list

Figure 1 Breeds currently used as human remains detection cadaver dogs based on a survey completed by ten brigades from Europe and Canada 62

Figure 2 Handlers' estimation of the importance of the physical and behavioral traits that must be considered during the selection of HRDDs human remains detection dogs. *The response distribution describes the percentage of handlers (out of 50) that estimate the importance of each trait (not important, fairly important, very important). The score estimates the importance of the traits during the selection according to equation 1*.....**64**

Figure 3 Proportions of dogs declared to be used as human remains detection dogs HRDD s in our survey *(N = 10 out of 16 brigades)* **belonging to the three common types of dog's skull conformation** *(skull pictures are reproduced with the kind permission of Tibor Csörg)*.....**66**

Figure 4. Types of operant conditioning used by the surveyed handlers *(y axes, N = 50)* **to train their human remains detection dogs with the pie chart representing the proportion of reinforcement and punishment technics used** *(light grey: punishment, dark grey: reinforcement)* **74**

Figure 5. Types of training aids used by the surveyed human remains detection dogs' brigades to train their dogs. *Colors in the pie charts represent the diversity of training aids used by the teams in the country where the chart is located. The bar chart represents the number of human remains detection dogs' teams (y axe) which use the different training aids*.....**75**

Figure 6 Diagram of the main objectives of the present thesis researches. *Numbers in Roman refer to the subsection of the different chapters. Ch3. I. assess the impact of the insect's colonization on the cadaveric volatile profile; Ch3. 1I. investigate the impact of the soil texture on chemicals released during underground decomposition; Ch3. III. investigate the impact of the water salinity on the cadaveric volatilome release at the water surface as well as the development of a method to characterize the dissolve cadaveric volatile compounds. Ch4. aims at characterizing the volatile profile of early deceased people. Ch5. I. aim at developing a method to assess detection dogs' efficiency; Ch5. II. decipher the olfactory cues which are perceive and which induce a positive response to detection dogs; Ch5.III. assess the impact of the chemical complexity of training aids on the ability to recognize the target odor* **84**

Figure 7 Temperature *(black)* **and relative humidity** *(grey)* **monitoring in the different comportments of the greenhouse..... 96**

Figure 8 Duration *(Number of days)* **of the decomposition of rats exposed or not to insects** *(without insect, with blowflies, with hide beetles and with both blowflies and hide beetles)* **during the entire decomposition process. Modalities sharing the same letter are not different from each other** *(threshold: $p < 0.05$)*..... **97**

Figure 9 Correlation plot representing chemical families of compounds released during decomposition of rats without insect (I), With L. sericata (II), With D. frischii (III) and with L. sericata and D. frischii (IV). *Colors illustrate the correlation between families of compounds (A: Alkanes, B: Sulphur Compounds, C: Aldehydes, D: Alcohols, E: Ketones, F: organic acids, G: Aromatic compounds, H: Amines, I: Alkenes)*..... **101**

Figure 10 PCA ordination plots. Visualization of the VOC profiles during decaying process, depending on the decomposition stage *(figures)* **and the modalities** *(colors)***.** *Each sampling is represented in two-dimensional plan constituted by two axes of the principal compounds analysis. Axes are combination of studied factors (insect presence and stage of decomposition characterized by a percentage of reliability)*. **103**

Figure 11 *(Left)* **Graves were set up in a greenhouse. A dead rat was placed in each grave except the two control graves.** *(Right)* **After two months, rats were exhumed and different layers of soil were collected**..... **109**

Figure 12 Non-metric multidimensional scaling representing the impact of a decaying mammal on the soil chemical features including pH, bioavailable compounds *(P: phosphorus; K: potassium; Mg: magnesium, Ca: calcium)***, conductivity, dry matter** *(DM)***, organic carbon** *(C_{org})* **and nitrogen amount** *(N)* **at different depths** *(soil layer 4 = L4, soil layer 3 = L3, soil layer 2 = L2, soil layer 1 = L1)* **of two gravesoil textures (***yellow-brown = sandy loam soil; green = loam soil)* **114**

Figure 13 Principal component analyses illustrating the impact of a buried decaying mammal *(see center of the figure)* **on the soil chemical features including pH, bioavailable compounds** *(phosphor; potassium; magnesium, calcium)***, conductivity, dry matter, organic carbon and nitrogen amount at different depths** *(Layer 4, Layer 3, Layer 2, Layer 1)* **within two soil textures separately analyzed: Left: Loam soil. Right: Sandy loam soil**..... **115**

Figure 14 Single compounds analysis illustrating the impact of a decaying mammal on pH, bioavailable compounds *(phosphor; potassium; magnesium, calcium, sodium)***, conductivity, dry matter, organic carbon and nitrogen amount, at different depths** *(Layers 1 to 4)***, expressed in percentage of dry matter** *(%DM)* **or in percentage of fresh matter** *(%FM)***. The two soil textures were separately analyzed: Left: Sandy loam soil. Right: Loam soil. All layers were compared to the control of the respective soil texture** *(Datasets sharing the same uppercase letter are not significantly different from each other (threshold: p-value < 0.05))* **.....118**

Figure 15 Principal component analyses illustrating the impact of a buried decaying mammal *(see center of the figure)* **on the volatile cadaveric compounds at different depths** *(Layers 1 to 4)* **within two soil textures separately analyzed: Left: Loam soil. Right: Sandy loam soil 121**

Figure 16 Stage duration in days in both water salinity condition *(dark grey : sea water ; light grey : soft water)* **for each stage of decomposition excepting the floating decay as we stop the experiment during this last stage.** *(NS: non signtificative ; NA : non applicable)* **.....131**

Figure 17 Linear discrinative analyses *(LDA)***. Visualization of the VOCs profile during the decaying process of submerged rats remains, depending on the decomposition stage.** *Each sampling is represented in a two dimensional plan constituted by two axes of the LDA***. 135**

Figure 18 Behavioral arena formed of half circle cinderblocks, in which plastic cups were placed containing distractive and target odors. The dog started the odor search trial at an equal distance from each cinderblock. The handler stayed next to the detection dog *(DD)* **to provide comments** *(motivation and starting)***, but was not allowed to guide the dog by pointing out any blocks. The experimenter stayed outside the arena to avoid the "clever Hans" effect, and followed the dog's behavior on a screen linked to the camera placed above the arena.....166**

Figure 19 Importance of different criteria on the performance of detection dogs *(DDs)***. These criteria were based on surveys completed by 25 DD brigade supervisors and 25 DD handlers.** *For a description of the criteria, see Table 1. More important criteria had higher ratings (modalities sharing the same letters are not different p-value > 0.05)* **.....169**

Figure 20 Coinertia analyses (I) including the correlation (II) between the evaluation of the handlers or supervisor *(top right correlation circle). A: Rapidity; B: Strategy level; C: Strategy efficacity; D: False negative; E: False positive; F:*

Sensitivity to low concentration; G: Sensitivity to high concentration; H: Guidance; I: Search efficacity; J: Ranking. **The data were measured during the behavioral tests** *(top left correlation circle). a: variance of angle between the start point and the target; b: number of false negative; c: number of false positive; d: mean of angle between the start point and the target; e: variance in eye contact; f: variance of frequency target zone visit; g: mean of distance; h: mean of frequency target zone visit; i: variance of search time; j: mean of search time; k: variance distance ; l: mean starting point angle ; m: mean precedent angle; n: variance starting point; o: variance previous angle, p: mean angle previous target; q: mean eye contact; r:variance previous target)*. **The correlation between the dimensions of each correlation circle is represented in the two circles.** *Red: PCA dimension of the experiment; blue: PCA dimension of the evaluation of handler and supervisor, respectively. (The contribution of each variable is provided on the correlation circle)* **..... 172**

Table list

Table 1 Summary of studies for the profiling of cadaveric human VOCs.....47

Table 2 Human cadaveric volatile organic compounds. *The column ''Human cadaver studies" refers to volatile collection performed on human remains. Last column identifies example studies identifying each specific VOC from remains of another animal species* **.....49**

Table 3 Bibliographic research performed for each section of the present review using Scopus®, Science Direct® and Google Scholar® between June and December 2019.....59

Table 4 Survey sent to handlers *(N = 50)* **of human remains detection dog** *(HRDDs)* **.....60**

Table 5 Selection methods of detection and service dogs.....70

Table 6 Analytical parameters of the TDU-GC-MS analysis *(adapted from Rosier et al., 2014)* **.....95**

Table 7 List of the 67 volatile organic compounds released by rat remains in absence *(-)* **or presence of insects** *(Δ)* **.....98**

Table 8 Analytical parameters of the TD30R-GC/MS analysis 111

Table 9 Differences between decomposition stages of surface, buried and submerged corpse 127

Table 10 List of volatile organic compounds released by rat remains decaying either in soft or sea water.....132

Table 11 Peak areas of the organic compounds collected from submerged rats before and after the optimisation of the sampling method *(* refers to compounds that were identified from submerged bodies by Irish et al 2019)* **.....142**

Table 12 Cadavers information.....15353

Table 13 List of identified cadaveric compounds from 20 human corpses experiencing the fresh decomposition stage. 15555

Table 14 statistical values for studied factors 15757

Table 15 Survey sent to DD handlers belonging to the Kynopol network. For each question, a grade from 1 *(strongly disagree)* **to 10** *(totally agree)* **had to be given. 16565**

Table 16 Descriptions of the variables collected per trial from video recordings of the arena. Ten trials of odor search were conducted across sessions. *(Parameters identified by * are presented in Supplementary figure 1)***..... 16767**

Table 17 Evaluation of detection dog *(DD)* **operational performance by handlers and brigade supervisors. For each question, a graduation from 0** *(no)* **to 10** *(absolutely)* **was used..... 16868**

Table 18 Means and standard deviations of each studied quantitative MAP variable 17070

Tableau 19 Formulation of the initial blend made of cadaveric odorant cues……..179

Tableau 20 Composition of solutions used to answer Q3 to Q5…….180

Chapter 1

General introduction

Introduction

« What happens after death? »

This question has been investigated by religious people, philosophers, novelists and of course by scientists. Despite the popularity of the afterlife question -for which only hypotheses can be raised- science has only generated limited knowledge, mainly focusing on the physical and chemical processes of the decomposition of a body. According to science, the question of the "after death" can only be partially answered.

The organic matter, of which a body is made of, decays to simpler molecules thanks to chemical mechanisms mediated by various biological processes. These reactions also lead to physical modifications: the body inflates, deflates, leaks and slowly disappears (Vass, 2001; Verheggen et al., 2017). Accordingly, the complete process can be divided in five stages of decomposition: (i) **fresh stage**, in which no major visual changes are observed; (ii) **bloated stages**, characterized by an inflation of the body, driven by symbiotic bacteria; (iii) **active decay stage**, identifiable by the deflation of the corpse and the presence of necrophagous entomofauna; (iv) **advanced decay stage**, during which insects are less present due to the lack of organic matter to feed on; (v) **dry remains stage**, identified by the only presence of bones, hairs and dried skin (Dekeirsschieter et al., 2009; Gennard, 2007). Several biotic and abiotic factors promote the chemical reactions occurring during the decomposition, leading to the production of various by-products (Campobasso et al., 2001; Carter et al., 2010; Kumara et al., 2009; Prado e Castro et al., 2013; Rivers & Dahlem, 2014b; Saukko & Knight, 2004; Verheggen et al., 2017). These factors can, in some particular cases, drastically modify the entire decaying process by drying the corpse (mummification) (Janaway et al., 2009b; Vass, 2001) or by changing fat in wax (saponification) (Dent et al., 2004; Dubois et al., 2018; Vass, 2001; Zurgani, 2018).

Volatile organic compounds (VOCs) are released over the course of the decomposition process, and have been the subject of many scientific works, mainly during the last 15 years. They constitute what is called the cadaveric volatilome, and what I like to name "the smell of death"! This odor is characterized by a wide diversity of molecules belonging to almost all chemical classes (*i.e.*, ketones, alkanes, aldehydes, carboxylic acids, sulfur containing compounds). In total, through the entire decaying process, several hundred compounds are released (Armstrong et al., 2016; Dekeirsschieter et al., 2012; Forbes, Perrault, et al., 2014; Perrault, Nizio, et al., 2015). While some are released continuously, others appear at a single stage of the decaying process (Armstrong et al., 2016; Cablk et al., 2012; Forbes et al., 2016). Hence, each stage of decomposition is characterized by a specific odor. The *fresh* stage and the *dry remains* stage are both typically associated with fewer molecules than the *bloated* and

active/advanced decay ones (Dekeirsschieter et al., 2009). We now know that the biotope in which corpse decayed impacts the release of cadaveric VOCs (Cablk et al., 2012; Dekeirsschieter et al., 2009; Rosier et al., 2015, 2016). In addition, all vertebrate species do not release the same VOC either. Most of the studies dedicated to the cadaveric VOCs used pigs as surrogate human models (Agapiou et al., 2015; Dekeirsschieter et al., 2012; Forbes & Perrault, 2014; Irish et al., 2019; Perrault et al., 2014). Only few studies focused on the human cadaveric volatilome (DeGreeff et al., 2012; Dubois et al., 2018; Perrault et al., 2017; Rosier et al., 2015; Ueland et al., 2021; Vass et al., 2004). A better understanding of the VOCs released by human cadavers could, among other applications, leads to improvements in the training of human remains detection dogs (HRDDs), that we often call "cadaver dogs".

Cadaver dogs are part of a larger category of working dogs called detection dogs, that include for instance explosive detection dogs, drug detection dogs and diseases detection dogs (Frederickx et al., 2011; Lit et al., 2011). Their work consists in the recognition of a specific odor (*e.g.* cadaveric odor for HRDDs) (Hackner & Pleil, 2017; Hayes et al., 2018; Lazarowski et al., 2019). Their role is to signal the presence of this odor to their handler. Before being able to do so, they follow a training program relying on operant conditioning: handlers teach their dog to express a particular behavior when facing the target odor, by providing a reward (*e.g.* toys, food) (Cornu et al., 2011; Frederickx et al., 2011; Johnen et al., 2013; Lazarowski & Dorman, 2014). Handlers should use an odor source representative to the odor released by the target source (*e.g.*, a cadaver) if they want their dog to be performant during its search. This training odor is usually called olfactory stimulus or training aids. These aids can be natural (*e.g.* gravesoil, cadaver clothes) or synthetic (*e.g.* Pseudo corpse sell by Merck®) (Cablk et al., 2012; Forbes, Rust, et al., 2014; Hoffman et al., 2009; Stadler et al., 2012). Despite the recent scientific interest for cadaveric volatilomes, and related works on their application for the training of HRDDs, very few scientific researches have investigated the relationship between the training procedure (including training aids' composition) and the resulting HRDDs efficiency.

The following chapters are devoted to reviewing the existing literature on the characterization of the smell of death and its valorization in the training of detection dogs.

Odor profile of human corpses: a review

This section is an adapted version of the article:

Martin C, Verheggen F (2018) Odor profile of human corpses: a review. Forensic chemistry, DOI: 10.1016/j.forc.2018.07.002

Abstract

The decaying process of vertebrates leads to the emission of a wide panel of volatile organic compounds (VOCs). While many studies use pigs or rats as surrogate human models, some studies have attempted to characterize the volatilome of human corpses, with very contrasting results. In this literature review, we summarize the genesis of the vertebrate cadaver volatilome, from the breakdown of macromolecules to specific VOCs. Furthermore, we list the analytical methods previously employed to collect, separate, identify and quantify human cadaveric VOCs. We also describe the advantages and disadvantages of these methods. Based on previously published reports, we formulate an up-to-date directory of the VOCs identified from human cadavers. We discuss the impact of the heterogeneity of the samples, and the limitations associated with sample size and the intrinsic characteristics of the corpses that were subjected to these studies. Finally, we consider the different applications that would benefit from improving the identification of the human volatilome.

Introduction

The decaying process of vertebrates has been intensively investigated. This process stems from the combined action of autolysis and putrefaction. Autolysis is driven by the activation of intracellular enzymes that impair tissues. The activity of insects and exogenous bacterial enzymes lead to putrefaction, which is much more destructive and quickly overtakes autolysis (Gill-King, 1997; Janaway et al., 2009a; Perrault, Nizio, et al., 2015; Rosier et al., 2016). The combined action of autolysis and putrefaction causes remains to undergo physical and chemical changes, including tissues liquefaction (Dent et al., 2004; Janaway et al., 2009a, 2009b; Pekka Saukko, 2004; Pinheiro, 2006). Based on physical changes, five stages of decomposition have been defined (Dekeirsschieter et al., 2009; H. B. Reed, 1958; Vass, 2001). The first stage of decomposition starts immediately after death and ends with the first signs of body inflation. At this point, the corpse enters the bloated stage (second stage). Body deflation characterizes the beginning of the active decay stage (third stage), during which the bulk of insect larvae feed on soft tissues. When bones are visible, the body reaches the advanced decay stage (fourth stage). Finally, the dry remains stage is reached (fifth stage) when the body is reduced to a heap of bones and hairs (Dekeirsschieter et al., 2009; Galloway, 1996; Sampat et al., 2016). Various biotic and abiotic factors impact the relative duration of each stage of decomposition (Byrd & Castner, 2001; Oliveira & Vasconcelos, 2010; Verheggen et al., 2017). Among them, elevated temperature and humidity can hasten the rate of decomposition as well as the development rate of necrophagous insects (Galloway, 1996; Janaway et al., 2009a). But extremes of temperature can prevent bacterial degradation and potentially lead to mummification, under low relative humidity (Charabidzé et al., 2014; Notter et al., 2009).

In addition to using physical changes to distinguish the stage of decomposition, chemical degradative reactions occur soon after death (Cablk et al., 2012). Chemical reactions degrade macromolecules (proteins, lipids and carbohydrates) from vertebrate corpses, resulting in the emission of hundreds of different volatile organic compounds (VOCs) belonging to almost all chemical classes (*i.e.*, alkanes, alkenes, sulphur compounds, nitrogen compounds, aromatic compounds, aldehydes and ketones) (Cablk et al., 2012; Dekeirsschieter et al., 2012). Studies focused on cadaveric VOCs have been primarily conducted on pigs, rats and birds, with pigs often being used as surrogate human models (Dekeirsschieter et al., 2009, 2012; Focant et al., 2013; Rosier et al., 2015, 2016).

Despite progress in the analysis of cadaveric VOCs, studies addressing the volatilome of human cadavers remain scarce. Highly contrasting results were obtained among these studies, mainly due to (1) the diversity of analytical methods used to

collect, separate and identify cadaveric VOCs and (2) major heterogeneity among samples (Cablk et al., 2012; Dubois et al., 2018; Vass et al., 2008a). This review summarizes the genesis of the human cadaver volatilome. The methods currently used to sample cadaveric VOCs are described, with the advantages and disadvantages of these methods being described in a comparative analysis. An up-to-date directory of the VOCs associated with human cadavers is created, along with suggestions for the potential applications of these VOCs.

Thanatochemistry and volatile organic compounds

Post-mortem VOCs emission involves the complex degradative mechanisms of macromolecules. The human body is approximately composed of 64% water, 20% proteins, 10% fat, 5% minerals and 1% carbohydrates (Janaway et al., 2009b). However, proteins, lipids and carbohydrates diversity, along with the relative abundance of these parameters, depend on many intrinsic factors (*e.g.*, genetics, diet and weight, microbiome) (Brasseur et al., 2012; Paczkowski & Schutz, 2011). The genesis of cadaveric VOCs depends on the macromolecules they originate from (Dent et al., 2004; Janaway et al., 2009a; Paczkowski & Schutz, 2011).

Proteins degradation

Proteins are present in all tissues, but their degradation does not occur at a uniform rate throughout the body, mainly due to heterogeneity in moisture, temperature and bacterial action. Indeed, decaying processes are promoted by humidity, with proteins degradation increasing with temperature (Dekeirsschieter et al., 2009; Janaway et al., 2009b; Paczkowski & Schutz, 2011). Moreover, proteins are resistant to degradation due to their ability to link with other molecules. For instance, in bones, mineral compounds (such as calcium) inhibit the degradation of proteins structures and contribute to their preservation, whereas brains proteins are quickly broken down (Gill-King, 1997; Janaway et al., 2009a, 2009b). During decomposition, amino acids are removed from the tertiary structure of proteins by microbial proteases and peptidases (Dekeirsschieter et al., 2009; Janaway et al., 2009b; Paczkowski & Schutz, 2011). Among the large bacterial community, *Pseudomonas* spp, *Bacillus spp* and *Micrococcus* spp. have significant proteolytic activity (Janaway et al., 2009b; Paczkowski & Schutz, 2011). Free amino acids lead to the emission of specific groups of VOCs (Dekeirsschieter et al., 2009; Paczkowski & Schutz, 2011; Vass et al., 2003). For instance, the degradation of leucine, isoleucine and threonine releases higher alcohols (branched chain alcohols and 1-propanol) through the Ehrlich pathway and the fermentation of microorganisms (yeasts and bacteria) (Agapiou et al., 2015; Boumba et al., 2008; Paczkowski & Schutz, 2011; Rosier et al., 2016). The degradation of arginine, lysine and tryptophan produces nitrogen compounds (*e.g.*,

indole) through decarboxylation and the activity of microorganisms (Dent et al., 2004; Paczkowski & Schutz, 2011). Sulfur containing amino acids (cysteine and methionine) undergo desulfhydralation and bacterial degradation to produce sulphur compounds, such as methantiol and dimethyl disulfide, which are typical biomarkers of vertebrates decomposition (Agapiou et al., 2015; Dekeirsschieter et al., 2009; Dent et al., 2004; Paczkowski & Schutz, 2011; Rosier et al., 2016). Aromatic compounds and organic acids are also released during the degradation of various amino acids, without being specific to some of them (Agapiou et al., 2015; Paczkowski & Schutz, 2011).

Lipids degradation

Lipids are widely present in the cell membranes, but are also found in adipose tissues, such as intramuscular fat and fat deposits under the skin. The lipid fraction of vertebrates is mainly composed of triglycerides (90–99%) (Janaway et al., 2009a). During decomposition, the hydrolysis of triglycerides produces glycerol and fatty acids. Glycerol degradation forms pyruvate, which is decomposed into volatile small chain alcohols and organic acids (Boumba et al., 2008; Paczkowski & Schutz, 2011). Fatty acids are broken down into several VOCs, but also undergo saponification, especially in basic conditions or with anaerobic bacteria (De Donno et al., 2014; Notter et al., 2009). Fatty acids are decomposed through hydrolysis and oxidation, both occurring simultaneously, but the relative importance of each is determined by environmental factors, such as access to light and oxygen. Fungi and bacteria might contribute to this part of the decaying process; however, none have been identified acting on the decomposition of fatty acids (Dent et al., 2004). The lack of detailed studies makes the metabolic products of lipid degradation difficult to clarify (Paczkowski & Schutz, 2011). Lipids degradation likely results in the emission of volatile alcohols, organic acids, ketones, aldehydes and esters (Boumba et al., 2008; Dent et al., 2004; Paczkowski & Schutz, 2011). Anaerobic bacterial hydrolysis of body fat leads to the formation of adipocere, a wax-like organic substance, leading to the formation of a permanent firm cast of fatty tissues that replaces putrefaction (Forbes et al., 2004; Moses, 2012; Takatori et al., 1996). Adipocere release mainly carboxylic acids and acid esters. Moreover, some sulfured compounds including dimethyl disulfide and dimethyl trisulfide, were identified in high quantities (Dubois et al., 2018; Vass et al., 2003).

Carbohydrates degradation

Microorganisms convert carbohydrates to free energy through two mechanisms: the Embden-Meyerhof-Parnas (EMP) glycolytic pathway and the Entner-Doudoroff (ED) pathway. The EMP pathway is the most common type of glycolysis that occurs in mammals, plants and many microorganisms, like bacteria and yeasts. The ED

pathway is widespread in GRAM negative bacteria (Boumba et al., 2008; Paczkowski & Schutz, 2011). Both pathways produce ATP and pyruvate. In yeasts and bacteria, the fermentation of pyruvate produces either to ethanol or acetic acid, in addition to other volatile organic acids (*e.g.*, butanoic acid) and alcohols (*e.g.*, propanol) (Boumba et al., 2008; Paczkowski & Schutz, 2011). In fungi, pyruvate fermentation also produces volatile organic acids. Bacteria contribute to two degradation processes, depending on oxygen availability: (1) under anaerobic conditions, organic acids and alcohols are released, whereas (2) under aerobic conditions, aldehydes, organic acids, and carbon dioxide are released (Boumba et al., 2008; Dent et al., 2004). The obligate anaerobic bacteria of the Clostridiaceae family quickly colonize dead bodies, because they are widely present in the gut and anaerobic soil layers. From pyruvate, they form alcohols (*e.g.*, ethanol, butan-1-ol) and organic acids (*e.g.*, acetic acid, butanoic acid). Facultative anaerobic bacteria of the Enterobacteriaceae family ferment pyruvate into organic acids and alcohols. However, bowel is not the only body part where bacteria are located. For instance, Streptococaceae and Enterococaceae are present in the respiratory system, where they ferment carbohydrate into lactic acid, acetic acid and ethanol. These bacteria produce organic and inorganic gases that cause corpses to inflate, termed the bloating stage. When the skin breaks, the inner fluids come into contact with oxygen, which allows aerobic bacteria (such as Bacillaceae and Pseudomonaceae) originating from air and soil to colonize the body. Because these bacteria cannot facilitate fermentation, they use the ED pathway to produce energy, leading to the production of metabolic products from the degradation of pyruvate (Boumba et al., 2008; Paczkowski & Schutz, 2011).

The wide diversity of VOCs released after death is explained by the diversity of macromolecules they originate from, as well as by the composition of the cadaver microbiome, which changes during the entire decomposition process (Debruyn & Hauther, 2017; Dent et al., 2004; Hauther et al., 2015). The heterogeneity in the chemical structure of VOCs makes it challenging to collect and separate them. Some published reports have attempted to do so on the human volatilome, with some degree of success.

Human subjects for VOCs analysis

Table 1 lists all 12 studies characterizing the cadaveric volatilome of decaying human bodies. The sources of VOCs differed among the 12 studies, and included grave-soil, human remains (bones or flesh) and whole human bodies. The intrinsic variability of each biological matrix makes it necessary to perform large numbers of replicates. However, human cadavers are not easily accessible for forensic studies. Consequently, most of these studies have used very small sample sizes (Statheropoulos et al., 2005, 2007; Stefanuto et al., 2015; Vass et al., 2004). Only one

study sampled cadaveric VOCs from 27 bodies, but did not cover all stages of decomposition (Degreeff & Furton, 2011). Studying human cadaveric VOC emissions on few corpses is likely not to be representative because many intrinsic and extrinsic factors influence cadaveric VOCs profiles (*e.g.*, bacterial colonization, decaying stage, insects' accessibility, abiotic conditions of the environment where the corpse was located). Because these studies have not standardized most biotic and abiotic parameters, it is difficult to obtain a "snap shot" of an average human corpse volatilome.

A small group of studies (Hoffman et al., 2009; Rosier et al., 2014, 2015, 2016; Vass et al., 2008a) have collected VOCs from human remains, including blood, flesh and bones collected on cadavers. These remains were collected during autopsies, obtained from medico legal center or from cadaver dog handlers who use them as training aids. Human remains are easier to obtain than entire bodies, because no consent is needed if the donation is anonymous. However, because the absolute and relative composition of each body part differs in terms of proteins, lipids and carbohydrates, VOCs from human remains are not likely to represent the diversity of cadaveric volatiles from whole human corpses (Paczkowski & Schutz, 2011; Rosier et al., 2015; Vass, 2001). Thus, it is better to use whole bodies as sources of cadaveric VOCs to characterize the human cadaver volatilome. Indeed, such analyses are not skewed as no intrusive experimentation is performed on the corpse before sampling the odors (Janaway et al., 2009b; Paczkowski & Schutz, 2011). However, the use of whole human bodies requires permits or people to have donated their bodies after death (Brasseur et al., 2012; Cablk et al., 2012; Presnell et al., 2013; Rosier et al., 2014; Statheropoulos et al., 2007; Wang et al., 2017). Moreover, sample size is important, as many factors affect the decaying process (Verheggen et al., 2017). Gravesoil volatile profiles have received attention to train search dogs to locate clandestine graves (Larson et al., 2011). Analysis of gravesoil VOCs might also inform us about the presence of cadavers (Focant et al., 2013; Vass et al., 2008a). Depending on the atmospheric pressure, gravesoil is good source of cadaveric VOCs (Larson et al., 2011; Vass, 2012). However, soil chemistry must also be considered because the VOCs that are released at the surface are dependent on the soil adsorbing ability of components with which they have mixed (Agapiou et al., 2015). The soil microbiome also differs among soil types, with bacteria being active organisms that directly impact the VOCs released by decaying bodies (Paczkowski & Schutz, 2011). Finally, each soil type has its own chemical background that might interact with cadaveric VOCs or hide them during the separation of chemicals (Dubois et al., 2018). Additional studies on gravesoil volatiles must be conducted to improve our understanding of the interactions between cadaveric VOCs and soil constituents.

Sampling of human cadaveric VOCs

Besides the importance of establishing baselines for the analysis of human samples (*e.g.*, sample size and homogeneity of the biotic/abiotic conditions), standard analytical methodologies are with respect to the sampling, separation, identification and quantification of human cadaveric VOCs.

Among the 12 identified studies, human cadaveric VOCs were trapped using a diversity of sampling methodologies. While most studies performed dynamic headspace volatile collection, others performed passive sorption using solid-phase microextraction (SPME) (Degreeff & Furton, 2011; Hoffman et al., 2009). SPME has the advantage of being a cheap, fast and easy-to-use sampling technique. However, the specific affinity and limited adsorbing capacity of the polymeric fiber (made of PDMS and/or Divinyl benzene) makes it necessary to calibrate the method with appropriate quantitative standards to allow absolute quantification (Romeo, 2009). SPME does not allow one to compare quantities of different compounds in the same sample. Dynamic sampling performed using appropriate adsorbent material is preferred for quantification purposes, and is usually performed to collect human cadaveric VOCs (Table 1) (Woolfenden, 1997).

The diversity of cadaveric molecules is wide. Thus, low selectivity sorbent material should be used. By using a combination of sorbents gathered in a single cartridge, most of the studies increased their chances to collect a representative blend of cadaveric VOCs. When a wide range of molecules is targeted during analyses, it is more suitable to use a combination of adsorbents of increasing strength. Several previous studies on the human cadaver volatilome made use of Tenax TA. However, this weak hydrophobic sorbent is more suitable for compounds that are less volatile than benzene (Woolfenden, 1997), with cadaveric VOCs having molecular weights that often reach 200 $g.mol^{-1}$ (Table 1). Tenax TA alone would, therefore, not allow the collection of all kinds of cadaveric VOCs. To optimize the procedure both qualitatively and quantitatively, it is, therefore, recommended to combine weak (Tenax TA) and strong sorbents, like Carbosieve or Carbopack, to collect all molecules regardless of their molecular weight and polarity (Table 1) (Woolfenden, 1997). STU-100 is a less common sampling method, which is recommended for training cadaver dogs and for field experiments (Degreeff & Furton, 2011). STU-100 involves the transfer of VOCs released by the sources (*i.e.*, cadaver) on an odor carrier (*i.e.*, gauzes) by passing the air through the carrier using an air pump. Then, the carrier is kept at a low temperature and the soaked VOCs are collected in laboratory on an SPME fiber before being desorbed and analyzed in gas chromatograph. This approach has the advantage of being easy to use on-site by non-scientists. However, very few studies have compared the adsorption and desorption performance of this method with

other more conventional ones. Legal proceedings suggest that STU-100 does not collect VOCs efficiently, and is a source of contaminants (Eckenrode et al., 2006). Thus, when considering the selectivity and adsorbing capacity of SPME, the efficiency of this sampling method is questionable. This approach is also based on the assumption that the gauze (odor carrier) is not selective during the process of trapping VOCs and during the collection of SPME.

Table 1 Summary of studies for the profiling of cadaveric human VOCs

Reference	Field studies	Study location	Matrix (effective)	Sorbent	Sampling type	Injection or pre-treatment	Analysis	Column	Detection	Method type
(Degreeff & Furton, 2011)	No	Laboratory, Miami, USA	Human bodies (27)	STU-100 (Dukal gauze/polyester)	Dynamic	SPME	GC	DB-225MS capillary (unknown dimension)	MS	Semi-quantitative
(Statheropoulos et al., 2005, 2007)	No	Laboratory, Athens, Greece	Human bodies (2) (1)	Carbograph2, carbograph1 and Carbosieve S-III	Dynamic	Thermo-desorption	GC	SPB-624 capillary (60m×0.25mm×1.4µm)	MS	Quantitative
(Stefanuto et al., 2015)	Yes	Texas, USA	Human bodies (4)	Tenax GR and Carbopack™ B	Dynamic	Thermo-desorption	GC×GC	1D column: Rxi-5Sil (30m×0.25mm×0.25µm); 2D column Rxi-17 (1m×0.15mm×0.15µm)	TOF-MS	Quantitative
(Vass et al., 2004)	Yes	Knoxville, Tennessee, USA	Gravesoil from buried human bodies (4)	Carbotrap, Carbotrap-C, Carbosieve S-III	Dynamic	Thermo-desorption	GC	DB-1 phase capillary (60m×0.32mm×1µm)	MS	Quantitative
(Vass et al., 2008a)	Yes	Knoxville, Tennessee, USA	Gravesoil of buried humans remains (4), bones (2)	Carbotrap, Carbotrap-C, Carbosieve S-III	Dynamic	Thermo-desorption	GC	Rtx-1PONA column (100m×0.25 mm×0.5 µm)	MS	Quantitative
(Vass, 2012)	Yes	Europe, Pacific rim, Canada, Africa, USA	Soil from human grave	Carbotrap, Carbotrap-C, Carbosieve S-III	Heating soil vials to release VOCs in the headspace	Headspace injection	GC	Rtx-1PONA column (100m×0.25 mm×0.5 µm)	MS	Semi-quantitative
(Dubois et al., 2018)	Yes	Unknown	Soil from death scene (n=2) and adipocere (n=1)	HS-SPME (PDMS/DVB)	Passive		GC×GC	1D column: Rxi-624Sil (30m×0.25mm×1.4µm); 2D column Stabilwax (1.8m×0.25mm×0.50µm)	MS	Quantitative
(Focant et al., 2013)	No	Texas and BE	Soil from human (n=?) graves	Tenax GR and Carbopack™ B	Dynamic	Thermo-desorption	GC×GC	1D column: Rxi-5Sil (30m×0.25mm×0.25µm); 2D column Rxi-17 (1m×0.15mm×0.15µm)	TOF-MS	Quantitative
(Rosier et al., 2014, 2015, 2016)	No	Laboratory, Leuven, BE	Human remains (6)	Tenax Ta	Dynamic	Thermo-desorption	GC	VF-624ms capillary, (60m×0.25 mm×1.4µm)	MS	Semi-quantitative
(Hoffman et al., 2009)	No	FBI laboratory, Quantico, USA	Human remains (14)	PDMS/DVB and SPME	Passive		GC	DB5-MS capillary (15m×0.25mm×0.25µm)	MS	Qualitative

Separating and identifying human cadaveric VOCs

Most of the 12 studies were conducted using regular "one-dimension" gas chromatography (GC) to separate the sampled cadaveric VOCs. Again, the diversity and abundance of cadaveric VOCs leads to another analytical issue; namely, the coelution of some VOCs, and the subsequent inability to identify and quantify minor constituents that co-elute with major constituents (Verheggen et al., 2017). This issue could be partially solved by using a longer capillary column, such as 100 m long columns (Vass et al., 2008b). The polarity of the stationary phase extends from non-polar to highly polar, based on the studies listed in Table 1. Due to the very high diversity of VOCs, the choice of the polarity of the capillary column does seem a crucial analytical question. In 2014, Rosier and al. (Rosier et al., 2014) performed the validation of a one-dimension gas chromatography method for cadaveric VOC separation. However, this method remains limited, in terms of separation capacity, for complex VOC blends, such as those likely characterising the human cadaver volatilome. Even if this method is repeatable and allows the identification of more compounds than other methods, the chromatograms still have coelution issues.

Two-dimensional gas chromatography (GC x GC–MS) allows most constituents to be collected at a higher resolution by avoiding coelution. As described by Verheggen et al. (2017), GC x GC–MS was developed and validated on samples from human surrogate models, such as pig carcasses. This method was performed by three studies on human cadaveric VOCs. For instance, one study was conducted directly on human cadavers (Stefanuto et al., 2015), while the other two studies were conducted on grave-soil samples (Dubois et al., 2018; Focant et al., 2013). GC x GC couples two columns in series using a modulator, which sends successive fractions of VOCs, previously separated on a classical one-dimension column, to a second column that is usually shorter (up to 2 m in length). As a result, up to several hundred compounds from complex samples that are not separated in the first dimension (first column) are separated in the second dimension. Moreover, the second dimension reduces background noise, improving the global sensitivity of GC x GC compared to conventional GC (Perrault, Nizio, et al., 2015).

Most of the studies performed on human cadaver (listed in Table 1) identified VOCs using mass spectrometry, and confirmed it with standards. To identify compounds, initial identification is made by comparing the spectra obtained after separation with spectra from a spectra library. Then, the injection of standard solutions confirms the identification. Kovats retention indices are less commonly calculated. Following GC separation, these indices help to identify VOCs by comparing known values with experimentally found retention indices, especially when large numbers of compounds need to be identified, and/or when standard solutions are not available.

Table 2 Human cadaveric volatile organic compounds. The column ''Human cadaver studies" refers to volatile collection performed on human remains. Last column identifies example studies identifying each specific VOC from remains of another animal species.

	Human cadaver studies										VOCs referred in animal decaying studies
	a	b	c	d	e	f	g	h	i	j	k	
Acids and esters												
Acetic acid	X							X				(Statheropoulos et al., 2011)
Acetic acid, ethyl ester				X								(Dekeirsschieter et al., 2012; Statheropoulos et al., 2011)
Acetic acid, propyl ester				X								(Statheropoulos et al., 2011)
Propanoic acid	X						X					
2-Methylpropanoic acid, , ethyl ester				X								
2-Methylpropanoic acid								X				(Dekeirsschieter et al., 2012)
Butanoic acid	X						X					(Dekeirsschieter et al., 2009, 2012)
Pentanoic acid							X					(Dekeirsschieter et al., 2009, 2012)
Butanoic acid, ethyl ester				X			X					(Dekeirsschieter et al., 2009, 2012; C. V. Hoermann et al., 2012; Kasper et al., 2015; Perrault, Stefanuto, et al., 2015)
Hexanoic acid							X	X				
Butanoic acid, methyl ester		X										(Cablk et al., 2012)
Butanoic acid, butyl ester							X					(Cablk et al., 2012)
Hexanoic acid, ethyl ester							X					
Hexanoic acid, pentyl ester							X					
Hexanoic acid, hexyl ester							X					
Hexanoic acid, 2-ethyl								X				(Dekeirsschieter et al., 2009, 2012; Kasper et al., 2015)
Octanoic acid								X				
1,2-Benzenedicarboxylic acid, diethyl ester						X			X			
Hexadecanoic acid, methyl ester			X			X			X			
Benzoic acid methyl ester								X				
Undecanoic acid, 10-methyl-methyl ester								X				
Undecanoic acid							X					
2-Ethylhexyl tetradecyl ester, oxalic acid								X				
Acetates												
Propyl propionate										X	X	(Rosier et al., 2015, 2016)
Propyl acetate	X											
Ethyl propionate	X									X	X	(Rosier et al., 2015, 2016)
Ethyl-2-methyl-propionate	X											
Propyl butyrate	X									X	X	(Kasper et al., 2015; Rosier et al., 2015, 2016)
Propyl-2-methylbutyrate	X											
Propylpentanoate	X											
Ethyl butyrate											X	(C. V. Hoermann et al., 2011, 2012; Kasper et al., 2015; Rosier et al., 2015)
Ethyl 3-methylbutyrate	X										X	(Rosier et al., 2015)
Ethyl pentanoate	X									X	X	
Ethyl octanoate	X											
Methyl salicilate								X				
Isobornyl acetate								X				
1,3-Diacetyloxypropan-2-yl acetate	X											
Alcohols												
Ethanol	X			X	X					X		(Dekeirsschieter et al., 2009, 2012; Perrault, Stefanuto, et al., 2015; Rosier et al., 2015)

2-(2-Methoxyethoxy)ethanol								X			
1-Propanol	X				X				X		(Rosier et al., 2015)
1-Propanol, 2-methyl	X			X						X	X	(Dekeirsschieter et al., 2012; Kasper et al., 2015; Rosier et al., 2015)
1-Butanol	X			X						X	X	(Rosier et al., 2015)
2-Butanol	X										X	(Perrault, Stefanuto, et al., 2015; Statheropoulos et al., 2011)
3-Methyl-1-butanol	X									X	X	(Rosier et al., 2016)
2-Methyl-1-butanol	X									X	X	(Rosier et al., 2015, 2016)
2-Ethyl-1-Butanol	X											
1,3-Butanol	X											
2-Butoxy ethanol								X				
1-Pentanol	X			X			X					(Cablk et al., 2012; Dekeirsschieter et al., 2012)
2-Pentanol	X											
2-Methyl-3-pentanol		X										
1-Octen-3-ol							X					(Kasper et al., 2015; Statheropoulos et al., 2011)
1-Hexanol				X			X					(Kasper et al., 2015)
1-Octanol							X	X				(Dekeirsschieter et al., 2012; Kasper et al., 2015)
2-Ethyl-1-hexanol			X		X		X	X				(Statheropoulos et al., 2011)
5-Methyl-2-(1-methylethyl)-cyclohexanol								X				
-Hexyl-1-octanol								X				
Furfuryl alcohol								X				
2-Pentadecyn-1-ol								X				
2-(2-Ethoxyethoxy) ethanol								X				
7-Octen-2-ol, 2,6 dimethyl								X				
1-Hexyl-1-decanol								X				
2-Hexyl ethanol								X				
5-Methyl-2,1-methylethyl cyclohexanol								X				
Aldehydes												
Acetaldehyde				X					X			(Agapiou et al., 2015; Dekeirsschieter et al., 2012)
Ethanal	X											
Propanal	X											
2-Methylpropanal	X								X			
Butanal									X			(Dekeirsschieter et al., 2009; Statheropoulos et al., 2011)
2-Methylbutanal									X			
3-Methylbutanal	X				X				X			(Perrault, Stefanuto, et al., 2015; Statheropoulos et al., 2011)
Pentanal				X					X			(Dekeirsschieter et al., 2009)
2-Hexenal							X					(Cablk et al., 2012)
Hexanal				X			X		X			(Cablk et al., 2012; Perrault, Stefanuto, et al., 2015)
Benzaldehyde		X	X				X	X				(Agapiou et al., 2015; Brasseur et al., 2012; Dekeirsschieter et al., 2009; Perrault, Stefanuto, et al., 2015; Statheropoulos et al., 2011)
2,4-Hepadienal							X					
2-Heptenal							X					(Cablk et al., 2012)
Heptanal							X		X			(Cablk et al., 2012; Dekeirsschieter et al., 2009, 2012; Kasper et al., 2015)
2-Octenal							X					(Cablk et al., 2012; Dekeirsschieter et al., 2012)
Octanal							X		X			(Agapiou et al., 2015)
2,4-Nonadienal							X					
2-Nonenal							X					(Cablk et al., 2012)
Nonanal			X		X	X	X					(Cablk et al., 2012; Dekeirsschieter et al., 2012)
Decanal			X		X		X	X				(Cablk et al., 2012)

Furfuraldehyde							X		
Halogen compounds									
Carbon tetrachloride*					X				
Tetrachloroethene					X		X		
1,1,2,-Trichloro-1,2,2-trifluoromethane*					X			X	
1,1-Dichloro-1-fluoroethane					X			X	
Dichlorotetrafluoroethane					X			X	
Dichlorodifluoromethane					X			X	
Tetrachloromethane				X					(Agapiou et al., 2015)
Chloromethane				X					
Chlorodifluoromethane				X					
1,2-Dichloroethene								X	
Tetrachloroethene					X			X	
Trichloroethylene								X	
Trichloromonofluoromethane								X	
Trichloroethene					X				
Chloroform					X				
1-Chloro-2-methoxy-benzene		X							
Cyclic hydrocarbon									
Phenol	X				X		X		(Brasseur et al., 2012; Caraballo et al., 2009; Dekeirsschieter et al., 2009; Rosier et al., 2014; Statheropoulos et al., 2011; Christian von Hoermann et al., 2016)
4-Methylphenol				X					(Dekeirsschieter et al., 2009, 2012)
Benzene				X		X		X	
Dimethylbenzenemethanol			X			X		X	
1,2,4-Trimethylbenzene				X					
1,2,3-Trimethylbenzene				X	X				
1,3,5-Trimethylbenzene				X					
1,2,3,4-tetramethylbezene							X		
Toluene				X		X	X	X	X	(Agapiou et al., 2015; Cablk et al., 2012; Statheropoulos et al., 2011)
o-Xylene					X				(Agapiou et al., 2015; Dekeirsschieter et al., 2012; Statheropoulos et al., 2011)
m-Xylene				X	X				(Agapiou et al., 2015; Statheropoulos et al., 2011)
p-Xylene				*X*	*X*		X			(Cablk et al., 2012; Dekeirsschieter et al., 2012)
Indole	X						X			(Brasseur et al., 2012; Cablk et al., 2012; Dekeirsschieter et al., 2009, 2012; Rosier et al., 2015)
2-Pentyl-furan							X			(Cablk et al., 2012; Dekeirsschieter et al., 2012; Perrault, Stefanuto, et al., 2015)
2-Methyl furan								X	(Agapiou et al., 2015)
2-Butylfuran				X					
1,2-Dimethyl benzene						X		X	
1,4-Dimethyl benzene						X		X	
1-Ethyl, 2-methyl benzene				X		X		X	
1-Ethyl, 4-methyl benzene				X					
2-Ethyl, 1,4-methyl benzene					X				
1-Methoxypropyl benzene			X			X		X	
Methylbezene					X				
Ethylbenzene		X		X		X		X	(Agapiou et al., 2015; Perrault, Stefanuto, et al., 2015)
(1-Methylethenyl)benzene					X				
Naphthalene					X	X	X	X	(Dekeirsschieter et al., 2012; Statheropoulos et al., 2011)
Styrene					X	X	X	X	(Perrault, Stefanuto, et al., 2015; Statheropoulos et al., 2011)

2-Ethylfurane				X							
Benzyl alcohol								X			
Propylbenzene				X							
1,2-Diethylbenzene				X							
d-Limonene				X	X						(Statheropoulos et al., 2011)
Terpinolene		X									
1-Methyl- 4-(1-methylethenyl)-cyclohexene	X										
Ketones											
1-Phenylethanone					X						(Dekeirsschieter et al., 2009; Rosier et al., 2014)
2-Propanone	X		X	X	X				X		X (Dekeirsschieter et al., 2012)
2-Hydroxybutanone	X										
3-Pentanone				X							(Statheropoulos et al., 2011)
2-Pentanone				X							X (Perrault, Stefanuto, et al., 2015)
4-Methyl-2-pentanone	X										
2-Butanone	X			X	X				X		X (Perrault, Stefanuto, et al., 2015; Statheropoulos et al., 2011)
Cyclohexanone				X			X				(Cablk et al., 2012; Dekeirsschieter et al., 2012; Statheropoulos et al., 2011)
2-Hexanone				X							(Dekeirsschieter et al., 2012)
4-Methyl-3-hexanone		X									
2-Heptanone	X			X			X				(Cablk et al., 2012; Dekeirsschieter et al., 2012)
4-Heptanone	X										
2-Octanone	X										
2-Nonanone	X				X						(Dekeirsschieter et al., 2012)
2-Decanone	X										
2-Tridecanone	X										
6-Methyl-5-heptene-2-one	X							X			
1-[4-(1-Hydroxy-1-methylethyl)phenyl] ethanone								X			
Geranyl acetone								X			
Sulfur compounds											
Cabon disulfide	X					X			X	X	
Hydrogen sulfide	X										
S-methylmethanethioate	X										
S-methylprapnethioate	X										
Carbon oxide sulfide				X					X		
Sulfur dioxide						X			X		
Methanethiol										X	(Dekeirsschieter et al., 2009; Rosier et al., 2014, 2015; Statheropoulos et al., 2011)
3-Methylthio-1-propanol										X	(Kasper et al., 2015; Rosier et al., 2015, 2016)
Dimethyl sulfide					X						(Statheropoulos et al., 2011; Christian von Hoermann et al., 2016)
Dimethyl disulfide	X			X	X	X	X		X		X (Cablk et al., 2012; Dekeirsschieter et al., 2009, 2012; Perrault, Stefanuto, et al., 2015; Rosier et al., 2015, 2016; Statheropoulos et al., 2011)
Dimethyl trisulfide				X	X	X		X	X		(Dekeirsschieter et al., 2012; Kasper et al., 2015; Perrault, Stefanuto, et al., 2015; Rosier et al., 2014, 2016; Statheropoulos et al., 2011)
Diethyl disulfide										X	X
Dimethyl tetrasulfide	X										
Methyl(methylthio)ethyl disulfide										X	X (Rosier et al., 2015, 2016; Statheropoulos et al., 2011)
Methylisobutyl disulfide										X	(Rosier et al., 2015)
Methylhexyl disulfide										X	(Rosier et al., 2015)
Methylethyl disulfide	X				X					X	(Rosier et al., 2015)
Methylpropyldisulfide				X							

Methylisopropyl disulfide	X							X		(Rosier et al., 2015)
Methyl allyl disulfide		X								
S-methyl-3-methylbutanethioate	X									
1-Methylthiohexane	X									
1-Methylthioheptane	X							X		(Rosier et al., 2015)
Methylthiopentane	X									
Methane, thiobis							X			
2.4-Dithiapentane	X									
Nitrogen compounds										
Methanamine							X			(Statheropoulos et al., 2011)
Methenamine			X		X					(Perrault, Stefanuto, et al., 2015; Statheropoulos et al., 2011)
2,5-Dimethyl-1H-pyrrole									X	(Dekeirsschieter et al., 2012; Rosier et al., 2016)
Pyridine	X							X	X	(Dekeirsschieter et al., 2009; Rosier et al., 2015, 2016; Statheropoulos et al., 2011)
Dimethyl pyridine									X	
2,5-Dimethylpyrazine								X		(Christian von Hoermann et al., 2016)
Trimethylpyrazine								X		(Dekeirsschieter et al., 2009; Christian von Hoermann et al., 2016)
Tetramethylpyrazine	X							X		(Christian von Hoermann et al., 2016)
Benzonitrile			X			X				(Brasseur et al., 2012; Dekeirsschieter et al., 2009; Perrault, Stefanuto, et al., 2015)
Methoxy phenyl oxime						X				
1-H-Indene, 2,4-dimethyl				X						
Nitroso methane		X								
Heptane nitrile	X									
Hydrocarbons										
2,2,3-Trimethylbutane				X						
2,3-Dimethylbutane				X						
2,2-Dimethylbutane				X						
2,5-Dimethyloctane									X	
2-Methylnonane									X	
2-Methylbutane							X			
Pentane				X			X			(Agapiou et al., 2015; Perrault, Stefanuto, et al., 2015; Rosier et al., 2016; Statheropoulos et al., 2011)
3-Methylpentane				X						
2-Methylpentane				X			X			
Hexane	X			X	X	X	X			(Brasseur et al., 2012; Cablk et al., 2012; Statheropoulos et al., 2011; Stefanuto et al., 2015)
2,4-Dimethylheptane				X						(Dekeirsschieter et al., 2012)
2,3-Dimethylpentane				X						
2,4-Dimethylpentane				X						
2-Methylhexane				X						
3-Methylhexane				X						
Heptane	X			X	X		X			(Dekeirsschieter et al., 2012; Perrault, Stefanuto, et al., 2015)
2,3,4-Trimethylpentane				X						
2-Methylheptane				X						
3-Methylheptane				X						
2,3-Dimethylhexane				X						
Octane	X			X	X		X			(Dekeirsschieter et al., 2012; Perrault, Stefanuto, et al., 2015)
2-Methyloctane				X						
Nonane	X						X			(Dekeirsschieter et al., 2012; Statheropoulos et al., 2011)
2,3-Dimethyloctane				X						
Tetramethylhexane					X					
Decane					X		X			(Agapiou et al., 2015; Cablk et al., 2012; Statheropoulos et al., 2011)

Undecane	X				X		X	X	(Cablk et al., 2012; Dekeirsschieter et al., 2012; Statheropoulos et al., 2011)
Trimethyldecane				X					
Tridecane							X		(Statheropoulos et al., 2011)
Tetradecane							X		(Dekeirsschieter et al., 2012; Perrault, Stefanuto, et al., 2015; Statheropoulos et al., 2011)
Pentadecane							X		(Rosier et al., 2016; Statheropoulos et al., 2011)
Hexadecane							X		(Frederickx et al., 2012; Perrault, Stefanuto, et al., 2015)
Heptadecane							X		
Methylcyclopentane				X					
Cyclohexane				X					
1,4-dimethylcyclohexane				X					
1-Ethyl-4-methylcyclohexane				X					
1,2,4-Trimethylcyclohexane				X					
1,3,5-Trimethylcyclohexane				X					
1,1,3,5-Tetramethylcyclohexane									
3-Ethyl-2methyl-heptane		X							
1-Propene				X					
2-Methylpropene				X				X	
2-Pentene								X	(Perrault, Stefanuto, et al., 2015)
1-Pentene				X					
1,3-Pentadiene				X					(Rosier et al., 2014)
1-Heptene				X					(Perrault, Stefanuto, et al., 2015; Statheropoulos et al., 2011)
1-Hexene				X					(Perrault, Stefanuto, et al., 2015; Statheropoulos et al., 2011)
2-Octene	X			X					
3-Octene				X					
4-Methyl-2-pentene				X					
1-Decene	X								
Isoprene				X					(Statheropoulos et al., 2011)
Undecene	X			X					18]
1-Pentadecene	X								

A directory of human cadaveric VOCs

The 12 studies produced lists of VOCs collected on decomposing human bodies, human remains and grave-soil. Some of these studies aimed to identify VOCs specific to human decomposition (Rosier et al., 2015, 2016). However, no consensus was obtained among these studies since, to date, no human specific markers have been identified (Cablk et al., 2012; Degreeff & Furton, 2011; Rosier et al., 2015, 2016; Vass, 2012).

How the stage of decomposition impacts the emission of cadaveric VOCs has been well characterized in pig carcasses (*Sus domesticus* L.) (Dekeirsschieter et al., 2009). However, no similar work has been performed on human cadavers. The stage of decomposition of sampled human cadavers is poorly described. The limited sample size also makes it difficult to identify the VOCs associated with each stage.

As identified from surrogate human models (Agapiou et al., 2015; Dekeirsschieter et al., 2009), almost all of the chemical families of compounds are found in the VOCs profile of human cadavers (Table 2). Dimethyl disulfide is probably the most common compound, and has been identified in both human cadavers and surrogate human models, such as piglets (Agapiou et al., 2015; Dekeirsschieter et al., 2009). A total of 211 VOCs was identified from human cadavers (Table 2), many of which have only been listed in one or two studies. This issue demonstrates the large heterogeneity among the samples studied in previous studies, and supports the fact that the chemical markers of human decomposition are likely difficult to identify. Overall, there is a general consensus that more studies must be performed on human cadavers to characterize the volatile compounds associated with human decomposition with greater accuracy.

Conclusion and perspectives

To date, the post-mortem volatilome of human cadavers has received limited attention, at least compared to other vertebrates. To elucidate the chemical complexity of this volatilome, powerful analytical tools and proper expertise are required. The difficulties associated with the lack of samples leads to a lack of understanding and consensus across studies.

Specialists in this field agree on the necessity to complete the characterization of the human cadaveric volatilome, at least with the most common constituents, per stage of decomposition. The identification of a group of chemical markers would clearly improve the training of HRDDs. Another challenge is the identification of factors that influence the cadaveric odor profile. VOC composition depends on the stage of decomposition, weight, age and gender of the cadaver, and the presence of wounds and access of insects to the body, among other factors (Martin & Verheggen, 2018a). Thus, the ability to detect human cadavers could be enhanced if some targeted VOCs were identified. Hence, it is important to determine the characteristics of the corpses and the conditions of death by analyzing VOCs profiles at crime scene and in gravesoil.

In conclusion, the characterization of the odor profile released by decaying human bodies is among the important challenges of today's forensic scientists. Indeed, part of the task of police forces consists of locating decayed human bodies, with the help of human remains detection dogs (HRDDs) (Oesterhelweg et al., 2008; Tipple et al., 2014; Verheggen et al., 2017). These dogs are often trained with synthetic mixtures of cadaveric volatiles that are commercially available, but are not necessarily representative of human decomposition (Stadler et al., 2012).

Cadaver Dogs and the Deathly Hallows— A Survey and Literature Review on Selection and Training Procedure

This section is an adapted version of the article:

Martin C, Diederich C and Verheggen F (2020) Cadaver Dogs and the Deathly Hallows—A Survey and Literature Review on Selection and Training Procedure. Animals, DOI: 10.3390/ani10071219

Abstract

Human remains detection dogs (HRDDs) are powerful police assets to locate a corpse. However, the methods used to select and train them are as diverse as the number of countries with such a canine brigade. First, a survey sent to human remains searching brigades ($N_{countries} = 10$; $N_{Brigades} = 16$; $N_{Handlers} = 50$; $N_{questions} = 9$), to collect their working habits confirmed the lack of optimized selection and training procedures. Second, a literature review was performed in order to outline the strengths and shortcomings of HRDDs training. A comparison between the scientific knowledge and the common practices used by HRDDs brigade was then conducted focusing on HRDDs selection and training procedures. We highlighted that HRDDs' handlers select their dogs by focusing on behavioral traits while ignoring anatomical features, which have been shown to be important. Most handlers reported to use a reward-based training, which is in accordance with training literature for dogs. Training aids should be representative of the target odor to allow a dog to reach optimal efficiency. The survey highlighted the wide diversity of homemade training aids, and the need to optimize their composition. In the present document, key research topics to improve HRDD works are also provided.

Introduction

Human remains detection dogs (HRDDs) are "canines specially trained to find human decomposition scent and alert their handler to its location" (Rebmann & David, 2000). In addition to be called cadaver dogs, HRDDs are most likely less known than other categories of scent-detection dogs such as explosive or drug detection dogs. However, they are used by law enforcements in many contexts including homicides, as well as natural and manmade disasters to search for human cadavers, body parts, or fluids (Komar, 1999; Migala & Brown, 2012; Nizio et al., 2017; Oesterhelweg et al., 2008; Rebmann & David, 2000; Riezzo et al., 2014; L. T. Rust et al., 2018). These activities are usually gathered under the term "necrosearch" (Van Denhouwe & Schotsmans, 2014).

During dog domestication, humans selected individuals upon their olfactory abilities, which were particularly important to the development of hunting dog breeds (Quignon et al., 2012). As a result, today's breeds of detection dogs show accurate, sensitive, and reliable olfactory abilities (Frederickx et al., 2011; Hall et al., 2013; R. J. Harper & Furton, 2007; Rendine et al., 2018; Riezzo et al., 2014; Sacharczuk et al., 2019). Dogs are able to recognize several hundred compounds due to a wide diversity of olfactory receptor cells present in their olfactory epithelium (Chen et al., 2012; Lesniak et al., 2008; Mombaerts et al., 1965; Quignon et al., 2005, 2012). In addition, polymorphism in olfactory receptors genes increases the number of molecules that can be bound, making dogs powerful biological detectors. However, not all dogs are suitable to perform detection work, as a result of *e.g.*, their head conformation, their olfactory sensibility, or the polymorphism of their olfactory receptor genes (Beebe et al., 2016; Chen et al., 2012; Frederickx et al., 2011; Lesniak et al., 2008; Polgár et al., 2016; Quignon et al., 2012; Sacharczuk et al., 2019).

The diversified environments in which HRDDs are deployed require dogs with specific skills. While some of them can be learned, several intrinsic traits would ease the training program. Handlers would benefit from identifying morphological, olfactory, and behavioral traits that indicate a dog's suitability to become an HRDD. However, poor information is available regarding the selection of HRDDs, including traits and procedures. The scientific literature mainly focuses on the selection of service dogs (Tomkins et al., 2011; Wilsson & Sundgren, 1997). As regards to the training procedures, dogs as pets are more studied compared to working dogs (Coppinger et al., 1998; Feng et al., 2016; Ziv, 2017).

The conditioning of detection dogs relies on the quality of the available olfactory stimulus used during the training, also called training aids (R. J. Harper et al., 2005). HRDDs should be trained with aids that mimic the smell of any human cadaver. Some

training aids are available on the market but were shown to be unrepresentative of the smell of a real decaying corpse (Stadler et al., 2012). The characterizations of the volatile organic compounds (VOCs) released by human corpses are scarce as they are complicated to carry out (Dekeirsschieter et al., 2012; Martin & Verheggen, 2018b). The cadaveric smell is composed of up to 800 molecules belonging to almost all chemical classes (*e.g.*, alkanes, ketones, aromatics, amines, and sulfur compounds) (Dekeirsschieter et al., 2012; Focant et al., 2013; Perrault, Nizio, et al., 2015; Stefanuto et al., 2015). Moreover, the decomposition of a corpse involves a wide range of interweave mechanisms making it highly variable and uneasy to understand (Agapiou et al., 2015; Dekeirsschieter et al., 2009; Janaway et al., 2009b; Martin & Verheggen, 2018b; Rosier et al., 2016). Numerous biotic (*e.g.*, tissues nature, animal species, necrophagous insects, microorganisms) and abiotic factors (*e.g.*, humidity, temperature, death location) affect the volatilome of a cadaver (Cernosek et al., 2019; Dubois, Stefanuto, et al., 2019; Finley et al., 2015; Martin et al., 2019; Rosier et al., 2016). Furthermore, a decaying corpse typically goes through five stages of decomposition respectively named fresh, bloated, active decay, advanced decay and dry remains, all characterized by different blends of VOCs (Dekeirsschieter et al., 2009, 2012; Statheropoulos et al., 2011). As a result, the volatiles released during the decomposition process differ greatly from one case to another (Dekeirsschieter et al., 2009, 2012; Dubois, Stefanuto, et al., 2019; Martin & Verheggen, 2018a; Nizio et al., 2017; Rosier et al., 2016), making it difficult to provide HRDDs' handlers with training aids mimicking human decomposition.

This review aimed to gather all available information on the selection and the training procedures of detection dogs. This research was carried out through two approaches: (i) a survey that was sent to human remains searching brigades across the world, in order to collect their working habits; (ii) a literature review carried out to outline strengths and shortcomings of the selection (anatomical and behavioral traits) and training of HRDDs. A comparison between the scientific knowledge and common practices used by law enforcement forces was then performed. Finally, based on this comparison, we have provided a guideline compiling the best scientific-based practices to improve HRDDs' works, as well as perspectives for further scientific researches in the field.

Methods

Bibliographic research

The bibliographic research was performed using Google Scholar®, Science Direct® and Scopus® between June and December 2019. First research was performed by focusing on the selection and training of cadaver dogs. The words' combination used

was the following: "("cadaver dog" OR HRDD OR "human remains searching dog" OR "human remains detection dog") AND (selection OR training)". This research only highlighted two publications. We therefore decided to extend our research to other types of detection dogs and gathered information that could be applied to HRDDs. We ended up conducting different bibliographic researches according to the section of the present review (Table 3).

Regarding the "anatomical traits" section of this work, we have extended the scope to wildlife detection dogs, because they have to face similar working environments as HRDDs: they mostly perform their searching activities outdoor, rather than indoor. Regarding the "methods of selection", "behavioral traits" and "olfactory traits" section of this work, we have extended the scope to all detection dogs, because they are not dependent of environmental searching conditions. Regarding the "training methods" section, all working dogs were taken into account as the methods can be considered equivalent. Finally, the "training aids" section focused only on HRDDs because the olfactory cues used during the training have to be representative of the smell of a decaying cadaver.

Table 3 Bibliographic research performed for each section of the present review using Scopus®, Science Direct® and Google Scholar® between June and December 2019.

Review Outline	Combinations of keywords
	Selection
Anatomical traits	Morphological: (selection AND suitable AND wildlife AND "detection dogs") Olfactory: (selection AND "detection dog" AND ((olfaction OR "Olfactory system") OR ("genetic marker"))
Behavioral traits	(Selection AND ("detection dog" OR "scent detection dog") AND "behavio(u)ral trait")
Methods	(Selection AND (method OR methodology) AND police AND forensic AND "detection dog" AND (method OR procedure OR methodology))
	Training
Methods	("human remains searching dog" OR "cadaver dog") AND ("training procedure" OR "training method" OR "training methodology")
Training aids	(("human remains searching dog" OR "cadaver dog") AND "training aid")

Survey

A survey made of nine questions based on information from the literature and provided by Belgian HRDDs' handlers (Table 4) was sent to the Kynopol Secretariat, who forwarded it to all affiliated human remains searching brigades around the world (LimeSurvey®). The survey was written in English. Sixteen brigades answered the survey between August 1st and September 20th, 2019. Response to the questions was

not mandatory. The handlers belonging to the brigades also had the possibility to add comments for each question listed in Table 4. Ten brigades out of 16 also described the HRDDs working in their brigades. Only the responses provided by HRDD's handlers were taken into account. Fifty handlers from ten countries answered the survey, including Canada, United-Kingdom, Portugal, Belgium, Sweden, Finland, Austria, Slovenia, Romania, and Cyprus.

Through the present document, the word 'brigade' is applied to the entire department (including handlers and HRDDs). The results of the survey are presented at the beginning of each part of the review (selection and training).

Table 4 Survey sent to handlers (N = 50) of human remains detection dog (HRDDs).

Survey Outline	Questions
	Selection
Anatomical traits considered during the selection of HRDDs	Q1: What are the physical characteristics you are paying attention to when you select a dog to train? (For each trait, select among: very important/important/not important) Size, Length and thickness of the fur, Ability to run, Muscle mass Q2: How do you assess the physical characteristics?
Behavioral traits considered during the selection of HRDDs	Q3: What are the behavioral characteristics you are paying attention to when you select your searching dogs? (For each trait, select among: very important/important/not important) Dominant, Social, Curious, Dynamic, Player, Independent, Obedient, Adventurous Q4: How do you assess these behavioral characteristics?
	Training
	Q5: What kind of training do you perform? Punishment (including take off the toy). If yes: What kind of punishment? (Electric shock/strike/other) Reward. If yes: Which kind of reward? (Toy/vocal encouragement/ food/ clicker) Q6: What is the duration of a training session (in minutes)? Q7: What kind of training aids do you use? Compress with human cadaveric fluids, introduced in a container with human organs and/or blood, with animal cadaveric fluids, Clothes wear by human cadaver, Gravesoil or Commercial kit Q8: Could you describe your trainings? Q9: If you use biological training can you describe how you obtain the final trainings aids?

Data analyses

A descriptive analysis of the survey responses has been performed. A subjective number between 0 and 100 was dedicated to each level of importance (not important = 0; important = 50 and very important = 100). We attributed a score to each selection parameters based on the answers of handlers (Equation 1). All the other answers were plotted by using Microsoft Excel (Version 16) or R Studio (RStudio, 2019). When possible, a chi-square test was performed to compare the proportions of response. However, due to a low sample size, statistical analyses were not often implemented.

Equation (1): Calculation of the score of importance of the different selection parameters evaluated in the survey (n = number of handlers choosing a specific level of importance (not important, important, very important) and N = total number of handlers responding to the survey).

$$\text{Importance score} = \frac{(n_{not\ important}\times 0)+(n_{important}\times 50)+(n_{very\ important}\times 100)}{N_{Handlers}}$$

Selection of human remains detection dogs

Results of the survey

The survey recorded a number of 7±2 HRDDs per country (data from seven countries). Moreover, based on our survey ($N_{Brigades} = 16$, $N_{Handlers} = 50$), we conclude that there is no standard procedure being applied for selecting dogs within a litter. All teams (except one) declared to implement homemade selection tests to assess the learning abilities of puppies during preliminary training sessions, taking into account some specific traits (*e.g.*, drive, independence). In most cases, the selection is based on the handler's own opinion during training or playing time only, without collecting any quantitative measures. Only two brigades (out of 16) perform normalized tests whose detailed description has not been provided. These two brigades assess the dog morphology as well as about 20 behavioral traits including what they call drives, courage, aggression, and nerve strength. Respondents mostly ignore morphological traits during the selection of HRDDs. They usually select puppies within the same breed (Figure 1). The four preferred breeds are: Malinois shepherd, German shepherd, English spaniel and Labrador retriever. Agility and stamina are the most important traits according to handlers, while dog size, fur length, and muscle mass are barely taken into account (Figure 2). Handlers from only two brigades pointed out that size and fur length are important traits that could influence dog performances. These two brigades mentioned that the perfect dog size is medium to tall while fur length has to be long. Among the brigades describing their HRDDs teams (10 brigades out of 16), males are more often used than females ($X_2 = 5.23$; p-value = 0.02). In addition, they prefer to not neuter their dogs ($X_2 = 16.09$; p-value < 0.001) with only 23% of HRDDs being neutered. All Canadian and English dogs are neutered. Neutered dogs are always males, no HRDD females are mentioned.

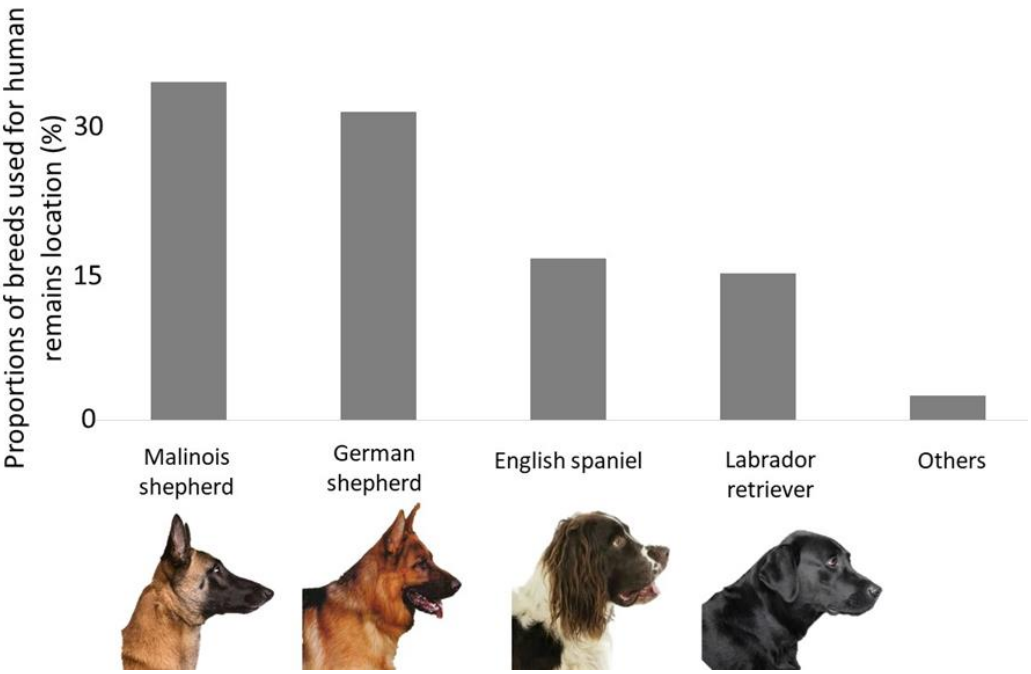

Figure 1 Breeds currently used as human remains detection cadaver dogs based on a survey completed by ten brigades from Europe and Canada.

None of the 50 questioned handlers select their dogs based on olfactory traits. Most handlers agreed about the importance of taking behavioral traits into account during puppies' selection. Handlers highlighted several important parameters: playfulness, curiosity, sociability, independence, and dynamism, with a score higher than 70. In addition, HRDDs should not lead the search and should follow the instruction of the handlers. Some handlers judged that a pronounced leadership is important for an HRDD. None of the surveyed handlers mentioned the importance to evaluate the ability of the dog to cope with stressful situations (Figure 2). Among the responding handlers, 36% assessed the drive during the selection.

Puppy selection remains crucial to obtain efficient adult HRDDs, as all dogs within the same breed or litter do not have the intrinsic qualities to perform such a complex work (Cobb et al., 2015; Hayes et al., 2018; Jamieson et al., 2017; Jezierski et al., 2014; Kerley & Salkina, 2007; Lesniak et al., 2008; Quignon et al., 2012; Sacharczuk et al., 2019). For this reason, a list of recruitment criteria must be defined. However, the survey reveals that no standard procedure is applied to choose dogs within a litter. Moreover, handlers mainly focus their selection on the behavioral traits while literature research suggests that physical characteristics are also important features to consider during the selection (Hussein et al., 2012; Jamieson et al., 2017; Polgár et

al., 2016; Smith et al., 2003; Wilsson & Sundgren, 1997). In the following paragraphs, we discuss the importance of four groups of features on the selection of a detection dog including (i) morphological traits, (ii) olfactory traits, (iii) behavioral traits, and (iv) the documented methods used to assess these three groups of traits.

Morphological traits

HRDDs perform search in a large diversity of environments, including urban (*e.g.*, collapsed building, garden) and wild environments (*e.g.*, woodlands, grasslands, mountains, beaches) (Agapiou et al., 2015; Komar, 1999; Leigh & Dominick, 2015; Oesterhelweg et al., 2008). Like wildlife detection dogs, HRDDs should be agile to move easily in rough fields and have steady stamina to stay efficient during a long period of time (Rebmann & David, 2000; Sinn et al., 2010). Because the dog's size (also called "dog body shape") and the coat length are both correlated with dog's mobility, they should both be considered during dog selection (Chesney, 1997; Hurt & Smith, 2009; Jamieson et al., 2017). Three types of build (large, medium, and small) and two types of coat length (short and long) are typically highlighted in the literature (Beebe et al., 2016). Handlers should prefer a medium built dog (*e.g.*, Springer spaniel, Labrador retriever, German shepherd) to ease their move in a rough environment. Another advantage of these breeds is that their thermoregulation is more efficient in various environments (*e.g.*, warm/cold conditions, rough field) (Chesney, 1997; Kerley & Salkina, 2007). Among medium-sized dogs, breeds having proportionally longer legs show better agility (Hurt & Smith, 2009; Reed et al., 2011). Large dogs may experience trouble to cool down in hot or strenuous environments whereas small dogs are known to get tired faster under these environments (Hurt & Smith, 2009; Polgár et al., 2016; Reed et al., 2011). Heat tolerance is not only regulated by the size of the dog but also by the length of the coat. A long coat will reduce the dog's mobility while it will be an obstacle to the smooth running of his work under hot weather. On the other hand, a short coat decreases the performance of the dog in case of cold conditions. The main point to focus on is the ability of the dog to regulate its own temperature during the searching time as it will impact its stamina (Bulanda, 2010; Chesney, 1997; Hurt & Smith, 2009; Polgár et al., 2016). In their book "Cadaver Dog Handbook", Rebmann et al. advised that the working environment should define dog size and coat length (Beebe et al., 2016; Rebmann & David, 2000).

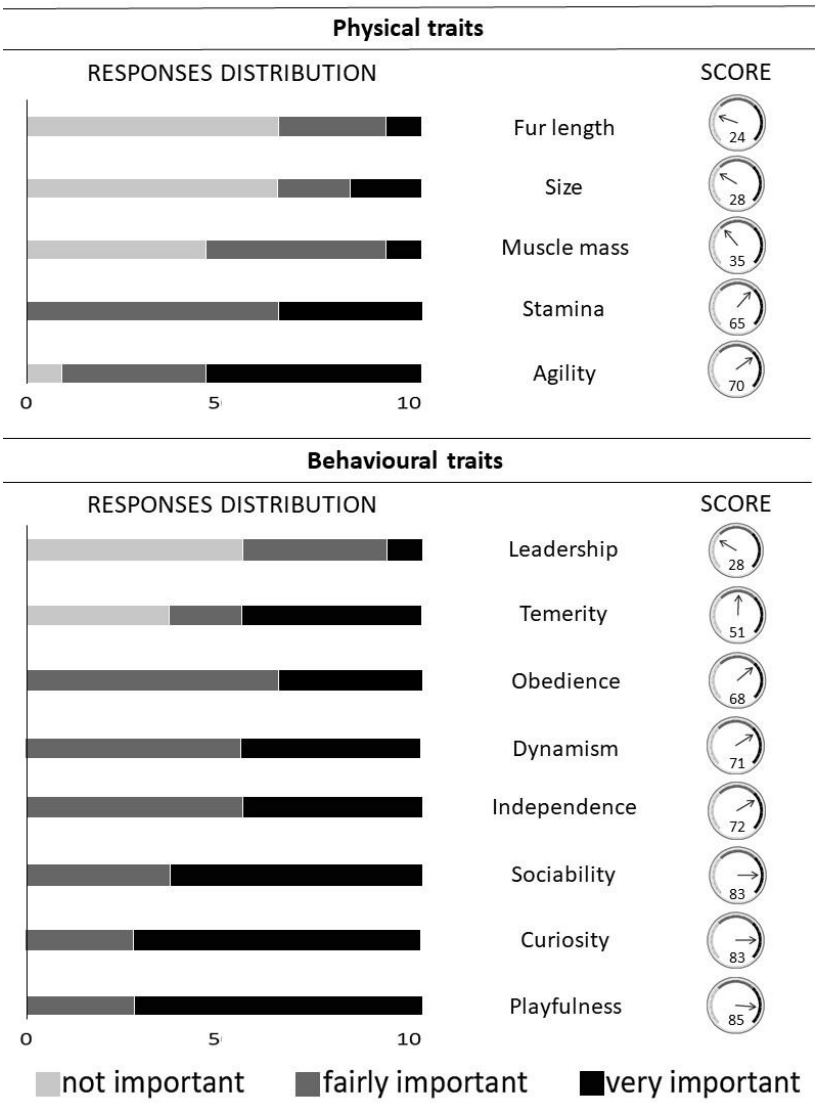

Figure 2 Handlers' estimation of the importance of the physical and behavioral traits that must be considered during the selection of HRDDs human remains detection dogs. The response distribution describes the percentage of handlers (out of 50) that estimate the importance of each trait (not important, fairly important, very important). The score estimates the importance of the traits during the selection according to equation 1.

While agility was classified as the most important trait, size and coat length were not being considered by the international teams having answered our survey when selecting HRDDs. Sex has not been identified to impact HRDD's efficiency. However, females seem to be less aggressive than males, suggesting that they would

be proficient specialist searching dog (N. J. Rooney & Bradshaw, 2004). However, our survey suggest that males are preferred over females. In addition, no differences were highlighted between neutered and unneutered dogs (N. J. Rooney & Bradshaw, 2004). However, neutered dogs live longer than the non-neutered ones (Moore et al., 2001). Finally, health and expected longevity of the dog have been identified as important factors, among others to not waste time with dogs that would not be able to work properly because of repeated sickness periods (Brady et al., 2018; Cobb et al., 2015; Jamieson et al., 2017; Thrailkill et al., 2018).

Olfactory traits

Some breeds have developed a more efficient olfactory apparatus than others, as a result of hundreds years of selection (Chen et al., 2012; Lesniak et al., 2008; Sacharczuk et al., 2019; Tacher et al., 2005). The efficiency of the olfaction is linked to the structure of the dog's skull. Three types of dog skull conformation can be described: brachycephalic, mesaticephalic, and dolichocephalic breeds (Figure 3). Although the exact description of these conformations is not clearly explained in the literature, researchers calculate the ratio between skull width and skull length, leading to average values of 0.81 for brachycephalic, 0.52 for mesaticephalic, and 0.39 for dolichocephalic (Hussein et al., 2012). The skull conformation will impact the orientation of the olfactory bulb and the volume of the nasal cavity. Brachycephalic breeds are characterized by a compressed nasal cavity, positioning their olfactory bulb in a ventral orientation and reducing the epithelial surface available for the capture of odors (Hussein et al., 2012). Brachycephalic breeds should, therefore, be avoided for olfaction-based work, and mesaticephalic and dolichocephalic breeds should be preferred (Hussein et al., 2012; Jamieson et al., 2017). The head conformation is a characteristic that should be taken into account to easily assess the olfactory capability of a dog: the larger the nasal cavity is, the more olfactory receptors cells (ORCs) are located in the epithelium (Craven et al., 2010; Dahlgren et al., 2011). During our survey, questioned handlers did not mention the structure of the head as a selection factor. However, no brachycephalic breed is used by the questioned teams (Figure 3). To be perceived by the dog's brain, odorant molecules must enter the nasal cavity and reach the olfactory epithelium where olfactory receptor cells (ORCs) bind them. The number of ORCs, which varies among dog breeds, is therefore another important parameter to consider. For example, bloodhounds have 300 million ORCs (the higher number among dog breeds), and German shepherds have 225 million ORCs (Craven et al., 2010; Dahlgren et al., 2011). Variations are observed among dog breeds but also among individuals within the same breed (Jezierski et al., 2014; N. J. Rooney & Bradshaw, 2004; Sacharczuk et al., 2019). Almost 1100 genes are responsible for ORCs synthesis (Quignon et al., 2012; Sacharczuk et al., 2019), which makes any selection effort based on genetic characteristics a difficult task (Chen et al., 2012;

Lesniak et al., 2008; Sacharczuk et al., 2019; Tacher et al., 2005). No genetical selection was mentioned in additional comments, suggesting that they base their selection on interesting genes. A third olfaction-associated parameter that should be considered when selecting a potential HRDD is the size of the ear and dewlaps. Large ears and dewlaps allow a dog to catch more volatile molecules focusing the scent around the nose (Beebe et al., 2016). The size of the ear and dewlaps were not mentioned in the answers of the survey. Yet, German shepherd dogs, which have a dewlap, and English spaniel dogs, which have large ears, are among the most common HRDDs (Figure 1).

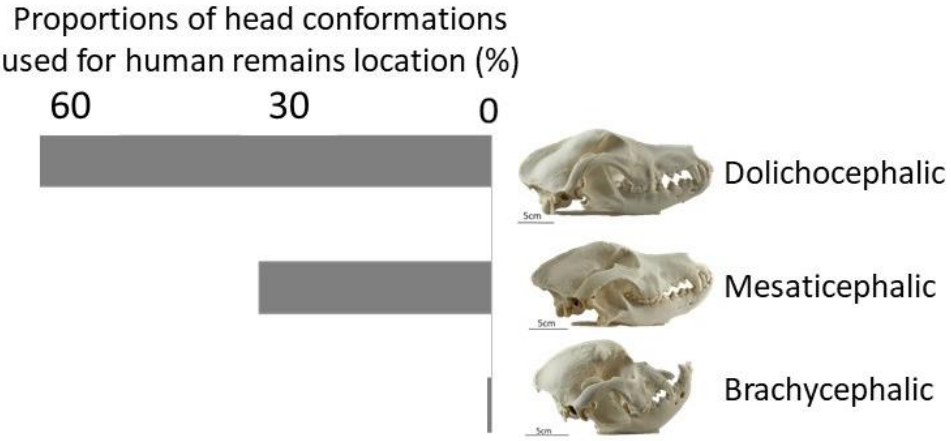

Figure 3 Proportions of dogs declared to be used as human remains detection dogs HRDD s in our survey (N = 10 out of 16 brigades) belonging to the three common types of dog's skull conformation (skull pictures are reproduced with the kind permission of Tibor Csörg).

Behavioral traits

Behavioral traits also play critical roles in the determination of the success or failure of future HRDDs' training (Beebe et al., 2016; Dahlgren et al., 2011; Lazarowski et al., 2018). The behavioral response of a dog facing a particular stimulus is driven by two main components: its temperament and its personality (Bray et al., 2019; Tomkins et al., 2011; Weiss & Greenberg, 1997). Temperament can be defined as the individual difference in behavioral responses, which is steady among time and context, that are grounded in affective state and its regulatory processes and which are evident from an early age (Diederich & Giffroy, 2006). It usually has genetic predisposition (Brady et al., 2018; Diederich & Giffroy, 2006; Jenkins et al., 2018; Lazarowski et al., 2018). Personality is the result of both a dog's genetics and experiences over time, which shapes the dog's behavioral response to situational events (Beebe et al., 2016). Two main behavioral traits should be considered in HRDDs: the drives and the nerve

strength (Brownell & Marsolais, 2000; DeGreeff et al., 2012; Jamieson et al., 2017; Wilsson & Sundgren, 1997).

Some authors consider the term "drive" as synonymous to motivation (Alexander et al., 2011; Brady et al., 2018; Cobb et al., 2015; Jenkins et al., 2018) which is defined as an innate impulse that prompts a canine action while others make the distinction between drive and motivation but without explaining the differences (Alexander et al., 2011; Beebe et al., 2016; Brownell & Marsolais, 2000; Jamieson et al., 2017; Kerley & Salkina, 2007). However, drive is a generic term that includes at least six sub-categories : (i) pack or social drive, (ii) play drive, (iii) food drive, (iv) prey drive, (v) hunt drive, and (vi) defense drive (Brownell & Marsolais, 2000; Lazarowski et al., 2018). Pack drive is the ability of the dog to work in cooperation with humans (Jamieson et al., 2017). Play drive is the desire to be entertained (Jamieson et al., 2017). Food drive is the desire to obtain food (Jenkins et al., 2018). Prey drive is defined as the willingness to engage in a competition game (Wilsson & Sundgren, 1997). Hunt drive is the ability to hunt and locate a prey, and also comes with a desire to be rewarded (DeGreeff et al., 2012; Larson et al., 2011). Defense drive is the tendency to defend itself or its handler (Wilsson & Sundgren, 1997). Most of the many behavioral traits listed in the literature (*e.g.*, trainability, personality, stress, fearfulness, and courage) could be related to one of the drives previously described (Christiansen et al., 2001). In addition, drive is linked to the concentration of the dogs, *i.e.*, its ability to remain focused on its searching work despite the presence of distracting scents or events (Hurt & Smith, 2009; Lazarowski et al., 2018). Drive is among the most important behavioral traits to consider during the selection of a scent detection dog according to the scientific literature (Branson Cobb & McGreevy, 2010; Brownell & Marsolais, 2000; Cablk & Heaton, 2006; Early et al., 2014). Indeed, the stronger drive is, the faster the training is and the better the final performances of HRDDs are (Beebe et al., 2016; Hurt & Smith, 2009; Lazarowski et al., 2018). Scientists however consider that some drive types are more important than others (Lazarowski et al., 2018): Play drive is one of the most important for any wildlife detection dog, including HRDDs. A high play drive dog will have a strong desire to train and receive its reward, which is essential for training and work (Hurt & Smith, 2009; Jamieson et al., 2017; Rebmann & David, 2000). Later, the dog will engage more easily with the searching work. A high play drive dog will be less distracted during the training and the work. Indeed, the dog will concentrate on getting the reward (*e.g.*, toy) (Beebe et al., 2016). Food drive is most likely as important as play drive when it comes to HRDD's selection. Indeed, using a food reward is supposed to bring similar result than a toy (Brownell & Marsolais, 2000; Lazarowski et al., 2018). We suggest to gather play and food drives under a single drive category: reward drive. The reward drive would be defined as the desire of the dog to get the reward. The

more the dog wants its reward, the more it will focus on its work and the less it will be distracted by other stimuli. Two other drives should be considered by handlers: hunt and prey drives (Brownell & Marsolais, 2000; Jezierski et al., 2014). Hunt drive will inform the handler on the motivation of the dog to locate a target without perceiving it by using its nose. It is strongly linked to the prey drive. However, some scientists highlighted that a too high prey drive can be catastrophic during the location of a victim: these dogs could attack the target when they find it (which is not desired during the search of decaying human bodies) (Cablk & Heaton, 2006). One last important drive is the pack drive or social drive: detection dogs have to be able to work with unfamiliar dogs and humans without expressing any anxiety or fear. Pack drive also gives an idea of the level of cooperation that can be expected between a dog and its handler. No study evaluated the impact of high pack drive level on HRDD efficiency. However, we can hypothesize that the better the ability of a dog to understand the gestures and orders of its handler (which is linked to the cooperation with the handler), the easier will be the training (Gácsi et al., 2009; Hurt & Smith, 2009). While a highly developed defense drive is a prerequisite for most police working dogs, it is not a required trait for detection dogs (Brownell & Marsolais, 2000).

The nerve strength is another important behavioral trait to consider. The nerve strength is the ability of the dog to deal with a stressful situation, such as a noisy sound (Beebe et al., 2016; Brownell & Marsolais, 2000). This parameter has to be specially considered for HRDDs as they are likely to work under highly stressful environment (Rebmann & David, 2000). Indeed, dogs exposed to stressful environment will reduce their activity level and are less efficient during learning session (N. Rooney et al., 2009b). HRDDs are trained to not react to auditory, tactile and visual stimuli, as they are likely to work in disaster environments. They must be able to work on unstable ground surfaces, tunnel, or crawl spaces. They are likely to be exposed to gunshots, rock collapse, and fire smoke (Brownell & Marsolais, 2000; Hurt & Smith, 2009). However, it was not confirmed by scientific research that the presence of such stimuli impacts their efficiency (Brownell & Marsolais, 2000; Lazarowski et al., 2018).

Handlers mentioned the drive in the additional comments but never named the types of drive, even if based on the literature review, the drive must be evaluated. Yet, as previously mentioned, none of the surveyed handlers detailed the drives they evaluated. However, they did evaluate some behavioral characteristics that may be related to the drive, such as playfulness, sociability, and curiosity. These traits can be easily associated with play drive, pack drive, and prey or hunt drives. During our review of the literature, we had to face the lack of homogeneity within the vocabulary used to describe behavioral traits which explain the wide diversity of terms used to describe a unique behavior. On the other hand, some handlers judged that a

pronounced dominant trait is important for an HRDD, which is not in accordance with the literature that states that a detection dog should always be led by its handler for more efficient results (Gácsi et al., 2009; Hurt & Smith, 2009; Jamieson et al., 2017). Finally, none of the surveyed handlers mentioned the importance to evaluate the ability of a dog to cope with stressful situations, suggesting that they most likely do not evaluate this ability. They should however train their dog to better resist stressful situations. Indeed, repeated exposure to stressful stimuli increase nerve strength (Brownell & Marsolais, 2000; Lazarowski et al., 2018; Lopes et al., 2015). However, dogs with a high food or play drive may have a higher nerve strength, since they are likely to keep focused on their target to receive their reward as soon as possible. As food and play drive are taken into account during the selection of HRDDs, they might select, simultaneously, higher nerve strength dogs (Rebmann & David, 2000). Standardized tests would be helpful for a more accurate evaluation of behavioral scent detection dog traits, including HRDDs, during their selection.

Selection procedures

Several methods of working dog selection are described in the literature and are summarized in Table 5. Most of these studies deal with the selection of service dogs (Duffy & Serpell, 2012; Tomkins et al., 2011; Wilsson & Sundgren, 1997). Even if several traits are important for both service and detection dogs, different works require different skills. For instance, while a guide dog should constantly stay close to its handler and keep looking at him to receive its instructions, a detection dog works at a distance and pays attention to its handler's verbal communication (Duffy & Serpell, 2012; MacLean & Hare, 2018). Most of these methods assess nerve strength and the above-described drive types (Beebe et al., 2016; Jamieson et al., 2017).

Table 5 Selection methods of detection and service dogs.

Characteristics	Description (according to authors)	Method				
		C-Barq	SDTC	IFT	M-B Scale	GDTP
Drive						
Causal reasoning	Use of visual and auditory cues to infer the location of hidden reward				Δ	Δ
Commitment to toy	Tendency to engage game with its handler (new toys or familiar toys)	Δ			Δ	Δ
Retrieval inhibition	Ability to inhibit prepotent motor response in object retrieval task					Δ
Energy level	Show enthusiasm and be always ready to play	Δ				
Excitation	Before a walk or when owner/visitor is coming home	Δ				
Gaze direction	Ability to use human gaze direction to locate hidden reward				Δ	Δ
Hiding-finding	Object permanence				Δ	Δ
Hunting behaviour	Tendency to track its prey directly or after a turnaround of 360°	Δ		Δ	Δ	
Odour control trials	Control trials ruling out ability to locate hidden food using olfaction				Δ	Δ
Perspective-taking	Tendency to obey/disobey a command depending on whether a human is watching				Δ	Δ
Play with stranger	Tendency to play with familiar toy and stranger			Δ	Δ	
Reaching	Ability to infer reward location based on experimenter's reaching towards baited location					Δ
Retrieval	Tendency to retrieve object and return it to in front of experimenter	Δ			Δ	Δ
Reward preference	Preference for food or toy reward					Δ
Rotation	Egocentric vs. allocentric use of spatial cues					Δ
Social referencing	Tendency to look at human face when joint social activity is interrupted				Δ	Δ
Spatial transpositions	Ability to track location of hidden reward across spatial transformations					Δ
Visual discrimination	Ability to learn arbitrary visual discrimination prediction reward location					Δ
Nerve strength						
Affect discrimination	Preference to approach unfamiliar human	Δ	Δ	Δ	Δ	Δ
Confidence on rough surface	Ability to stay confident on rough surface				Δ	
Confined space	Ability to come from a confined space and enter a lighted area and dark area				Δ	
Fearfulness	Shaking/salivating/agitation/loss of appetite when they are left on its own	Δ				
Laterality: first step	Forelimb preference when initiating a step off a platform	Δ			Δ	Δ
Sociability towards other canines	Preference to approach unfamiliar canine	Δ		Δ	Δ	
Sound sensitivity	Ability to stay confident when confronted to several sounds	Δ	Δ	Δ	Δ	
Stability	Ability to stay confident and stable on unstable surface				Δ	
Surface sensitivity	Ability to travel across a slick surface				Δ	
Threating situation	With stranger, unfamiliar dog or object		Δ	Δ		

Touch anxious	Tendency to stay calm when manipulated by human	Δ	Δ		
Visual sensitivity	Ability to stay relaxed and confident in an area full of smoke	Δ		Δ	
	Trainability				
Arm pointing	Ability to use human arm pointing to find a hidden reward				Δ
Detour navigation	Navigation of shortest route around an obstacle				Δ
Inferential reasoning	Ability to infer the location of hidden reward through the principle of exclusion				Δ
Marker cue	Ability to infer location of hidden reward when human uses a novel communicative marker				Δ
Memory-distraction	Memory for location of reward across delays while dog's attention is discarded				Δ
Odour discrimination	Discrimination and memory for which of two locations is baited using olfaction				Δ
Response to command	Ability to sit/stay	Δ			
Spatial perseverations	Ability to inhibit previously established motor pattern when environment changes				Δ
Working memory	Memory for location of reward across temporal delays				Δ
	Personality				
Attachment	Sign of attention to the owner	Δ			
Begging for food	When people are eating	Δ			
Contagious yawing	Tendency to yawn during auditory exposure to human yawning vs. control sounds				Δ
Dynamism	Hyperactive or restless	Δ			
Escapes	Take each opportunity to escape	Δ			
Olfactory interest	Rolls when facing smelly substance	Δ			
Owner direct aggression	Tendency to be aggressive with owners during daily tasks (batch, eating, game…)	Δ			
Steal behaviour	Steal food	Δ			
Unsolvable task	Help seeking from human vs. independent behaviour when facing unsolvable task				Δ
	Morphological				
Physical exam	Examination of the body tension		Δ		
	Others				
Barks	To alarm or when excited	Δ			
Coprophagy	Eat its own faeces or of another animal	Δ			
Licking	Itself or people or object	Δ			
Pull on leash	During walk	Δ			

Sensory bias	Prioritization of visual vs. olfactory information when senses pitted against one another		Δ
Transparent obstacle	Ability to inhibit direct approach to experimenter when a detour is required		Δ
Urinates	During night, when owner approaches, on object	Δ	

Δ *indicates that the method takes the characteristic into account; C-Barq (Canine Behavioral Assessment and Research Questionnaire; survey), IFT (In For Training; tests), SDTC (tests developed by the Swedish Dog Training Center), M-B scale (tests developed by Brownell and Marsolais 2000) and GDTP (tests developed by the Guide Dog Training Program) M-B scale is the only test having been performed on detection dogs* (Bray et al., 2019; Brownell & Marsolais, 2000; Duffy & Serpell, 2012; MacLean & Hare, 2018; Tomkins et al., 2011).

Two of these selection procedures were scientifically assessed: based on C-Barq assessment (survey on the personality of the dog, (Cook et al., 2014) and on IFT (In-For-Training, exercises or tests, (Duffy & Serpell, 2012)). Both procedures were shown to be accurate and improved the selection of service dogs (Bray et al., 2019). However, the evaluation of these selection procedures did not document scent detection dogs. They were based on the observation of an evaluator, breeder or handler, making the tests subjective. In addition, no metrics were provided to decide after the selection procedure which dogs should be selected. Finally, these documented procedures mostly focus on behavioral traits without taking anatomical and olfactory traits into account. To validate a selection method, it is required to demonstrate its predictive value (*i.e.*, efficiency of the detection dogs). However, no performance assessment tests and skill measurements were found in the literature for HRDDs, most likely explaining why handlers continue to perform homemade selection procedures. We strongly recommend their developments. This lack of interest in the scientific community could be explained by the small number of HRDDs in each country, as revealed by our survey which shows that 7 ± 2 HRDDs are operational per country (data from seven countries). A common agreement on the terms used to describe the behavioral traits has to be adopted as well. These recommendations should help to standardize the HRDDs selection and improve their final efficiency. Finally, the dog genetic (through genetic markers) could also be considered during the selection, because morphological and behavioral traits are inherited (Lesniak et al., 2008; Tacher et al., 2005). The identification of specific markers would ease the selection and could help avoiding subjectivity during the selection.

Training of human remains detection dogs

Results of the survey

During their training sessions, most surveyed handlers declared using reinforcement rather than punishment (Figure 4). A minority of them, however, still mentioned the practice of punishments. When they do so, they do not give the reward to their dog (negative punishment). They are a minority to the handlers who use positive punishment (Figure 4). Among handlers practicing positive reinforcement, no generalization can be made. They reported to use either toys, clicker, food, or encouragement as positive stimuli. Most of them use a combination of the different rewards. Handlers train their dogs on average for 33.3 ± 3.2 min, 3.2 ± 0.4 times per week. Handlers did not explain the way their training sessions are organized. No information was provided about how the training session ends.

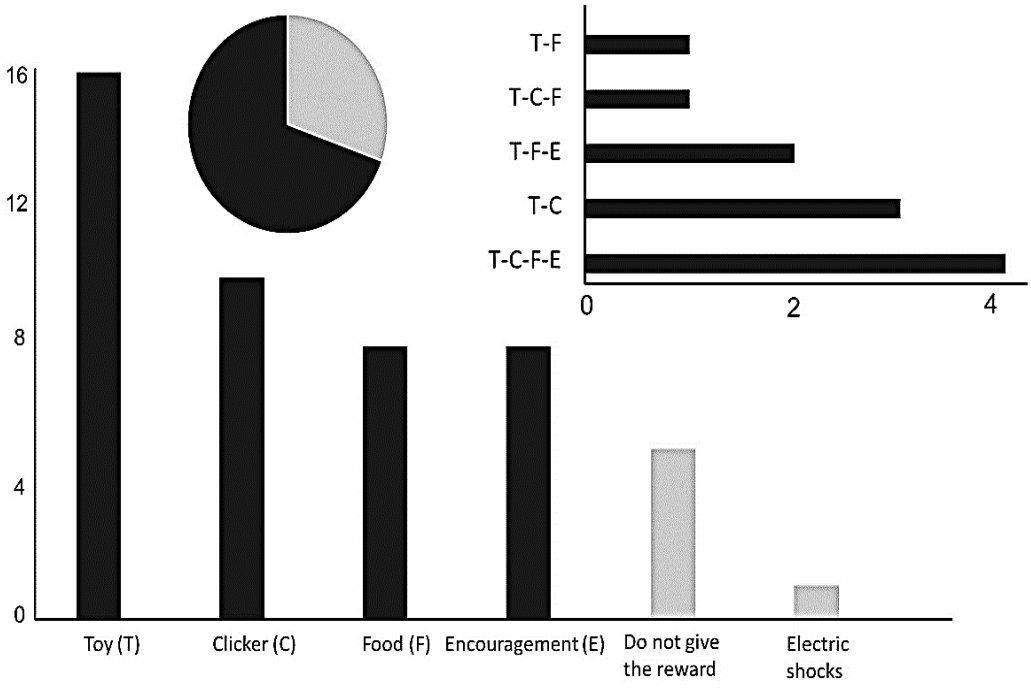

Figure 4. Types of operant conditioning used by the surveyed handlers (y axes, N = 50) to train their human remains detection dogs with the pie chart representing the proportion of reinforcement and punishment technics used (light grey: punishment, dark grey: reinforcement).

Olfactory stimuli used during training were very different among teams (Figure 5). They reported to only use homemade natural aids (human or swine origin). Most of the handlers used aids of human origin. Commercial aids available on the market were not used.

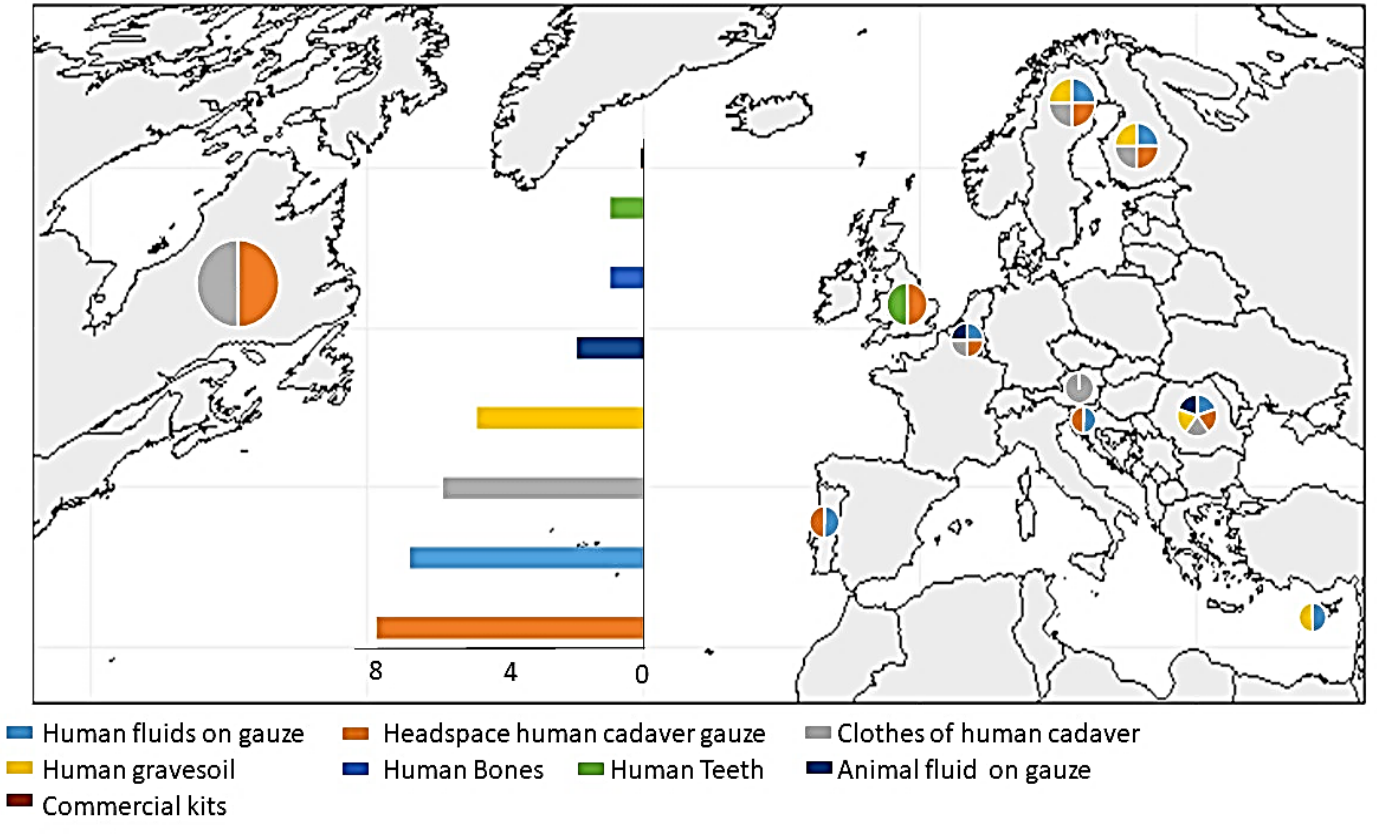

Figure 5. Types of training aids used by the surveyed human remains detection dogs' brigades to train their dogs. Colors in the pie charts represent the diversity of training aids used by the teams in the country where the chart is located. The bar chart represents the number of human remains detection dogs' teams (y axe) which use the different training aids.

Training methods

Most of the research performed on dog training procedures is dedicated to pets (Feng et al., 2016; Ziv, 2017). Documented HRDDs' training methods are scarce and when available, they focus on case studies (Rendine et al., 2018). Moreover, none of them were associated with an evaluation of the performances and behavioral outcomes of the dogs. Training methods were based on operant conditioning during which a dog learns that its response to a command has consequences. The dog will repeat the behavioral response to the specific stimulus that leads to the higher benefit (Doré & Mercier, 1992; Rendine et al., 2018). Reinforcements are associated with improved abilities to learn (E. J. Blackwell et al., 2008; Ziv, 2017). To type of reinforcement can be used: the positive reinforcement and the negative one. The latter may increase aggressiveness and fear and should therefore be avoided (Rendine et al., 2018; N. Rooney & Cowan, 2011; Ziv, 2017). Moreover, a positive reinforcement leads to the creation of a strong relationship between a dog and its handler. While negative reinforcement is associated with distraction and lower obedience (Rendine et al., 2018) . Despite these observations, the type of reinforcement remains a matter of debate among handlers: it is still unclear whether aversive and reward-based methods lead to different levels of efficiency in working dogs (Ziv, 2017).

In a recent publication (Topoleski et al., 2018), the Federal Bureau of Investigation (FBI) raised the importance of End of Session Cues (EoSC). EoSC is a (series of) stimulus that informs the dog about the coming end of the training session. EoSC must be clearly and repeatedly introduced at the end of each training session to avoid the dog to associate events that follow the training session (*e.g.*, return in the cage) as part of the training. If EoSC are not applied, the dog could reinforce unwanted behavioral responses. For instance, going back to the cage immediately after a training session could be considered by the dog as a punishment. Instead, a training session should end in a positive way, for example a playing period, which will likely increase dogs' efficiency (Affenzeller et al., 2017; Topoleski et al., 2018).

Aids

There is a wide diversity of materials that can be used to train HRDDs, including natural aids and synthetic ones (Stadler et al., 2012). As far as the authors know, no publication comparing the efficiency of various training aids on dogs' performances is available. So far, scientists seem to agree that the natural aids are the most efficient and reliable ones that can be used to train HRDDs (Hoffman et al., 2009; Nizio et al., 2017). However, they are difficult to obtain due to ethics, legislation, and biohazard risk for both human and dogs (DeGreeff et al., 2012; Oesterhelweg et al., 2008; Stadler et al., 2012). As already mentioned, the chemical profile released by a cadaver was deeply investigated by research who highlighted their highly variability (Agapiou et

al., 2015; Dekeirsschieter et al., 2009, 2012; Dubois, Stefanuto, et al., 2019; Martin et al., 2019), making HRDD work hard to perform, and the choice of the training aid difficult to make. Dogs must be able to recognize a wide range of chemical compounds associated to cadavers that may be fresh or putrefied, entire or torn into pieces, buried or not (DeGreeff et al., 2012). Gravesoil and cadavers' clothes seem to be among the most representative sources of the smell of a decaying corpse, because they accumulate fluids and are imbued by cadaveric odors (Alexander et al., 2015; Nizio et al., 2017). When they use cadaver clothes, handlers must pay attention to choose cotton clothes, because cotton better adsorbs cadaveric VOCs than composite tissues. Moreover, even if the clothes accumulate the odor released during the decomposition process, it is hardly recommended that the handler uses textiles associated with a different *postmortem* interval to present to the dogs an overview of the different situation that can be met (Nizio et al., 2017). Regarding gravesoil as training aids, one should pay attention to use a control soil to avoid the dog to be trained on non-targeted chemical substances (related to soil, and not to a cadaver) (Alexander et al., 2015). The use of cadaver pieces as training aids (*e.g.*, bones, flesh) should be avoided. They are not only difficult to obtain; they also do not cover the entire diversity of volatile chemicals released by a cadaver (Dekeirsschieter et al., 2012). The use of cadaver pieces as aids makes them unavailable for the court to perform additional analyses, and the preservation of forensic evidences can be compromised (Caraballo, Mendel, et al., 2016). A solution could be the use of human surrogate model (*e.i.,* pig). However, animal decomposition leads to different volatile signatures among species. The cadaveric VOCs released by decaying pigs are, for instance, different from those released by decaying human bodies (Rosier et al., 2015, 2016). Among the most reliable solution of training aids lies the Scent Transfer Unit 100TM (STU-100), which allows to trap the scent of a decaying corpse by pulling the surrounding headspace air. The air passes through a gauze which traps the volatile molecules and can then be used as training aids. This technique does not compromise the integrity of the material (*e.g.*, corpses, clothes, soil…) and meets ethical and biohazard recommendations. It provides a reliable training aid because it is representative of a dead body, adsorbing 60 to 85% of the total post-mortem VOC profile (Eckenrode et al., 2006). Commercial aids, also called synthetics aids, are not representative and reliable, because they do not contain compounds that have been previously reported within the headspace of human decaying bodies (Stadler et al., 2012).

Our survey revealed that handlers mostly use human origin aids which is in accordance with the scientific recommendations. However, they also meet biohazard and ethics problems. Those who use a human surrogate model to train their dogs have to face to the potential differences existing between these model and human corpses.

None of the handlers mentioned the use of commercial aids. In our opinion, developing a reliable synthetic aid is a promising perspective that would ease the training efforts of HRDDs' brigades. It requires a fine characterization of human post-mortem volatilome, and the identification of its key components, common to any decomposing corpse (Martin & Verheggen, 2018b). HRDD olfaction should also be better understood to avoid including compounds that cannot be perceived by the dog olfactory system. We recommend further researches to identify the chemical compounds that can be perceived by HRDDs, and to provide the composition of a synthetic training aid that would be cheap, easy to make, and lead to high levels of performance (Alexander et al., 2015; Hoffman et al., 2009; Jia et al., 2014; Stadler et al., 2012).

Conclusions

In addition to the recommendations made above, we suggest some perspective of research and suggestions to the HRDDs' brigades.

First, there is a lack of validated methods to be applied regarding the selection of puppies. We suggest the existing methods of selection to be validated. A selection method based on anatomical, olfactory, and behavioral traits should be developed. As no quantifiable data were recorded during the selection performed by handlers, this method should include measurable data to avoid subjectivity and to allow dog handlers to select the most promising individuals. So far, no bio markers indicating potential good detection dogs are available. One should therefore investigate the possibility to use, during the dog selection, biomarkers associated with high dog's performances. To validate methods or potential biomarkers, it is important to develop a protocol for measuring dog performances. As no consortium is dealing with the vocabulary used to describe behavioral traits of detection dogs, we also recommend a common agreement on the vocabulary to be used.

Regarding training procedures, one should compare the impact of conditioning methods (including the use of clickers) on HRDDs' performances. The olfactory aids used during the training of HRDDs are of prime importance. The impact of the composition of a HRDD training aid (natural or synthetic) on the dog performance has never been evaluated. In our opinion, the development of a synthetic aid mimicking the human cadaver volatilome is most likely one of the most promising perspectives, as this would provide handlers with a training tool that is reliable, ethical, easy to obtain and use.

Chapter 2

Objectives

Over the past ten years, the cadaveric volatilome has received increasing interest by the scientific community. We now have more information of the odors released during the decaying process of vertebrate remains. Also, while applications are emerging, very few studies have evaluated the potential of cadaveric compounds in the training of human remains detection dogs.

In this thesis, we therefore have set two specific, but complementary objectives: (1) we aimed at evaluating the impact of the environmental conditions on the cadaveric volatilome; and (2) we aimed at evaluating the potential of synthetic cadaveric odors in the conditioning of human remains detection dogs (HRDDs) (Figure 1).

The cadaveric volatilome released during the decaying process is composed of several hundreds of compounds belonging to a wide diversity of chemical classes (*e.g.*, carboxylic acid, ketones, sulfur containing compounds, nitrogen containing compounds*)*. Scientists agree to refer to some specific compounds as decaying biomarkers, this is the case for dimethyl disulfide, for instance. However, some other compounds are less common. Indeed, the cadaveric profile is impacted by several biotic and abiotic parameters, such as the decaying stage and the vertebrate species of the remains. **In chapter III,** we investigate the effect of some environmental parameters on the cadaveric volatilome: the presence of necrophagous insects, and the medium in which the corpse decay (*i.e.*, in the water, in the soil, at the surface). To reach this objective, rats (*Rattus norvegicus*) were selected as surrogate human model, as it was not possible to work on human remains under controlled conditions. It is well known that necrophagous insects are key actors fastening the decaying process. However, their impact on the volatiles released by colonized remains were not assessed. Hence, the first part of third chapter, dedicated to the open-air decomposition, aims at characterizing the odor profile released by dead rats under different conditions of insect colonization (*i.e.*, without insect, with Diptera, with Coleoptera and with both orders). The second part dedicated to the underground decomposition, examines the chemical and volatile changes over time occurring throughout the soil column in two common soil-texture types (*i.e.*, sandy loam and loam). Finally, as the volatilome of immerged remains has received less attention, we decided to characterized the volatile compounds released at the water surface by rats decaying in water of two different salinity levels (*i.e.*, sea water and soft water). In addition, we developed a method to characterize the volatiles that did not reach the surface and stay dissolved in the water during the decomposition.

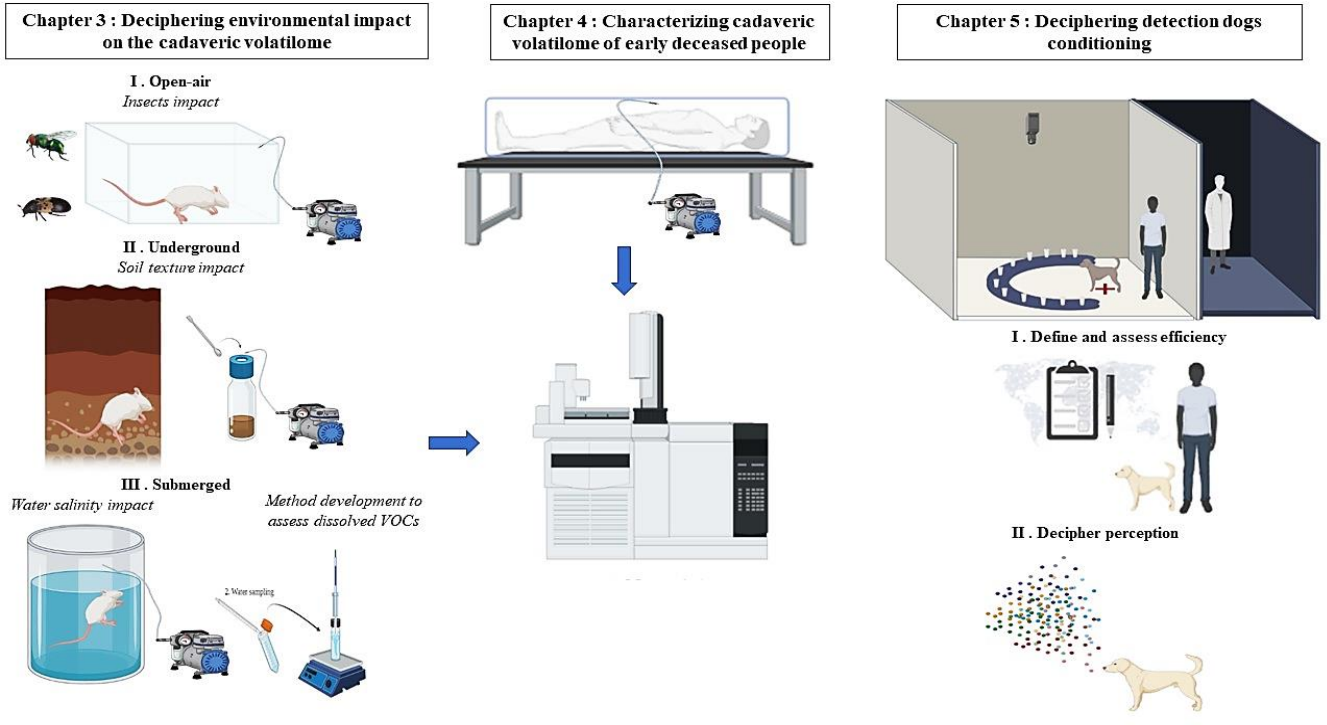

Figure 6 Diagram of the main objectives of the present thesis researches. Numbers in Roman refer to the subsection of the different chapters. Ch3. I. assess the impact of the insect's colonization on the cadaveric volatile profile; Ch3. 1I. investigate the impact of the soil texture on chemicals released during underground decomposition; Ch3. III. investigate the impact of the water salinity on the cadaveric volatilome release at the water surface as well as the development of a method to characterize the dissolve cadaveric volatile compounds. Ch4. aims at characterizing the volatile profile of early deceased people. Ch5. I. aim at developing a method to assess detection dogs' efficiency; Ch5. II. decipher the olfactory cues which are perceive and which induce a positive response to detection dogs; Ch5.III. assess the impact of the chemical complexity of training aids on the ability to recognize the target odor.

In **chapter IV**, we collected, identified and quantified the volatile organic compounds released by 20 human corpses at the fresh stage. We also assessed the impact of some parameters, such as the presence of lividity or the gender of the cadaver. Characterizing the cadaveric volatilome by using surrogate models helps to answer fundamental questions. However, as the smell of decomposition is not the same across vertebrate species, deciphering the odor released by human remains is crucial. The smell associated with the decomposition of a human body has been the subject of a limited number of studies, most using body parts or limited sample size.

Dogs are used in many professional contexts, including the detection of odor sources. A particular application of detection dogs is the location of human remains. Despite the interest for the use of dogs during detection tasks, scientific data allowing a better understanding of their efficiency or their ability to perceive some specific odors are poor. Hence, the first part of **chapter V** aims to first define what is an efficient detection dogs and second to develop a method assessing their efficiency based on this definition. The second part of chapter V aims to decipher the chemical basis of detection dog olfaction by assessing the post-training recognition spectrum.

Chapter 3

Environmental impact on the cadaveric volatilome

Introduction

The decomposition of vertebrate remains is driven by the combined action of autolysis and putrefaction. Autolysis is the consequence of intracellular enzymes' action while putrefaction mainly relies on both insect and exogenous bacteria activity (Gill-King, 1997; Janaway et al., 2009a; Presnell et al., 2013). Both autolysis and putrefaction lead to physical and chemical changes. Physical changes allow to define five stages of decomposition: fresh, bloated, active decay, advanced decay and dry remains (Vass, 2001). This classical process can also be impacted by environmental parameters leading to more particular processes as mumification (*i.e.*, presence of dry and leathery tissues) or saponification (*i.e.*, production of fatty acid methyl esters creating adipocere) (Cabirol et al., 1998; Janaway et al., 2009a; Ueland et al., 2021). Mumification occurs in dry environment while saponification usually occurs in wet and low oxygenated environment (Janaway et al., 2009a).

During the decomposition, volatile organic compounds (VOCs) are released due to the degradation of bodies' building blocks (Janaway et al., 2009b). A wide diversity of compounds can be associated with the decomposition. These compounds belong to almost all chemical classes (*e.g.*, aromatics, ketones, sulfur containing compounds) (Dekeirsschieter et al., 2009). Several biotic and abiotic parameters impact the cadaveric VOCs released (*e.g.*, decaying stage, storage conditions, climate) (Dekeirsschieter et al., 2009; Deo et al., 2020; Forbes, Rust, et al., 2014). Volatile profile has been investigated mainly during openair decomposition and under burial environment (Agapiou et al., 2015; Cablk et al., 2012; Dekeirsschieter et al., 2009; Forbes et al., 2016; Nizio et al., 2017; Rosier et al., 2015, 2016; Vass et al., 2008a). However, volatiles associated with submerged decaying bodies have almost not been investigated (Irish et al., 2019).

We have therefore decided to investigate chemicals released during the decomposition under three scenarios. First, the impact of insect on the volatile profile released during openair decomposition was investigated. Second, we assess the impact of decaying rats on both chemical and volatile profile of soil. Finally, we characterized the volatile profile released by immerged remains.

To investigate these three scenarios, we used dead rats as model as human remains do not allow to standardize the experimental conditions. Even if pig were already identified as the best model to investigate human cadaveric volatiles (Rosier et al., 2015, 2016), in a bioethics point of view, rats are more recommended. Indeed, only rats already used during other experiments were collected. No animals were killed for the purpose of this work. These rats underwent the same experiments which is

supposed to not impact the decaying process.

Impact of necrophagous insects on the emission of volatile organic compounds released during the decaying process

This section is an adapted version of the article:

Martin C, Vanderplanck M, Boullis A, Haubruge E and Verheggen F (2019) Impact of necrophagous insects on the emission of volatile organic compounds released during the decaying process. Entomologia Generalis, DOI: 10.1127/entomologia/2019/0663

Abstract

After death, corpses undergo a complex decomposition process, during which volatile organic compounds (VOCs) are released. Several groups of organisms, including insects, use these VOCs to select their mating and feeding sites. While the presence of insects on a corpse influences the decaying process, we do not know whether insects' impact the cadaveric VOC profile. Using decomposing rats exposed to dipterans (*Lucilia sericata*) and/or coleopterans (*Dermeste frischii*), we assessed how the presence of insects impacted the cadaver volatilome by using dynamic sampling. As expected, the decomposition of rats in presence of insects was faster than in absence of insects. All rats went through the five decaying stages with the exception of rats decomposing without insects. The composition of their volatile profiles differed among decomposition stages. We also found that insects do not affect the volatilome of decomposing rats, and no compound were associated to the presence of specific insect groups.

Introduction

Thanatochemistry is the branch of forensic science that investigates the chemical reactions that occur during the decomposition of a cadaver (Dermengiu et al., 2010; Salam et al., 2012; Tumram et al., 2014). After death, a corpse undergoes a complex process called decomposition, which includes the mechanisms of autolysis and putrefaction (Dekeirsschieter et al., 2009; Pinheiro, 2006). Autolysis impairs cells, tissues and organs through aseptic chemical processes. In comparison, putrefaction is the consequence of the activity of endogenous and exogenous, anaerobic and aerobic bacteria. Moreover, fungal and insect activity also contributes to the putrefaction (Campobasso & Introna, 2001; Dent et al., 2004; Pinheiro, 2006; Saukko & Knight, 2004). The joint action of autolysis and putrefaction leads to physical and chemical changes on the body, allowing to define five decaying stages: fresh, bloated, active decay, advanced decay and dry remains (Amendt et al., 2010; Dekeirsschieter et al., 2009; Grassberger & Frank, 2004; Rivers & Dahlem, 2014b; Vass, 2001). Several abiotic and biotic factors affect the process of decomposition, including temperature (Adlam & Simmons, 2007; Amendt et al., 2010; Campobasso et al., 2001; Rivers & Dahlem, 2014b), moisture (Campobasso et al., 2001), and availability of oxygen (Dent et al., 2004), as well as the diversity of microorganisms, insects and scavengers exploiting the corpse (Campobasso et al., 2001).

During decomposition, volatile organic compounds (VOCs) originating from the chemical degradation of macromolecules (*e.g.*, proteins, lipids and carbohydrates) are released (Rivers & Dahlem, 2014a; Vass et al., 2004). These VOCs belong to a wide range of chemical families (*e.g.*, alkanes, alkenes, aromatic compounds, alcohols, sulphur compounds, nitrogen compounds, and carboxylic acids) and form the cadaveric volatilome (Dekeirsschieter et al., 2009; Martin & Verheggen, 2018b; Paczkowski & Schutz, 2011; Pirrone & Albertini, 2017; Rosier et al., 2016; Verheggen et al., 2017). The volatilome of a decaying corpse differs among species. For example, pig remains are distinguishable from human remains based on five esters (Rosier et al., 2015). Several previous studies have collected and identified the odors released by cadavers during decomposition (Agapiou et al., 2015; Boumba et al., 2008; Dekeirsschieter et al., 2009; Rosier et al., 2015; Stefanuto et al., 2015; Vass, 2012; Verheggen et al., 2017). These odors differ with the stage of decomposition, and have been evaluated in relation to various biotic and abiotic factors. Previous studies also showed that the environment in which the corpse is decaying impacts the VOCs released by cadavers (Dekeirsschieter et al., 2009).

Because insects are important actors in decomposition processes, the present study aimed to assess how insects (*i.e.*, flies and beetles) influence the cadaveric VOCs profile.

Material & Methods

Insect rearing

To assess the impact of insects on the VOC profiles released by decaying rats, we used *Lucilia seriacta* (Meigen) (Diptera: Calliphoridae) as dipteran species model and *Dermestes frischii* (Kugelan) (Coleoptera: Dermestidae) as coleopteran species models. Indeed, both Calliphoridae and Dermestidae are commonly found on decaying carcasses (Abdullah et al., 2017; Charabidzé & Gosselin, 2014; Mayer & Vasconcelos, 2013; Pérez-Marcos et al., 2016; Pohjoismäki et al., 2010).

Blowflies pupae were placed in net cages (45 × 45 × 80 cm) inside an incubator (Snijders Scientific®), and were maintained at 23.0 ± 0.1 °C and 73.7 ± 0.4% relative humidity (RH) under a 12:12 h:h (light:dark) photoperiod (Rosenbaum et al., 2015). After the adults emerged, water and sugar were provided in a Petri dish, along with beef liver as a protein source. When eggs were observed, the Petri dish was removed and placed on sand. Liver was supplied during the entire development of the larvae. At the end of their lifecycle, the larvae migrated to the sand to pupate. Pupae were then stored in a fridge before being transferred to the same incubator to complete the metamorphosis (Clark et al., 2006; Martin & Verheggen, 2018a; Shiravi et al., 2011; Tarone & Foran, 2006).

D. frischii were reared on wood chips in a plastic box (50 × 30 × 40 cm) and were kept in darkness under laboratory conditions (22.1 ± 0.1 °C et 36.4 ± 0.1% RH). Beef liver was introduced daily to ensure mating and feeding. Pieces of polystyrene were provided for larval pupation (Menezes et al., 2006; Rąkowski & Cymborowski, 1981; Richardson & Goff, 2001).

Decomposing rats

Twelve female laboratory rats (3-5 months old; 291.6 ± 12.1 g; kept in identical laboratory conditions) were euthanized under the supervision of a veterinarian, before being frozen for several days. After thawing, the rats were placed in separate glass vivariums (40 × 30 × 30 cm) containing a mixture of 200 g of dry sand (required for the pupation of *L. sericata* larvae), 25 g of wood chips, 10 g of polystyrene (required for the pupation of *D. frischii*) and sugar (required to feed *L. sericata*). Each vivarium was placed in a net cage (45 × 45 × 80 cm). The 12 rats were left to decompose under four modalities: the absence of insects (n = 3); the presence of 10 newly emerged adults of *L. sericata* (sex ratio 1:1, n = 3); the presence of 10 adults of *D. frischii* of less than two months (sex ratio 1:1, n = 3); and the presence of both insect models (10 newly emerged adults of *L. sericata* and 10 adults of *D. frischii* of less than two months (sex ratio 1:1, n = 3)). All rats were left in the same greenhouse composed of

four sections separated by plexiglass walls with each 'rat-insect' association being allocated in the same greenhouse section to avoid the cross-contamination of VOCs. Temperature and humidity were monitored during the decomposition.

Volatile collection and analysis

The headspace of each decaying rat was sampled by using a dynamic "push-pull" pump system (Volatile Assay System®, PVAS11). The pushed (charcoal filtered) airflow was set at 1.2 L/min and the pulled air flow was set at 0.7 L/min. This overpressure prevented VOCs from the greenhouse to enter the vivarium during sampling. The VOCs from the headspace were trapped on a 60 mg Tenax TA® cartridge (Gerstel®, Germany) made of a microporous polymer of 2,6-diphenylen oxide, which was placed at the exit of the vivarium. VOCs were sampled twice a week during the first month and once a week until the dry remains stage to ensure that each stage was sampled at least once. After sampling, the cartridges were kept in a fridge at 4 °C with silica gel crystals to avoid water adsorption, until analysis (Rosier et al., 2015; Statheropoulos et al., 2011). Within a week, the VOCs were thermally desorbed in a gas chromatograph (Agilent Technology® 7890A) coupled with an automatic thermaldesorber (ATD; Agilent Technology®). After desorption, the VOCs were cryo-focused at -80 °C in a glass liner (CIS4; Agilent technology®). The liner was then heated at 260 °C with a temperature ramp of 12 °C/sec. See Table 1 for all analytical parameters. VOCs were identified on a mass spectrometer (Agilent Technology® 5975C, inert XL EI/CIMS with triple axis detection). All compounds were identified by interpreting their mass spectra and by injecting standards when available (Rosier et al., 2014, 2015, 2016). Blanks were also performed for each modality by collecting the VOCs from the headspace of vivarium containing everything but the rat (including wood substrate, insect diet and insects).

Table 6 Analytical parameters of the TDU-GC-MS analysis (adapted from Rosier et al., 2014)

TDU	GC-MS
Desorption temperature: 350 °C/4min	GC 7890 A
Trap temperature: -80 °C – 260 °C	**Carrier gas** : Helium
Transfer line temperature : 40 °C	**Column :** VF-624ms 60m x 0,25mm x 1,4um
Desorption mode : splitless	**Initial temperature** : 40 °C
	First ramp : 1 °C/min until 80 °C
	Second ramp : 3 °C/min until 120 °C
	Third ramp : 5 °C/min until 250 °C hold during10 min
	Detector : MS (MS 5975C)
	Mass scan : m/z 35-350

Statistical analyses

After aligning the different peaks of the chromatogram by using the GCAligner 1.0 program (Dellicour & Lecocq, 2013) and removing VOCs identified in the blank, we calculated the relative abundance of each compound based on the peak areas. Correlation plot was performed on data matrix for each modality to observe the variation of correlation due to the presence of insects ("corrplot" command, R-package Cran). Data were analyzed via multivariate analysis in R 3.0.2 program (R Core Team, 2013) after arcsine transformation allowing to transform finite data (percentages) into infinite data. To detect differences in the fragrance profiles among the different insect modalities, a permutational multivariate analysis of variance (*i.e.*, permMANOVA) was performed using an Euclidian distance matrix and 999 permutations ("adonis" command, R-package vegan, (J. Oksanen et al., 2017)). As this analysis is tough to the violation of data normality, only homoscedasticity was checked using the "betadisper" function. When a *p*-value was significant, pairwise comparisons were performed. The *p*-values of theses comparisons underwent Bonferroni's adjustment to avoid type I errors due to multiple analyses. Indicator compound analysis was also performed using the "indval" function from the labdsv package (Roberts, 2016) to identify the VOCs that were indicative of a single decomposition stage or insect modality. The analysis produced a *p*-value and an indicative value based on the abundance and relative frequency of each VOC. *p*-values were adjusted with a Holm correction to avoid type I errors due to multiple analyses.

Results

The evolution of temperature and relative humidity in the four greenhouse sections over the entire period of decomposition is provided (Figure 7). No difference is detected between greenhouse compartments (Temperature: $F_{742,7} = 0.027$, *p*-value =

1; Humidity: $F_{742,7} = 1.170$, *p*-value = 0.456).

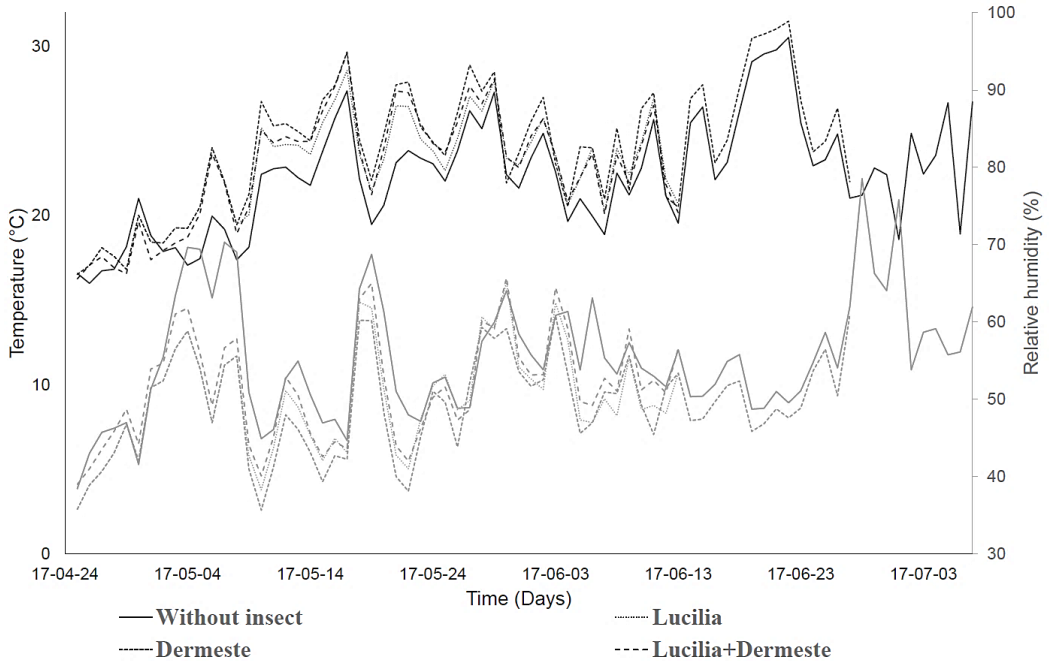

Figure 7 Temperature (black) and relative humidity (grey) monitoring in the different comportments of the greenhouse.

All rats from all four modalities passed through each of the five decaying stages, except for those decaying without insect, which were flattened and dry. The final stage differed with respect to modality. In the absence of insects, rats became mummified (dried undecomposed cadaver). In the presence of just blowflies, most rat tissues were consumed by larvae, with the bones being stacked under the skin, which became leathery on the upper side of the cadaver. In the presence of beetles (with and without blowflies), the cadaver ended up as a heap of bones and hairs. Besides the expected difference in the stages of decomposition (GLMM; decomposition stage effect, $F_{3,32} = 5.98$; *p*-value = 0.002), we found that insects significantly impacted the rate of decomposition (GLMM; modality effect, $F_{3,32} = 6.80$; *p*-value = 0.001). The decomposition rate was significantly faster in presence of blowflies (23.3 ± 0.3 days in the presence of *L. sericata*; 27.6 ± 0.7 days in the presence of both *D. frischii* and *L. sericata*) compared to the modalities without blowflies (67.3 ± 2.8 days without insects; 61.0 ± 5.3 days in the presence of just *D. frischii*) (Figure 8).

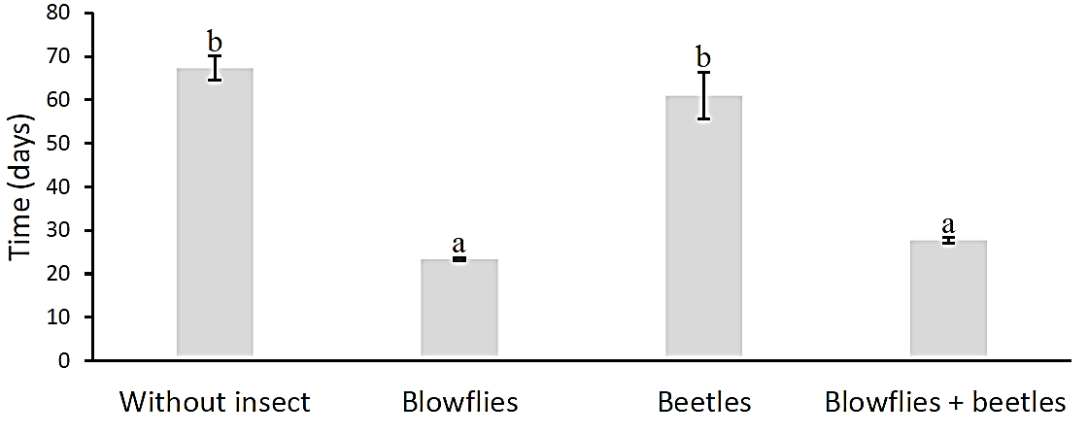

Figure 8 Duration (Number of days) of the decomposition of rats exposed or not to insects (without insect, with blowflies, with hide beetles and with both blowflies and hide beetles) during the entire decomposition process. Modalities sharing the same letter are not different from each other (threshold: p-value < 0.05)

Volatile organic compounds

67 different VOCs belonging to nine chemical classes were collected and identified (Table 7). Based on the relative abundance of the VOCs, the main compounds present in samples, regardless of insects' modalities and decomposition stages, is the dimethyl disulfide with a mean relative abundance of 27.66 ± 0.36 %. It reached 46.60 ± 4.28 % during the active decay stage and down to 10.69 ± 3.88 % during the bloated stage. However, it is almost constant among insect modalities (without insect: 28.59 ± 6.12 %; with *L. sericata*: 26.15 ± 6.76 %; with *D. frischii*: 33.97 ± 5.35%; with *L. sericata* and *D. frischii*: 18.85 ± 5.52 %). Even if we identified a wide diversity of molecules, three compounds only accounted for 50 % of the total quantity VOCs: dimethyl disulfide, 3-mmethyl-butanal (14.80 ± 1.92 %), 2-methyl-butanal (8.06 ± 1.32 %). We found dimethyl trisulfide in higher concentration in rats decaying in presence of *D. frischii* (3.44 ± 0.76 %) than for rats decaying under the others insect's modalities (without insect: 1.87 ± 0.82 %; with *L. sericata*: 0.78 ± 0.45%; with *L. sericata* and *D. frischii*: 0.86 ± 0.34 %) ($F_{3,57}$ = 3.219; *p*-value = 0.029).

Table 7 List of the 67 volatile organic compounds released by rat remains in absence (-) or presence of insects (Δ)

Volatile organic compounds	Current experimentation				Literature
	Without insect	With *L. sericata*	With *D. frischii*	With *L. sericata* and *D. frischii*	
Alkanes					
Hexane	Δ	Δ	Δ	Δ	(Cablk et al., 2012; Caldwell et al., 2011; Statheropoulos et al., 2011)
Nonane	Δ	Δ	Δ	Δ	(Dekeirsschieter et al., 2012; Statheropoulos et al., 2011)
Undecane	Δ	Δ	Δ	Δ	(Dekeirsschieter et al., 2012; Statheropoulos et al., 2011)
3-Methyl-octane	Δ	Δ	Δ	Δ	
Dodecane	Δ	Δ	Δ	Δ	(Cablk et al., 2012; Dekeirsschieter et al., 2012; Statheropoulos et al., 2011)
Tetradecane	Δ	Δ	Δ	Δ	(Dekeirsschieter et al., 2012; Statheropoulos et al., 2011)
Sulphur compounds					
Dimethyl-sulfoxyde	Δ	Δ	Δ	Δ	
Methanethiol	Δ	Δ	Δ	Δ	(Dekeirsschieter et al., 2009; Statheropoulos et al., 2011)
Ddimethyl disulfide	Δ	Δ	Δ	Δ	(Cablk et al., 2012; Dekeirsschieter et al., 2009, 2012; Hoffman et al., 2009; Statheropoulos et al., 2011)
Methylethyl-disulfide	-	Δ	Δ	Δ	(Statheropoulos et al., 2011)
Dimethyl trisulfide	Δ	Δ	Δ	-	(Vass *et al.*, 2008; Statheropoulos *et al.*, 2011; Dekeirsschieter *et al.*, 2012)
2-Thiaheptane	Δ	Δ	Δ	Δ	
Methional	Δ	Δ	Δ	Δ	(Statheropoulos et al., 2011)
Dimethyl tetrasulfide	-	Δ	Δ	-	
Aldehydes					
Methyl-propanal	Δ	Δ	Δ	Δ	(Dekeirsschieter et al., 2009, 2012; Statheropoulos et al., 2011)
3-Methyl-butanal	Δ	Δ	Δ	Δ	(Statheropoulos et al., 2007, 2011)
2-Methyl-butanal	Δ	Δ	Δ	Δ	
Pentanal	Δ	Δ	Δ	Δ	(Statheropoulos et al., 2005)
Hexanal	Δ	Δ	Δ	Δ	(Cablk et al., 2012; Dekeirsschieter et al., 2012; Lorenzo et al., 2003; Tolliver, 2005)
Heptanal	Δ	Δ	Δ	Δ	(Dekeirsschieter et al., 2012)
Benzaldehyde	Δ	Δ	Δ	Δ	(Cablk et al., 2012; Dekeirsschieter et al., 2009, 2012; Statheropoulos et al., 2005, 2011)

Octanal	Δ	Δ	Δ	Δ	(Cablk et al., 2012; Dekeirsschieter et al., 2012; Hoffman et al., 2009)
Octen-2-al	Δ	Δ	Δ	Δ	(Cablk et al., 2012; Dekeirsschieter et al., 2012; Hoffman et al., 2009)
Nonanal	Δ	Δ	Δ	Δ	(Cablk et al., 2012; Dekeirsschieter et al., 2012; Hoffman et al., 2009; Tolliver, 2005)
Decanal	Δ	Δ	Δ	Δ	(Cablk et al., 2012; Caldwell et al., 2011; Degreeff & Furton, 2011; Vass et al., 2008a)
Alcohol					
Propan-1-ol	Δ	Δ	Δ	Δ	
2-Methyl-propan-1-ol	Δ	Δ	Δ	Δ	(Statheropoulos et al., 2011)
Butanol	Δ	Δ	-	-	(Dekeirsschieter et al., 2012)
3-Methyl-butanol	Δ	Δ	Δ	Δ	(Dekeirsschieter et al., 2012)
2-Methyl-butanol,	Δ	Δ	Δ	Δ	
3-Methyl-butan-1-ol, -, acetate	Δ	Δ	Δ	Δ	
2-Methyl-butan-1-ol, acetate	Δ	Δ	Δ	Δ	
Hexenol	Δ	Δ	Δ	Δ	
2-butoxy ethanol	Δ	Δ	Δ	Δ	(Statheropoulos et al., 2011)
Heptan-2-ol	Δ	Δ	Δ	Δ	
1-Octen-3-ol	Δ	Δ	Δ	Δ	
Ketones					
Butan-3-one	Δ	Δ	Δ	Δ	
Butan-2-one	Δ	Δ	Δ	Δ	(Statheropoulos et al., 2005, 2007)
Butan-2-one, 3-hydroxy-	-	-	Δ	-	
3-Methyl-butan-3-one	Δ	Δ	Δ	Δ	
3-Methyl-pentan-2-one	-	Δ	-	-	
4-Methyl-pentan-2-one	Δ	-	Δ	-	
Hexen-2-one	Δ	Δ	Δ	Δ	
Octan-3-one	Δ	Δ	Δ	Δ	(Dekeirsschieter et al., 2012)
Octan-2-one	Δ	Δ	Δ	Δ	(Dekeirsschieter et al., 2012)
3-Octen-2-ol	Δ	Δ	Δ	Δ	(Caldwell et al., 2011)
Acetophenone	Δ	Δ	Δ	Δ	
Organic acids and esters					
Borate, trimethyl	Δ	Δ	Δ	Δ	

Acetic acid	Δ	Δ	Δ	Δ	(Caldwell et al., 2011; Degreeff & Furton, 2011; Statheropoulos et al., 2011)
Ethyl propionate	Δ	Δ	Δ	Δ	(Dekeirsschieter et al., 2012)
Methyl-3-methylbutanoate	Δ	Δ	Δ	-	
Ethyle-3-methylbutanoate	Δ	Δ	Δ	Δ	
Butylpropanoate	-	-	Δ	-	
Methyl acetate	Δ	Δ	Δ	Δ	(Dekeirsschieter et al., 2009)
Butanoïc acid	Δ	Δ	Δ	Δ	
Aromatics					
Toluene	Δ	Δ	Δ	Δ	(Cablk et al., 2012; Degreeff & Furton, 2011; Dekeirsschieter et al., 2012; Hoffman et al., 2009; Statheropoulos et al., 2005, 2011)
Isobutylbenzene	-	-	Δ	Δ	
Propylbenzene	Δ	Δ	Δ	Δ	(Statheropoulos et al., 2005, 2011)
Limonene	Δ	Δ	Δ	Δ	
Ethanol-Benzene	Δ	Δ	Δ	Δ	
Nitrogen compounds					
Trimethylamine	-	-	-	Δ	
Alkenes					
Oct-2-ene	Δ	Δ	Δ	Δ	
Non-1-ene	Δ	Δ	Δ	Δ	
Unknown					
Unknown 1	Δ	Δ	Δ	Δ	
Unknown 2	Δ	Δ	Δ	Δ	
Unknown 3	-	-	Δ	Δ	
Unknown 4	-	Δ	-	-	

We build up a correlation plot to illustrate the impact of insects on the families of compounds released during the decomposition (Fig. 9). While aldehydes and sulfur containing compounds are released simultaneously in absence of insects, their emission is differed in presence of *L. sericata* and *D. frischii*.

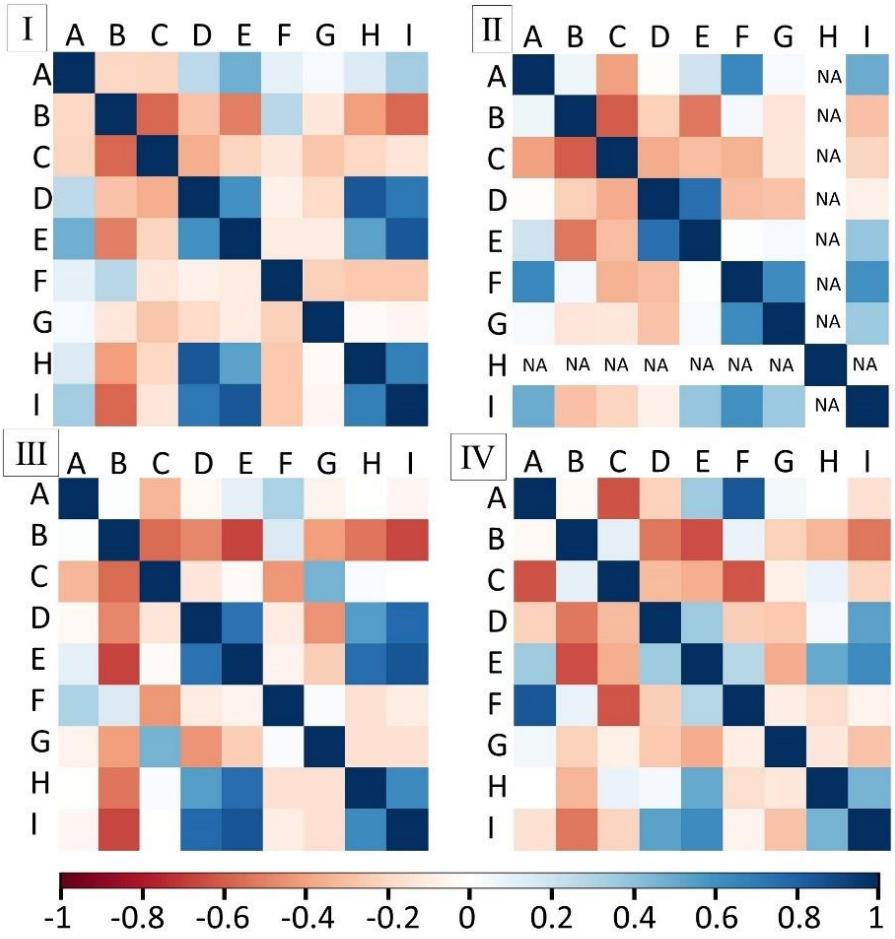

Figure 9 Correlation plot representing chemical families of compounds released during decomposition of rats without insect (I), With L. sericata (II), With D. frischii (III) and with L. sericata and D. frischii (IV). Colors illustrate the correlation between families of compounds (A: Alkanes, B: Sulphur Compounds, C: Aldehydes, D: Alcohols, E: Ketones, F: organic acids, G: Aromatic compounds, H: Amines, I: Alkenes).

As expected, statistical analysis revealed that VOC profiles differed among the decaying stages ($F_{3,57} = 7.896$; p-value < 0.001), all stages being significantly different

from each other (pairwise comparisons, *p*-value < 0.05). However, no clear discrimination may be visually assessed on the PCA ordination (Figure 10) and no indicative compound has been highlighted. Regarding, the impact of insects on the cadaveric volatilome, without considering the decaying stage, no effect was statistically detected ($F_{3,57}$ = 0.760; *p*-value = 0.681). Impact of insects' presence was then analyzed considering each decomposition stage (combination decomposition stage*modality leading to 16 different qualitative levels) and a significant difference was detected ($F_{13,47}$ = 3.020; *p*-value < 0.001). Presence of *L. sericata* and *D. frischii* impacted the VOC profile released during the fresh stage compared to rats decaying without any insect (*p*-value = 0.013) while no significant impact was detected in presence of either *L. sericata* or *D. frischii* alone. The same observation was made during the bloated stage, with a significant impact of the simultaneous presence of blowflies and beetles (*p*-value < 0.001). However, no indicator compound was associated to a specific insect order present on the decaying corpse for a given decomposition stage.

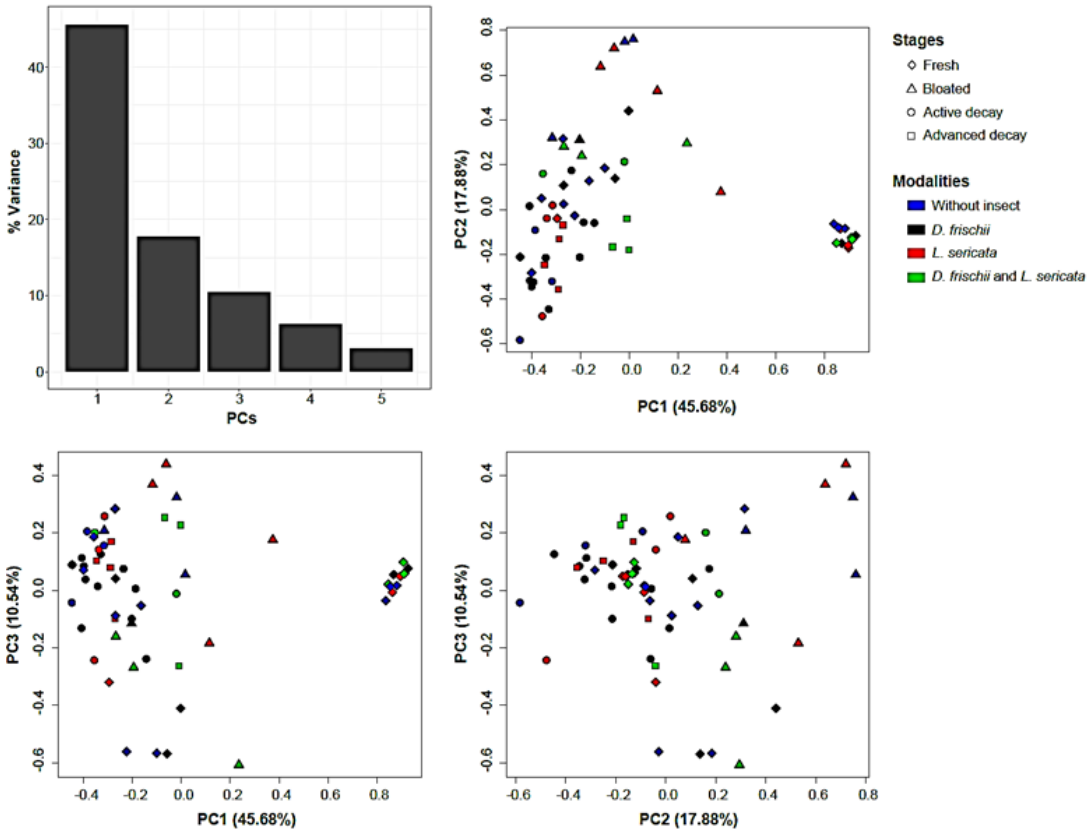

Figure 10 PCA ordination plots. Visualization of the VOC profiles during decaying process, depending on the decomposition stage (figures) and the modalities (colors). Each sampling is represented in two-dimensional plan constituted by two axes of the principal compounds analysis. Axes are combination of studied factors (insect presence and stage of decomposition Characterized by a percentage of reliability.

Based on the relative abundances of the nine chemical classes, the decomposition stage had a significant impact ($F_{3,57} = 8.766$; *p*-value < 0.001) while insect modalities did not have any effect, regardless of the decomposition stage ($F_{3,57} = 0.874$; *p*-value = 0.554). Considering each decomposition stage (16 level-combination variable), a significant difference was detected ($F_{13,47} = 3.113$; *p*-value < 0.001). Pairwise comparisons on chemical VOC families lead to the same results obtained with the individual VOC analysis. Actually, the simultaneous presence of both insects affected the VOC profiles released by decaying rats during fresh (*p*-value = 0.045) and bloated stages (*p*-value = 0.001) compared to those released by rats decaying without insects. However, no indicator compound was associated to a specific insect order present on

the decaying corpse for a given decomposition stage.

Discussion

The decomposition process of a corpse is affected by various biotic and abiotic factors, with environmental conditions and the causes of death being the most commonly studied factors (De Donno et al., 2014; Dekeirsschieter et al., 2009; Dent et al., 2004; Lynch-Aird et al., 2015; McIntosh et al., 2017; Notter et al., 2009; Tomberlin & Adler, 1998). This study confirms that the presence of dipterans and coleopterans directly impacts the decomposition rate. Rats decomposing with insects passed through the five stages of decomposition described in the published literature (*i.e*., fresh, bloated, active decay, advanced decay and dry remains). In contrast, rats decomposing in the absence of insect dried. In this last modality, the only biological actors of decomposition are microorganisms (Amendt et al., 2004; Pinheiro, 2006). Thus, in the absence of insects, no macro-organisms were feeding on soft tissues, resulting in no bones being observed. Because the exposition of bones characterizes the advanced stage of decay, this stage was not observed (Goff, 2010). Consequently, corpses were mummified (*i.e.*, soft tissues were preserved). Low humidity and heat promote mummification, leading to the formation of a dry unskeletonized cadaver (Campobasso & Introna, 2001; Schotsmans et al., 2017).

Decomposition in the presence of flies does not lead to the skeleton being revealed, because the bones stick to the dried skin. Indeed, when corpses reach the advanced stage of decay, they become less attractive to flies (Charabidzé et al., 2015). Because hide beetles feed on the dry remains (including skin and cartilage), their presence allows the corpse to reach the skeleton stage (Charabidzé & Gosselin, 2014; Hefti et al., 1980; Huchet, 2008).

The degradation of proteins, lipids and carbohydrates leads to the emission of VOCs (Brasseur et al., 2012; Dent et al., 2004; Statheropoulos et al., 2007). More than 60 compounds were identified from the headspace of decomposing rats. The fact that rats were placed in an unnatural environment (*i.e.*, inside a vivarium, placed inside a greenhouse), with a limited diversity of microorganisms and insects, might explain the limited diversity of VOCs collected in our study compared to other studies (Agapiou et al., 2015; Boumba et al., 2008; Dekeirsschieter et al., 2009; Hoffman et al., 2009; Rosier et al., 2015; Statheropoulos et al., 2005, 2007). However, most of the VOCs commonly reported in the published literature were found in our study, including sulphur compounds, such as methanthiol; dimethyl disulfide, methylethyl disulfide and dimethyl sulfide (Cablk et al., 2012; Dekeirsschieter et al., 2009; Hoffman et al., 2009; Rosier et al., 2015; Statheropoulos et al., 2011; Vass et al., 2004). Dimethyl disulfide and methanthiol were collected under all modalities.

Methylethyl disulfide was only detected from rats placed in the presence insects. All of these compounds originate from the degradation of sulfured amino acids (methionine and cysteine) (Dent et al., 2004).

VOCs collected are the products released due to the degradation of tissues of the corpse. Previous studies revealed that each tissue released specific families of compounds. Alkanes derive from the decomposition of muscles, bones and fat (Cablk et al., 2012). Aldehydes are typical decomposition by-products and include benzaldehyde, nonanal and decanal (Boumba et al., 2008; Caldwell et al., 2011; Dekeirsschieter et al., 2009; Statheropoulos et al., 2011), which were all identified under all modalities in our study. Aldehydes are associated with the degradation of carbohydrates, more specifically from the degradation of pyruvate by pyruvate decarboxylase (Boumba et al., 2008; Dekeirsschieter et al., 2009). Alcohols were released at the bloated stage in our study. Alcohols are products of carbohydrate fermentation, amino acids degradation and lipid oxidation. Ketones were produced in different quantities across all modalities. They are released during the degradation of lipids and carbohydrates (Agapiou et al., 2015). Finally, carboxylic acids and aromatic compounds were also collected, and exhibited high diversity (Agapiou et al., 2015; Dekeirsschieter et al., 2009; Hoffman et al., 2009).

By monitoring the cadaveric VOCs from decaying rats in the presence and absence of insects (Dipteran and Coleopteran) over a two-month period showed that insects do not have an important impact on the odors released during decomposition. Different odors were produced at some decaying stages; however, indicator compounds could not be identified. Two-dimensional chromatography could provide additional information by revealing the presence of additional compounds (Dekeirsschieter et al., 2012; Stefanuto et al., 2016; Verheggen et al., 2017). The background VOC signal present in the greenhouse could have also hidden interesting compounds (Dekeirsschieter et al., 2009).

The insect presence on a corpse is known to impact the process of decay; however, their impact the cadaveric VOC profile has never been investigated. Most of the differences highlighted in this study were made between rats decaying without insects and rats decaying in presence of both insect's species. While no specific compound is released as a result of necrophagous insect feeding, we found that insects have an impact on the emission of the most important chemical classes. Two-dimensional chromatography could reveal some VOCs not detected in this study, making it possible to deeper understand the impact of insects on the VOCs emission.

Forensic taphonomy: characterization of the gravesoil chemistry using a multivariate approach combining chemical and volatile analyses

This section is an adapted version of the article:

Martin C, Maesen P, Minchilli D, Francis F and Verheggen F (2021) Forensic taphonomy: characterization of the gravesoil chemistry using a multivariate approach combining chemical and volatile analyses. Forensic science international, DOI: 10.1016/j.forsciint.2020.110569

Abstract

Soil thanatochemistry, defined as the study of the chemical changes occurring during the decomposition of buried corpses, is a young and inadequately documented field of research. In this study, we aim to determine the effects of decomposition on soil physico-chemical properties by combining pedological, chemical, and volatile analyses of soil layers surrounding buried animals. We examined chemical and volatile changes over time occurring throughout the soil column in two common soil-texture (sandy loam and loam). We buried dead rats and let them decompose for two months. After their excavations, we characterized the physico-chemical conditions of three soil layers above the rats and one layer below, including (i) pH, dry matter, and electrical conductivity, (ii) organic carbon and total nitrogen, (iii) bioavailable nutrients (K, Na, Mg, Ca, and P), and (iv) volatile organic compounds. Multivariate analyses (permMANOVA) revealed that a decaying rat is associated with changes in soil chemical characteristics in both soils. However, the observed changes were not homogenous throughout the soil columns. Conditions in soil layers nearest the cadavers changed most during decomposition. We generated a predictive model by combining chemical and volatile analyses (10% error rate), allowing us to identify key gravesoil indicators that could be used to reveal the former existence of a buried corpse in loam and sandy loam soil (indicators in order of importance): organic carbon, calcium, pH, conductivity, dimethyl-disulfide, and nitrogen.

Introduction

Forensic taphonomy focuses on the study of postmortem processes that impact the preservation, observation, or recovery of dead organisms, allowing to reconstruct circumstances surrounding their deaths (Carter et al., 2006; Haglund & Sorg, 1997; Ubelaker, 1997). Forensic taphonomy is a young discipline and is considered to be a key component of forensic anthropology by providing a broader and deeper conceptual framework incorporating postmortem processes (Dirkmaat et al., 2008). In this context, the interest for vertebrate decomposition has increased (Carter et al., 2006; Fancher et al., 2017; Stokes et al., 2009; Szelecz, Koenig, et al., 2018; Tumer et al., 2013).

Many studies have focused on various stages of decomposition in open air over time (Aitkenhead-Peterson et al., 2012; Boumba et al., 2008; Bull et al., 2009; Forbes & Perrault, 2014; Larson et al., 2011; Macdonald et al., 2014; Meyer et al., 2013; Szelecz, Lösch, et al., 2018; Vass, 2001; Vass et al., 1992). However, underground decomposition has received much less attention (Meyer et al., 2013), especially relative to physico-chemical conditions. Data from such studies would be valuable because soil scientists have suggested that gravesoil chemistry could help determine post-mortem intervals (Adserias-Garriga et al., 2017; Carter et al., 2008; Fancher et al., 2017; Iqbal et al., 2017; Szelecz, Lösch, et al., 2018; Turner & Wiltshire, 1999; Vass et al., 1992). However, soil thanatochemistry, defined as the study of the chemical changes occurring during decomposition, is also an immature discipline with little documentation (Meyer et al., 2013). The available literature on soil chemistry surrounding a buried corpse is typically focused on either chemical or volatile organic compound (VOC) analyses (Aitkenhead-Peterson et al., 2012; Forbes, Rust, et al., 2014; Macdonald et al., 2014; Perrault et al., 2014) and thus far, no studies have investigated such changes using a multivariate approach combining data on characteristics of both chemical and volatile components.

Studies focusing on chemical changes in soils related to the decomposition of buried remains usually highlight changes in the concentration and diversity of chemicals (e.g., pH, nitrogen, organic carbon, calcium, and magnesium). Nitrogen has been suggested as a target compound for predicting post-mortem intervals (PMI) (Fancher et al., 2017) and an increase in nitrogen concentrations has usually been observed during decomposition (Aitkenhead-Peterson et al., 2012; Carter et al., 2008, 2010; Fancher et al., 2017; Tumer et al., 2013). In addition, variations in pH have already been well documented (Aitkenhead-Peterson et al., 2012; Benninger et al., 2008; Fancher et al., 2017; Hopkins et al., 2000; Stokes et al., 2009; Tumer et al., 2013). In contrast, previous studies have not observed any consistent trends in the variation of pH with decomposition because pH may either increase or decrease throughout the

decomposition process (Fancher et al., 2017) and is mainly related to the release of ammonium (NH_4^+) in soil during a corpse's decomposition (Fancher et al., 2017; Tumer et al., 2013). Increases in the amounts of calcium, phosphorus, and magnesium have also been reported during decomposition, but only during latter stages (Aitkenhead-Peterson et al., 2012; Benninger et al., 2008; Fancher et al., 2017; Tumer et al., 2013). Changes in soil volatilomes associated with buried remains have received less attention than soil mineral composition (Brasseur et al., 2012; Dubois et al., 2018; Forbes et al., 2016; Nizio et al., 2017; Vass et al., 2004). Common cadaveric compounds, such as sulfur compounds, alkanes, alcohols, and ketones have been identified in grave soils (Brasseur et al., 2012; Dekeirsschieter et al., 2009; Dubois et al., 2018; Forbes & Perrault, 2014; Perrault et al., 2014). Despite recent interest in chemical and volatile changes occurring in gravesoils, we have found no study investigating integrated chemical changes in gravesoils throughout a soil column or for changes related to variations in soil texture.

In this study, we link the decomposition of buried mammals to soil physico-chemical properties by analyzing changes in pedological, chemical (including bioavailable compounds, pH, conductivity, and organic carbon concentrations), and volatile compounds (including any released volatile organic compounds). Furthermore, we examine these changes relative to soil texture and soil depth.

Material and methods

Biological material

We were provided dead laboratory rats (3–5 months old, 208.9 ± 16.4 g) by the faculty of veterinary medicine at the University of Liège (uLiège, Belgium). No rats were killed for our experiments (Ethics agreement n°18-2021); they had been used in a previous experiment (which could not be communicated to the authors of the present research). Rats had been euthanized and kept frozen for eight weeks prior to our experiment.

We used six rat cadavers for our experiments, a sample size we determined to be sufficient for applying a multivariate approach to our data as the conditions of application of the statistical analyses were met. Before being buried, we defrosted the rats in a hot water bath (≈ 40 °C).

Site description

Our experiment took place in a greenhouse at Gembloux Agro-Bio Tech, Belgium. The greenhouse was 5 m long, 3 m wide, and divided into fifteen 1×1 m squared plots (Figure 11). To avoid cross contamination, we placed graves 1 m apart. We assessed

the decomposition in two textures of soils: a loam and a sandy loam. We allocated four graves to each soil texture. A dead rat was placed in three graves, whereas the fourth grave was used as a control (mixed soil, but no rat buried). All graves were 40 × 20 cm in area and 30 cm deep. First, we collected soils from each dug grave and placed them in separate containers. In one set of containers (n = 4), we added sand (granulometry: 0.7 mm) at a ratio of 60/40 (v/v; soil/sand) to increase the granulometry of the soil, which we then defined as sandy loam (Staff, 2017). The texture of soil in the other containers (n = 4), which we defined as loam soil, remained unchanged, but the soil was mixed in a similar way as for sandy loam. We homogenized the soil in each container by stirring it by hand (gloves were used). Then we introduced 10 cm of soil into each of the eight graves. Rats (n = 6) were then placed in graves, three rats in sandy loam soil and three in loam soil. Then, we added 20 cm of soil of the same texture to each grave (Figure 1). In both control graves (sandy loam and loam), we placed a data logger probe (EL-USB-2+®) at a depth of 20 cm to measure temperature every hour throughout the entire experiment.

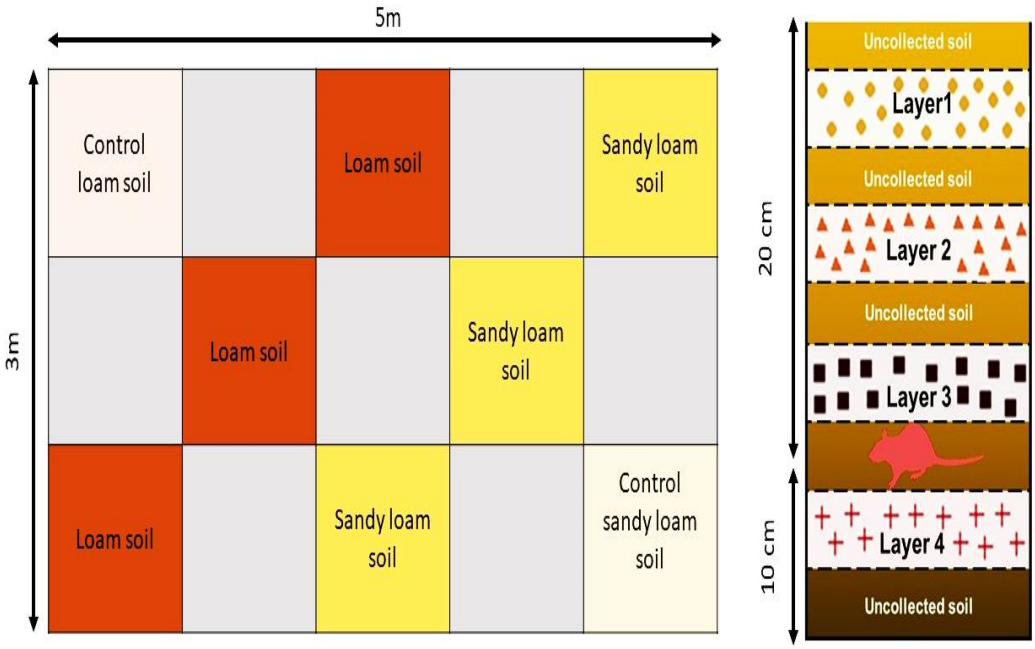

Figure 11 (Left) Graves were set up in a greenhouse. A dead rat was placed in each grave except the two control graves. (Right) After two months, rats were exhumed and different layers of soil were collected.

Exhuming rats and sampling grave soils

After two months, we exhumed the rats and performed chemical analyses on four

soil layers in each grave (Figure 11). First, we removed and discarded the top 4 cm of soil. Then we collected 1 kg of soil from the next 3 cm of soil, soil layer 1 (SL1), and discarded any surplus soil. We then removed the next 3 cm of soil, and then collected another 1 kg of soil (SL2). We repeated that procedure to collect soil layer 3 (SL3). When we reached the buried rat, we removed all of its remains. We then collected a fourth sample of soil (SL4), consisting of the 3 cm of soil directly below the remains. All samples were immediately placed in PTFE sealed glass bottles and stored at -80 °C until analyzed to prevent chemical and volatile changes that would occur after removing soil.

Gravesoil chemical analysis

For each sample, we removed soil moisture by placing our soil samples in a desiccator at 60 °C for two days. After two days, we weighed the samples and defined these weights as soil dry matter. After drying, we also measured electrical conductivity and soil water pH on 1:2.5 (w: w) soil to water extracts (Pansu & Gautheyrou, 2006; Szelecz, Koenig, et al., 2018) by first calibrating a pH-meter with standard solutions at pH of 4, 7, and 10. To measure conductivity, we calibrated the pH-meter using a standard solution with a conductivity of 140 µScm-1. After calibration, we measured pH and conductivity in the water extracts. We dosed bioavailable nutrients (phosphate, calcium, potassium, magnesium, and sodium) by mineralizing 0.5 g of dry soil in 5 ml of aqua regia [1:3 v:v (nitric acid : chloridric acid)] for 2 h under reflux. We performed a 1 % (0.5 g in 50 mL) dilution before analyzing the samples. We obtained phosphate, calcium, potassium, magnesium, and sodium concentrations with an atomic absorption spectrometer (AAS) (Perkinelmer AAnalyst200®, Waltham, Massachusetts, USA). We obtained all dosages from calibration curves using the following wavelengths for each chemical: λ_{K} (766.49 nm), λ_{Na} (589.00 nm), λ_{Ca} (422.67 nm), λ_{Mg} (285.21 nm), and λ_{P} (285.21 nm). We determined nitrogen concentrations using the Kjeldahl method and organic carbon content with the Walkley-Black wet digestion method using sulfochromic oxidation technique.

Gravesoil physical analysis

We sampled both soil textures to determine the clay, silt, and sand fractions in the loam and sandy loam. We applied the pipette method on samples to obtain soil particle sizes (Ranst et al., 1999). Because we had homogenized the loam and sandy loam before we filled the grave, we decided that one sample for each soil type would be sufficient to represent a soil's texture.

Gravesoil volatile analysis

To obtain the cadaveric volatilome of each soil layer, we placed 20.0 ± 0.5 g of each

soil layer into a 400 mL sealed glass chamber (d = 10 cm, h = 5 cm). Then after equilibrating for 20 min, we dynamically sampled the headspace air at ambient temperature (23 °C). We adjusted air flow at 200 mL/min (GilAir® plus pump, Sensidyne®, St. Petersburg, Florida, USA) and trapped the cadaveric VOCs from the soil on a hydrophobic TenaxTa/Carbograph tube (Markes International®, Llantrisant, UK) which allow to trap both hydrophilic and hydrophobic VOCs (Perrault et al., 2014). The air entering the chamber had been filtered with a charcoal filter to avoid contamination from the laboratory. After 120 min of sampling, we sealed the tubes and stored them in a refrigerator at 4 °C to avoid desorption of the compounds. We then analyzed tubes with a gas chromatograph and a mass spectrometer (QP2020 NX, Shimadzu®, Kyoto, Japan). We conducted thermodesorption on a TD30R module (Shimadzu®, Kyoto, Japan). We separated compounds on a Rtx-5ms capillary column (30 m x 0.25 mm x 0.25 µm; Agilent technology®; Santa Clara, California, USA). Table 8 lists all analytical parameters. We aligned all chromatograms with GCAligner® software (Dellicour & Lecocq, 2013).

Table 8 Analytical parameters of the TD30R-GC/MS analysis

TD30R	**GC-MS (QP2020 NX)**
Temperature: 280°C during 8min	**Carrier gas**: Helium
Trap temperature: -30°C – 280°C	**Column:** Rtx-5ms (30mx0.25mmx0.25µm)
Transfer line temperature: 250°C	**Initial temperature**: 40°C held during 3min
Desorption mode: split	**First ramp:** 4°C/min until 220°C
Slip ratio: 3	**Second ramp:** 10°C/min until 300°C held during 2 min
	Mass scan: m/z 30-350
	Ion source temperature: 200°C
	Detector voltage: 0.1kV

Statistics

We performed all statistical analyses with R version 3.4.2 (R Foundation for Statistical Computing). Our goal was to evaluate the accuracy of multidisciplinary analyses to highlight the presence of a buried corpse in different soil types. Because we intended to assess the impact of a cadaver on the chemical and volatile composition of a gravesoil, we first performed the same multivariate statistical analysis on the chemical and volatile results separately. Then, we merged the two data sets and analyzed both sets of data together. We also performed univariate analyses on the chemical data set (bioavailable nutrients, organic carbon, nitrogen concentrations, pH, and conductivity).

We conducted principal component analyses (PCA) on the various data sets [“Fviz_pca_ind” command, R-packages: ggplot2, Factoextra (ver. 1.0.3)]. We

conducted three PCAs: one on each texture of soil and a third on the combined data set. We also conducted a permutational multivariate analyses of variance (i.e., permMANOVA) using an Euclidian distance matrix and 999 permutations ("adonis" command, R-package vegan, (J. Oksanen et al., 2017)) to test for potential differences in the chemistry of both treatments (with and without a cadaver) among soil layers and between soil textures. We adjusted *p*-values using Bonferroni's correction to avoid increases in type error I due to multiple testing. When the returned *p*-value was significant (p-value < 0.05), we performed a multivariate pairwise analyses to identify which modality was different from the others ("pairwise.adonis" command, R-package vegan, (J. F. Oksanen et al., 2020). Because this pairwise analysis is robust in correcting for violations of data normality, we only checked for homoscedasticity using the "betadisper" function. When homoscedasticity was not observed, we replaced the Euclidean method with the Bray-Curtis method in the permMANOVA procedure. With a Bray-Curtis analysis, we replaced the PCA with a non-metric multidimensional scaling (NMDS) algorithm ("orditorp" command, R-package vegan, (J. Oksanen et al., 2017)) to respect the statistical assumptions for the data. The *p*-values of these comparisons underwent Bonferroni's adjustment to avoid type I errors due to multiple analyses. We identified indicator compounds with a random forest command (R-package randomForest), which evaluated the proximities among data points by generating 500 trees and using a number of variables at each branch-of-three.

We also performed two-way ANOVAs on data from the chemical analyses (bioavailable nutrient, organic carbon, nitrogen amount, pH, and conductivity) to identify any compounds that might have been impacted by the presence of a corpse.

We also analyzed the third data set (combined chemical and volatile data) by selecting the marker compounds revealed by the random forest analyses previously performed to see if the accuracy of the predictive model could be increased by measuring fewer chemical and volatile compounds.

Results

Environmental conditions

Two months after beginning the experiment, we exhumed all rats and estimated their decomposition stage based on qualitative data (Dekeirsschieter et al., 2009). In both soil textures, rats decomposed to the dry stage (i.e., only hair, dry skin, and bones remaining). However, we observed that rats in loam were moister than rats interred in sandy loam (*i.e.*, soil stuck to remains), whereas rats in sandy loam were drier (less soil stuck to remains). The average temperature during the experiment reached 16.5 ± 0.1 °C at 20 cm depth, for both soil textures.

Physical conditions

For both soil textures, we determined the portion of clay (0–2 µm), coarse silt (2–20 µm), fine silt (20–50 µm), and sand (>50 µm). Loam was composed of 33.3% sand, 24.6% coarse silt, 24.8% fine silt, and 17.3% clay. Sandy loam was composed of 63.2% sand, 13.5% coarse silt, 13.2% fine silt, and 10.1% clay.

Chemical conditions

Our initial analysis merged data from both loam and sandy loam soils. We applied the Bray-Curtis method because the assumptions for applying permMANOVA with Euclidean methods (homoscedasticity) were not met. For the same reason, we display results in Figure 12 following NMDS (stress value = 0.06). The presence of remains was related to the chemical properties in both soil textures ($F_{1,31}$ = 6.42; *p*-value = 0.011), but the conclusions are different for both soil textures ($F_{1,31}$ = 12.12; *p*-value = 0.004). As a consequence, we decided to analyze the impact of a cadaver on the two soil types independently.

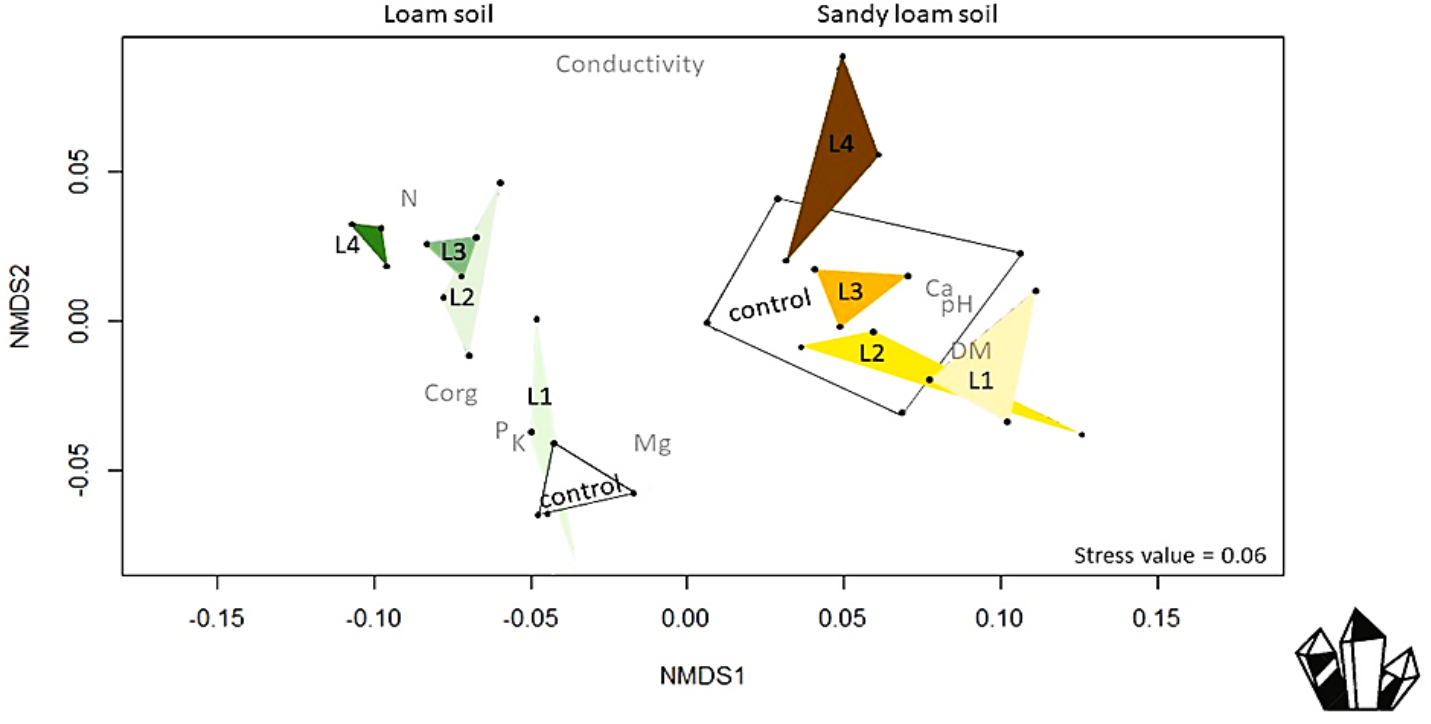

Figure 12 Non-metric multidimensional scaling representing the impact of a decaying mammal on the soil chemical features including pH, bioavailable compounds (P: phosphorus; K: potassium; Mg: magnesium, Ca: calcium), conductivity, dry matter (DM), organic carbon (C_{org}) and nitrogen amount (N) at different depths (soil layer 4 = L4, soil layer 3 = L3, soil layer 2 = L2, soil layer 1 = L1) of two gravesoil textures (yellow-brown = sandy loam soil; green = loam soil).

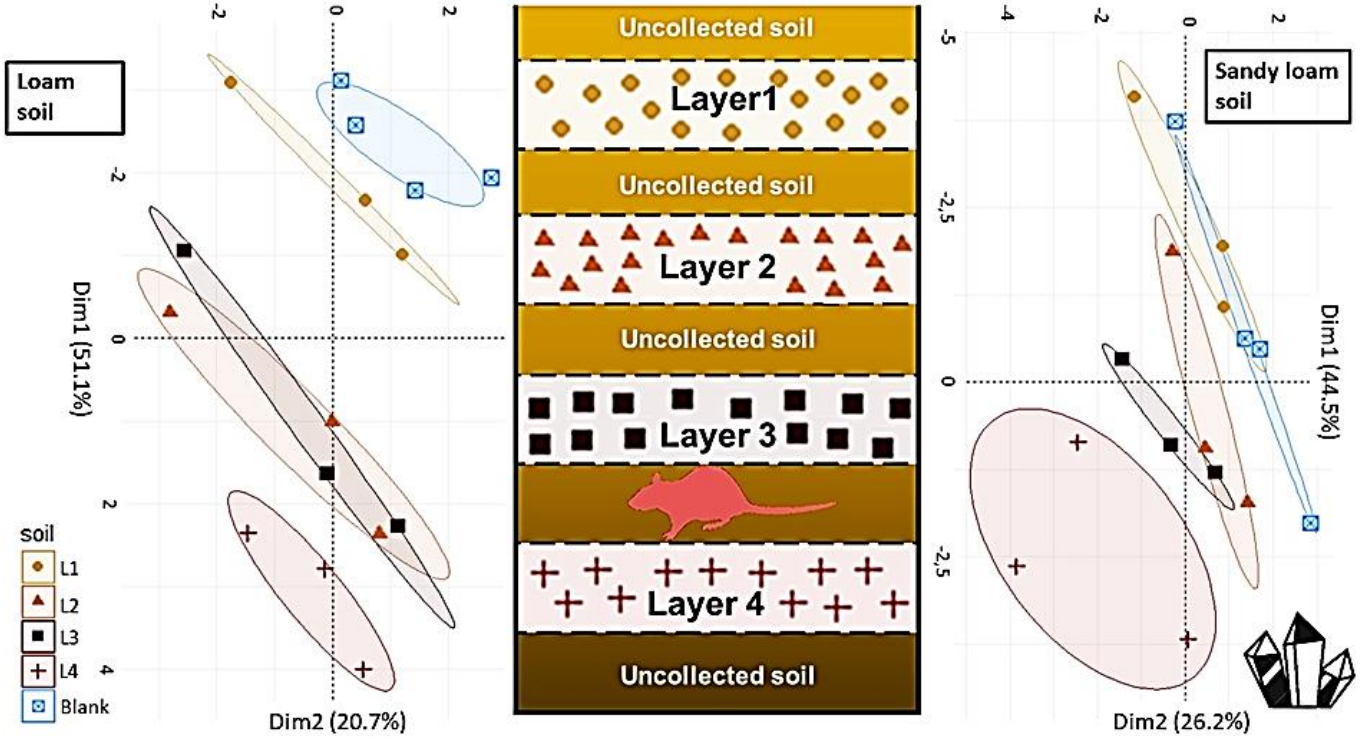

Figure 13 Principal component analyses illustrating the impact of a buried decaying mammal (see center of the figure) on the soil chemical features including pH, bioavailable compounds (phosphor; potassium; magnesium, calcium), conductivity, dry matter, organic carbon and nitrogen amount at different depths (Layer 4, Layer 3, Layer 2, Layer 1) within two soil textures separately analyzed: Left: Loam soil. Right: Sandy loam soil.

In our separate analyses of decomposition in the two soil textures (loam and sandy loam), the sample distributions enabled us to perform a permMANOVA with a Euclidean method (Figure 13). In both the loam and sandy loam, the corpse impacted the chemistry of the soil in which it was interred (loam: $F_{4,15} = 5.92$, *p*-value = 0.011; sandy loam: $F_{4,15} = 6.11$, *p*-value = 0.007). However, the two PCAs show that soil layers were not impacted similarly in the two soil textures. Specifically, differences in soil chemistry in the loam was more pronounced than in the sandy loam, mainly in SL4 (*i.e.,* the layer located under the remains) (Figure 13). Generally, the conditions in the loam were more affected by the carcass than they were in the sandy loam. The chemical properties of the soil layers located above the cadavers (SL2 and SL3) in both soil textures were also associated with the presence of a cadaver (no overlap with the control). The chemical properties of SL1 (the topmost layer, nearest the soil surface) were not related to the presence of a corpse located deeper in the soil (*i.e.,* 20 cm).

The univariate analyses we performed on the two soil textures revealed that chemical parameters were significantly related to the presence of a corpse, including amount of dry matter (loam: $F_{4,11} = 5.23$, *p*-value = 0.013; sandy loam: $F_{4,11} = 15.49$, *p*-value < 0.001), nitrogen concentrations (loam: $F_{4,11} = 32.89$, *p*-value < 0.001; sandy loam: $F_{4,11} = 4.51$, *p*-value = 0.021), pH (loam: $F_{4,11} = 23.36$, *p*-value < 0.001; sandy loam: $F_{4,11} = 105.80$, *p*-value < 0.001), and conductivity (loam: $F_{4,11} = 5.23$, *p*-value = 0.013; sandy loam: $F_{4,11} = 15.49$, *p*-value < 0.001). The chemical parameters that were not related to the presence of a cadaver include concentrations of phosphorus (loam: $F_{4,11} = 1.91$, *p*-value = 0.18; sandy loam: $F_{4,11} = 0.87$, *p*-value = 0.513), magnesium (loam: $F_{4,11} = 0.44$, *p*-value = 0.78; sandy loam: $F_{4,11} = 1.03$, *p*-value = 0.431), potassium (loam: $F_{4,11} = 1.98$, *p*-value = 0.167; sandy loam: $F_{4,11} = 0.41$, *p*-value = 0.80), and sodium (loam: $F_{4,11} = 0.464$, *p*-value = 0.761; sandy loam: $F_{4,11} = 0.546$, *p*-value = 0.706). We found two chemical parameters that were soil dependent: organic carbon (loam: $F_{4,11} = 19.18$, *p*-value < 0.001; sandy loam: $F_{4,11} = 1.49$, *p*-value = 0.274) and calcium (loam: $F_{4,11} = 1.69$, *p*-value = 0.221; sandy loam: $F_{4,11} = 5.00$, *p*-value = 0.015). All mineral measurements are presented in Figure 14.

To help us identify potential indicator compounds (those that are the most related to the presence of a corpse), we generated a predictive model for which we evaluated its accuracy by an error rate. The model that merged soil indicators from both soil types had an error rate of 25 %, indicating that key indicators (in order of importance) for the presence of a cadaver in the soil were calcium, pH, organic carbon, and conductivity. When we evaluated the two soil textures with two different predictive models, the error rate declined to 12.5 % (for both soil textures). Then the soil indicators became (in order of importance): calcium, concentration organic carbon, pH, conductivity (for sandy loam) and amount of organic carbon, conductivity,

nitrogen concentration, and pH (for loam).

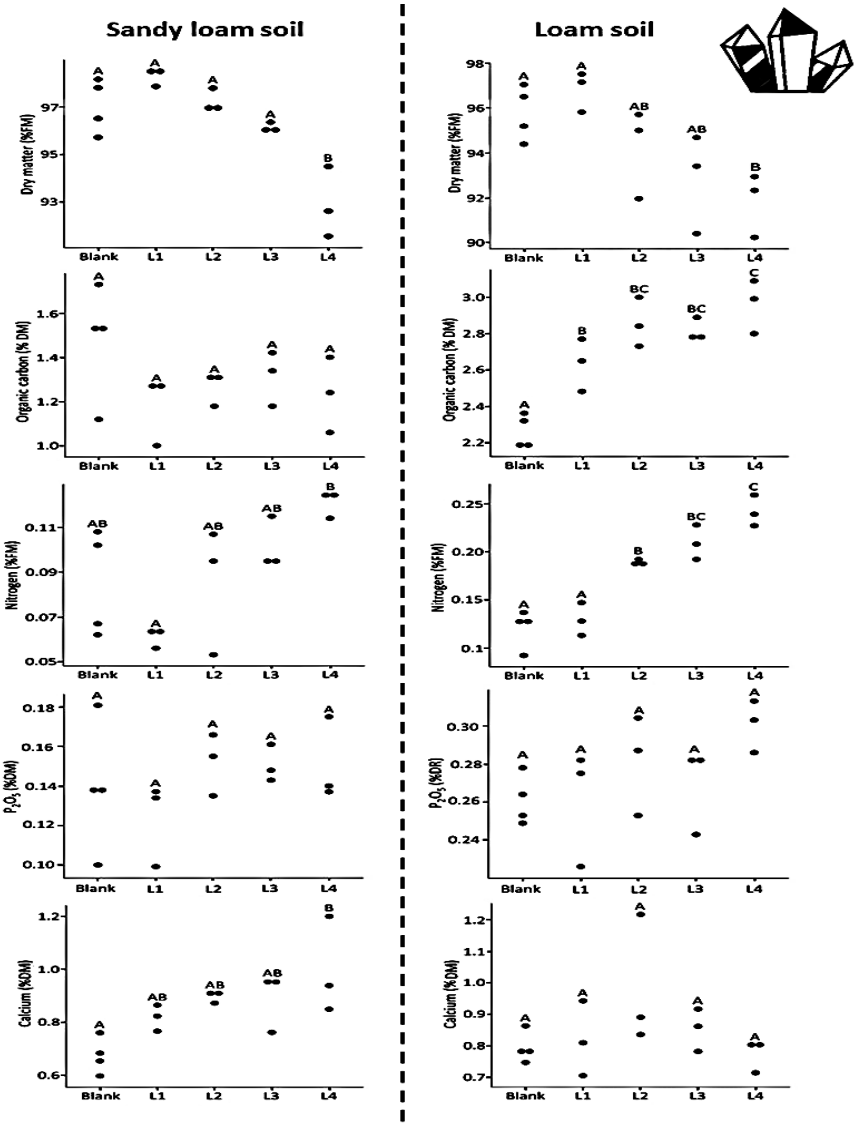

Figure 14 Single compounds analysis illustrating the impact of a decaying mammal on pH, bioavailable compounds (phosphor; potassium; magnesium, calcium, sodium), conductivity, dry matter, organic carbon and nitrogen amount, at different depths (Layers 1 to 4), expressed in percentage of dry matter (%DM) or in percentage of fresh matter (%FM). The two soil textures were separately analyzed: Left: Sandy loam soil. Right: Loam soil. All layers were compared to the control of the respective soil texture (Datasets sharing the same uppercase letter are not significantly different from each other (threshold: p-value < 0.05)).

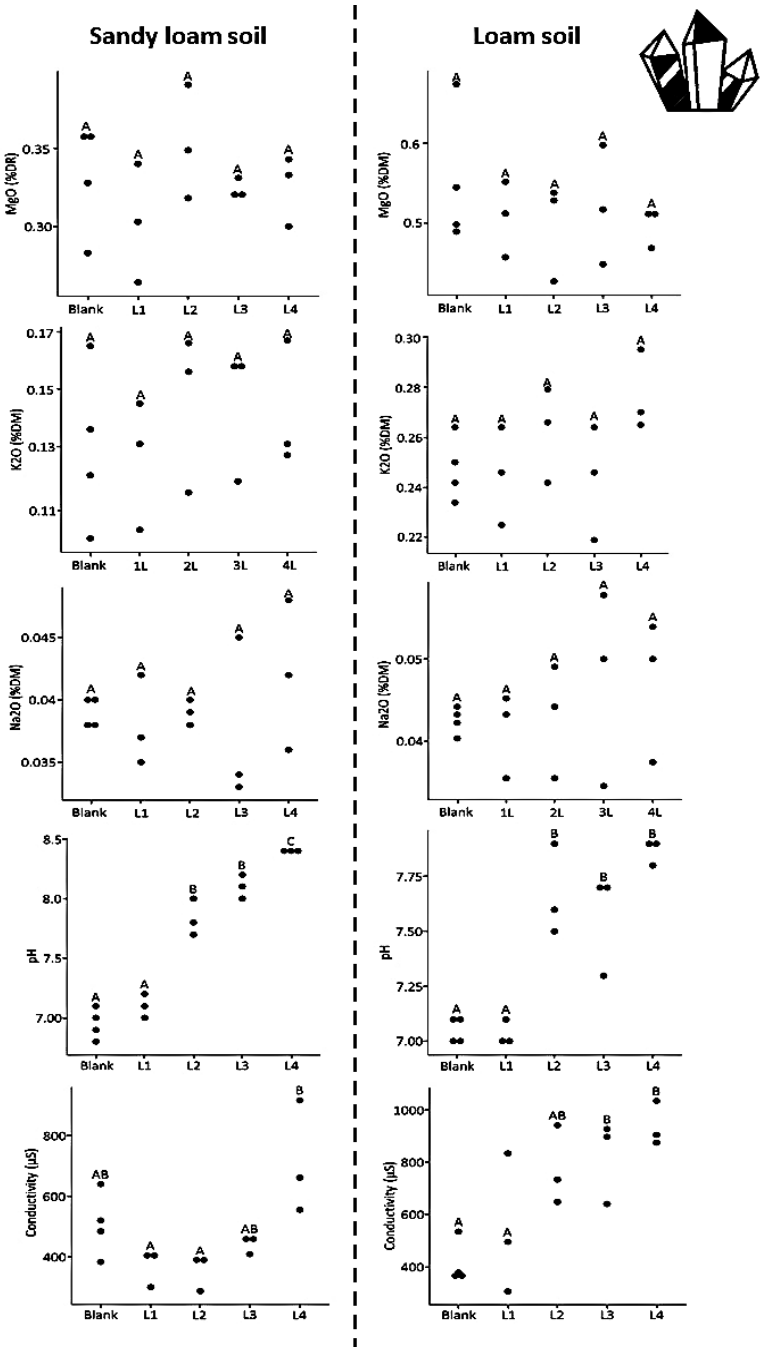

Figure 14 (continued)

Volatile analysis

The presence of animal remains was related to the soil's volatilome, in both soil texture types ($F_{1,31} = 3.64$, p-value = 0.009). In addition, we observed no relationships between soil texture and cadaveric volatilome ($F_{1,31} = 0.98$, p-value = 0.397). The volatilome of each layer of soil was related to the presence of a cadaver, but not similarly in both soil textures ($F_{9,31} = 2.63$, p-value = 0.001). For this reason, we analyzed both soil textures separately (Figure 15). Distributions of the data allowed us to perform a permMANOVA with a Euclidean method.

Soil volatilome was related to the presence of remains, but the relationship differed by soil layer (loam: $F_{4,15} = 3.04$, p-value = 0.004; sandy loam: $F_{4,15} = 2.60$, p-value = 0.011). A careful evaluation of the PCAs indicating that the volatilomes of all soil layers were related to the presence of animal remains in loam, whereas SL4 was only related to volatilomes in sandy loam.

As with the chemical data, we identified volatile compounds as potential indicators (those that were the most associated with the presence of a corpse) by generating a predictive model. The model that merged volatile data from both soil textures exhibited an error rate of 16 % and identified key chemical indicators for the presence of a cadaver buried in soil (in order of importance): dimethyl-disulfide, 2-isopropyl-5-methyl-heptan-1-ol, 2-4-dimethyl-hept-1-ene, 2,5-dimethyl-octane, and dimethyl-trisulfide. When we generated predictive analyses by separately analyzing the two soil textures (loam vs. sandy loam), the error rate increased to 30% and identified dimethyl-disulfide as the only effective indicator of animal decay (but only in sandy loam). For loam soil, we identified the following compounds as potential indicators (in order of importance): dimethyl-disulfide, 2,3,7-trimethyl-octane, and 2-isopropyl-5-methyl-heptan-1-ol.

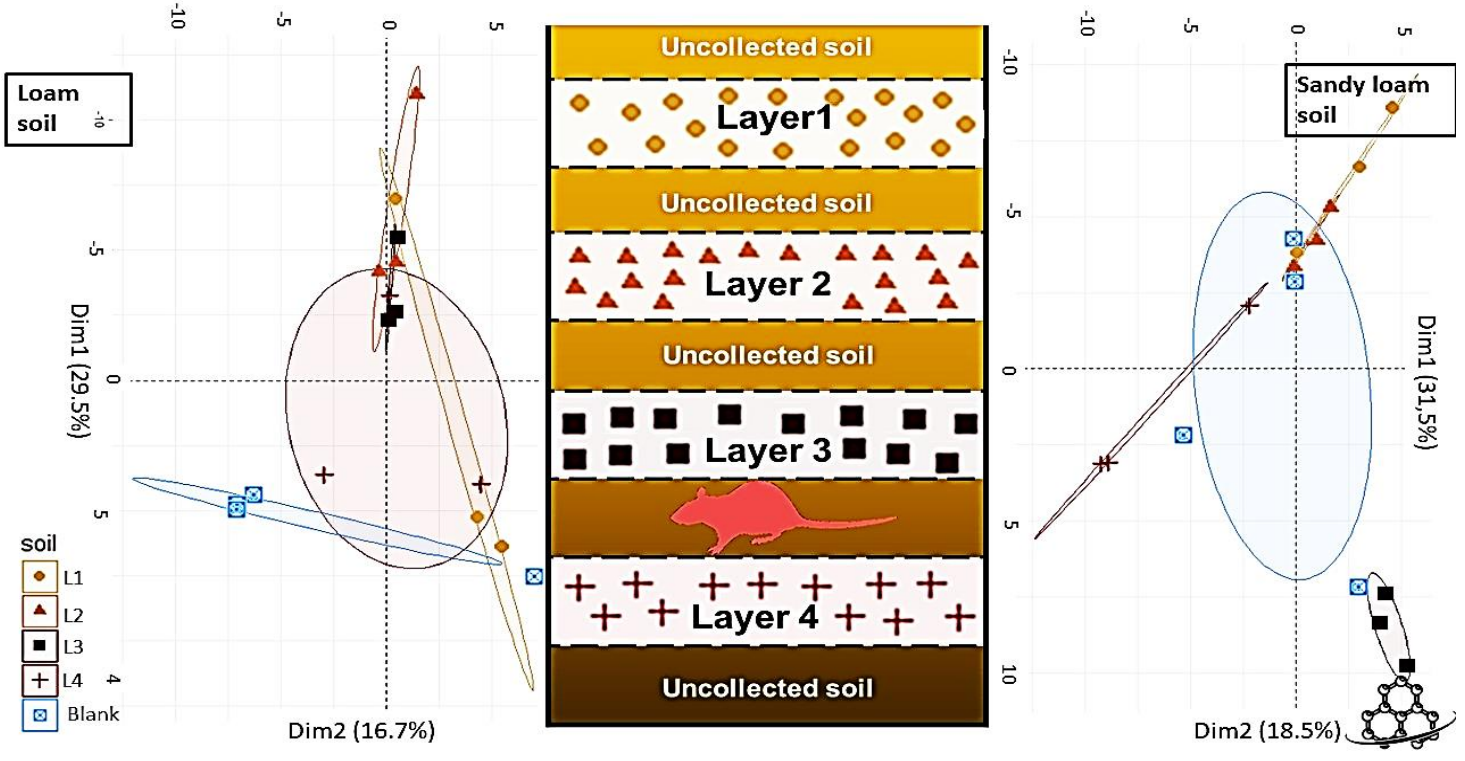

Figure 15 Principal component analyses illustrating the impact of a buried decaying mammal (see center of the figure) on the volatile cadaveric compounds at different depths (Layers 1 to 4) within two soil textures separately analyzed: Left: Loam soil. Right: Sandy loam soil.

Integrated soil chemistry conditions

In merging our chemical and volatile analyses and performing the same statistical analyses as listed above, we obtained similar results. The presence of a decaying rat in a soil is related to changes in the chemical characteristics of the soil (all soil textures and layers combined) ($F_{9.31}$ = 2.63, *p*-value = 0.001). This result was similar for both soil types: loam ($F_{4,15}$ = 3.04, *p*-value = 0.008); sandy loam ($F_{4,15}$=2.60, *p*-value = 0.012). SL4 (located just below the cadaver) was the layer most related to soil physico-chemical conditions. The chemistry of SL3 (layer directly above the cadaver) was significantly related to rat remains presence, but SL2 and SL 1 were not. When we performed a predictive analysis on the data set that combined both volatile and mineral data, the error rate reached 19 %, whereas when both soil textures were analyzed separately, we found that model error rates were 19 % for loam and 25 % for sandy loam. In contrast, combining mineral and volatile analyses decreased the error rate of the model to 10% and allowed us to identify key features that indicate the presence of a corpse in a soil (in order of importance): organic carbon concentration, calcium concentration, pH, conductivity, dimethyl-disulfide concentration, 2-isopropyl-5-methyl-heptan-1-ol concentration, and 2,3,7-trimethyl-octane concentration.

Discussion

In this study, we intended to determine the effect of mammal decomposition on burial soil physico-chemical properties by taking into account the chemical and volatile changes that occur during decomposition throughout a soil column. Herein, we briefly provide our insights on the impact of a decaying corpse on soil texture, soil chemistry, and volatile organic compounds (the reverse assumption was not considered in this work, *i.e.,* whether the soil texture impacts decomposition). Then we describe the feasibility of using one or more of these characteristics as gravesoil indicators.

Soil texture

Sandy loam is characterized by a higher porosity than loam, resulting in a higher drainage of cadaveric fluids (Dekker & Ritsema, 1994; Dias, 2011). Because we did not add water to the graves during the experiment, the remains were the only source of soil moisture below the rat cadavers. Due to the lower porosity of loam, all soil layers located around the cadaver (immediately below and above it) were moister than control soils, presumably due to the radial diffusion of body fluids from the decaying animals. The animal remains decaying in sandy loam were also drier than those decaying in loam, suggesting that moisture is absorbed more rapidly in sandier soils,

as also supported by other studies (Bohun et al., 2010; Carter et al., 2010; Dekker & Ritsema, 1994). The fact that cadaveric fluids drained differently (relative to soil texture) probably explains why the physico-chemistry of all soil layers were impacted differently by decaying remains. We know from previous studies that soil composition impacts the decomposition process of a corpse (Tumer et al., 2013). Because all rats reached the same decaying stage by the end of the experiment, the proportion of sand in soil probably had only a limited influence on the decomposition process.

Chemical conditions

Soil inorganic chemical properties of both soil textures were related to the presence of rat remains. The chemical properties of clayey soils have also been shown to be indicative of the presence of animal remains (Fancher et al., 2017; Tumer et al., 2013). Our study shows that decaying remains are not homogeneous throughout a soil column. This heterogeneity is probably due to the drainage of bodily fluids into soil as it decomposes (Carter et al., 2010; Dekker & Ritsema, 1994; Dent et al., 2004). Nitrogen, pH, and conductivity are all higher in sandy loam and loam surrounding a cadaver, and so can potentially be used to estimate post-mortem intervals (PMIs) (Benninger et al., 2008; Fancher et al., 2017). The elevation of nitrogen concentrations near a buried corpse has already been reported in previous studies (Aitkenhead-Peterson et al., 2012; Carter et al., 2008, 2010; Fancher et al., 2017; Tumer et al., 2013) and could be explained by the fact that animals are rich in nitrogen (32 $g.kg^{-1}$) from nucleic acids, amino acids, sugars, and proteins (Aitkenhead-Peterson et al., 2012; Benninger et al., 2008; Widdowson, 1950). We found that nitrogen concentrations were higher in loam. These results could be explained by the fact that loams generally support a higher diversity of microbes than sandy loam and soil microbes are known to participate to the release of nitrogen (Aitkenhead-Peterson et al., 2012; Dent et al., 2004; Hopkins et al., 2000; Stokes et al., 2009; Szelecz, Koenig, et al., 2018; Tumer et al., 2013). We found that soil pH is also increased by the presence of a cadaver, as has been well documented already (Aitkenhead-Peterson et al., 2012; Benninger et al., 2008; Fancher et al., 2017; Hopkins et al., 2000; Stokes et al., 2009; Tumer et al., 2013). Although some studies have found a decrease in pH near buried cadavers (Aitkenhead-Peterson et al., 2012; Benninger et al., 2008; Szelecz, Koenig, et al., 2018), most published studies have found that there is an increase in soil pH near a decaying animal (Benninger et al., 2008; Carter et al., 2010; Hopkins et al., 2000; Szelecz, Koenig, et al., 2018). Nitrogen concentrations and pH are associated: nitrogen is predominantly released as NH_4^+, which impacts pH. Nitrogen also increases conductivity in a soil (Aitkenhead-Peterson et al., 2012; Fancher et al., 2017; Stokes et al., 2009; Tumer et al., 2013), probably partly explaining the increase in conductivity we found in our present study.

Although we found that pH, conductivity, and nitrogen were altered during animal decay in both soil textures, other measured parameters were only altered in one or the other texture. For example, organic carbon concentrations are higher in loam soils. Several studies have also observed variations in organic carbon in a gravesoils, but most studies failed to determine the origin of such variation (Benninger et al., 2008; Fancher et al., 2017; Macdonald et al., 2014). In our opinion, the amount of organic matter in gravesoils is associated with the quantity of cadaveric fluids in the soil. Because cadaveric fluids are better retained in loams than in sandy soils, we speculate that more organic matter should occur in loamy gravesoils.

Calcium concentrations in soils also increase in the presence of a cadaver, but we found this to be true only in the sandy loam soil. Calcium is usually released at the end of the decomposition process (*i.e.*, during diagenesis) (Fancher et al., 2017; Szelecz, Koenig, et al., 2018). However, we doubt that diagenesis had begun after only two months of decomposition. Thus, the increase in calcium we observed could be due to the release of calcium from small pieces of bones that remained in the soil samples or because the calcium located in the muscle is released in the soil. However, our study does not focus on variations in phosphorus, magnesium, potassium, and sodium that occur at the latest stage of decomposition (Aitkenhead-Peterson et al., 2012; Benninger et al., 2008; Fancher et al., 2017; Tumer et al., 2013).

Volatile conditions

We found that the presence of a buried corpse impacted the VOCs in both soil textures, as also observed in previous reports (Brasseur et al., 2012; Dubois et al., 2018; Forbes et al., 2016; Nizio et al., 2017; Vass et al., 2004). At the depth of sampling (<20 cm from a cadaver), we found that the impact of decaying corpses on the volatilome was similar in the two soil textures. In both soil textures, we found previously-reported cadaveric VOCs (including sulfur compounds, alkanes, alcohols, and ketones) that were not present in our control soils (Brasseur et al., 2012; Dekeirsschieter et al., 2009; Dubois et al., 2018; Forbes & Perrault, 2014; Perrault et al., 2014). We also found that the cadaveric VOCs is not homogenous throughout the soil column (Brasseur et al., 2012; Vass, 2012); that is, the distribution of VOCs varied with depth for both soil textures. These depth profile differences with soil type can be explained by their dissimilar soil porosities (Vass, 2012). Although we found that some compounds were more abundant close to cadavers (*e.g.*, dimethyl-trisulfide), we detected other compounds in similar amounts throughout the soil profiles (*e.g.*, heptan-1-ol and 2-isopropyl-5-mehtyl). In sandy loam, the most impacted soil layer (in terms of VOCs released) was the stratum located directly under the remains, suggesting that the VOCs we detected mainly originated from cadaveric fluids, which were likely drained to the lowest part of the grave. In loam, the cadaveric VOCs were

more likely to diffuse homogenously through the column (upward and probably also laterally), suggesting that cadaveric VOCs diffuse around animal remains in less porous soils, as indicated in other studies (Adserias-Garriga et al., 2017; Boumba et al., 2008; Dent et al., 2004; Tumer et al., 2013). Polarity may also explain the adsorption of cadaveric VOCs on soil particles (Brasseur et al., 2012).

Prediction of clandestine graves

There is a great interest in finding compounds that can predict the presence of gravesoils, with pH and nitrogen concentrations most commonly identified as being useful (Benninger et al., 2008; Carter et al., 2008, 2010; Janaway et al., 2009a; Meyer et al., 2013; Perrault et al., 2014; Rosier et al., 2015). However, a more complete understanding of the variation in other chemical and volatile compounds would help improve the potential use of such compounds as indicators of burial sites (Carter et al., 2010). For example, our model identified calcium and organic carbon as potentially reliable predictors. However, because both indicators change in one of the soil textures, they should not be considered as interesting as suggested by our model.

Conclusions

This study is the first to reveal the impact of a buried cadaver on the composition of chemicals and VOCs in surrounding soil layers. Our results suggest that volatile compounds in burial soil are more reliable indicators of buried remains than other target chemicals usually used in forensic investigations (The error rate associated with our model for volatile compounds was twice as low as the model commonly used for chemical analyses of potential gravesites). However, we found that a combination of both volatile and chemical models is even more accurate in predicting the presence of a buried cadaver. The impact of decaying mammals on the chemistry of a grave is primarily related to the soil texture in which the animal is buried and is secondarily related to the depth at which the remains are buried. Future studies dedicated to the understanding geothanatochemistry should focus on targeted compounds, such as nitrogen or dimethyl-disulfide, and should pay closer attention to early stages of decomposition (*i.e.*, freshly buried corpses) to identify suitable indicators for establishing PMIs.

On the Styx bank - Characterization of the headspace cadaveric volatiles released by immerged decaying rats

This section is an adapted version of the article:

Martin C, Malevic M and Verheggen F (2023) On the Styx bank - Characterization of headspace cadaveric VOCs profile released by submerged decaying rats, *in prep*

Abstract

The cadaveric volatilome of terrestrial decomposition (including buried corpses) have been extensively studied in recent taphonomy studies. However, the volatiles associated with immerged vertebrate remains have received much less attention. This decaying process is particular, as illustrated by the succession of the decaying stage which are very different from the terrestrial decomposition. Indeed, five stages can be identified: fresh, early floating, floating decay, deterioration, and sunken remains. Because the decomposition process is different from terrestrial decomposition, we expect different cadaveric volatiles to be released by submerged remains. We therefore aimed to characterize the volatile compounds released by underwater decomposition and reaching the surface. Rat cadavers were place individually in glass chambers filled with water. Volatiles released at the surface were than collected three times a week during a month. Two types of water were used (fresh and marine water) to assess the impact of the salinity level on the cadaveric volatilome. More than 30 volatile compounds were identified with the majority being already reported in the headspace of cadaver decaying in terrestrial environment. Sulphur containing compounds were the most abundant, with dimethyl disulfide being the major one. We did not highlight any impact of the salinity level on the volatile profile. The later was impacted by the decaying stage. Skatole is the best candidate to discriminate the two first stage of decomposition from the third one.

Introduction

The characterization of the volatile cadaveric compounds has been performed for years on both human and surrogate models (*e.g.*, *pig*). Despite this interest, researches mainly focus on terrestrial decomposition, either in above or below ground conditions (Dubois, Stefanuto, et al., 2019; Forbes, Rust, et al., 2014; Perrault et al., 2014; Rosier et al., 2016; Verheggen et al., 2017). Volatiles associated with submerged vertebrate remains have received less attention (Dalal et al., 2020; Haefner et al., 2004). The mechanisms involved in the decomposition of immerged remains are specific to this environment (Dalal et al., 2020; Haefner et al., 2004; Heaton et al., 2010). Indeed, the stage succession (Table 9) is different from terrestrial decomposition as well as associated necrophagous insects which speed up the decomposition process in terrestrial environment (Amendt et al., 2004; Dalal et al., 2020; Martin et al., 2019; Tomberlin & Adler, 1998). Moreover, the lack of oxygen and the constant temperature in water induce a slower decomposition process promoting anaerobic decomposition (Dubois et al., 2018; Haefner et al., 2004; Keiper & Casamatta, 2015).

Table 9 Differences between decomposition stages of surface, buried and immerged corpses (Paczkowski & Schutz, 2011)

Decaying stages	Above/below ground decaying	Under water decaying
Fresh	No evident signs of decomposition	
Bloat / Early float	Inflation of carcass	Breaching water-line
Active / floating decay	Deflation of carcass	Inflation of carcass
Advanced decay / deterioration	Drying of soft tissue	Deflation of carcass
Skeletonisation / Sunken remains	Dried tissue	Sinking

Thus far, it is commonly admitted that the cadaveric volatilome is composed of hundreds of molecules belonging to almost all chemical classes (Dekeirsschieter et al., 2012; Verheggen et al., 2017). Despite an extensive literature on aerial cadaveric volatile organic compounds (VOCs), VOCs released by immerged bodies have received very limited attention. A recent study of adipocere volatilome came out (Dubois et al., 2018), suggesting that the formation of adipocere is associated with humid environment and is sometimes observed on immerged remains (De Donno et al., 2014; Moses, 2012). Adipocere formation is a particular process which preserves the body integrity and is usually observed in the late decomposition (Moses, 2012). With the exception of the particular case of adipocere volatilome, only one paper was

found (Caraballo, Sc, et al., 2016; Irish et al., 2019). They both investigated the cadaveric volatilome of immerged and non-immerged remains, and highlighted twice more VOCs in openair decomposition (Irish et al., 2019). Dimethyl disulfide participate to the majority of the total profile abondance, while the profile is more diversified during terrestrial decomposition (Caraballo, Sc, et al., 2016). Both studies used solid-phase-microextraction (SPME) to characterize the cadaveric VOCs released by decaying remains. Even though SPME is a cheaper and faster sampling technique, it does not allow to perform quantitative analyses of the results.

The objective of the present work was to characterize cadaveric VOCs released during the decomposition of immerged rat remains by using dynamic headspace sampling coupled with gas chromatography analyses. Moreover, we assessed the impact of the water salinity on the release of cadaveric VOCs.

Material and methods

Biological material

We were provided with 8 dead laboratory male rats (3–5 months old, 266.5 ±10.4 g) by the faculty of veterinary medicine of the University of Liège (Belgium). No rats were killed for this specific experiment (Ethics agreement n°18-2021); they had been used in a previous assay (which could not be communicated to the authors of the present research). Rats had been euthanized and kept frozen for about ten weeks prior to our experiment.

Rat decomposition setup

Three 15 L open glass tanks were filled to the top with soft water (collected in a water source in Sugny, Belgium); and three other tanks were filled with sea water (collected in the sea in Middelkerke beach, Belgium). Four rats were unfrozen in a hot water bath set at 40 °C. Two rats were individually introduced into two tanks containing soft water, two other rats were individually introduced into two tanks containing sea water. A third tank in both water conditions was filled to the top and kept without rat and considered as a control. All rats were left undisturbed to decompose for one month. All glass tanks were placed in a room set at 14 °C. A net was placed on the top of the glass tanks to prevent insects' colonization of the remains. This experiment was then replicated one month later using the remaining four rats. The duration of each stage of decomposition was recorded during the month of experiment. Stages were assigned based on Payne and King's (1970) (Payne, 1965).

Odor collection

One hour before sampling, a glass lid was placed to seal the tanks allowing the

equilibration of the VOCs in the headspace. The lid had several openings which were kept closed during the hour of accumulation. After that delay, one of the openings was connected to the sampling tubes (hydrophobic Tenax Ta/Carbograph sorbent (Gerstel, Germany)) connected with a pump system (PVAS11; Volatile Assay Systems, Rensselaer, New York). On another opening, a charcoal tube was placed to filter entering air. All additional openings were kept sealed. The pump was set at 200 mL/min. The sampling lasted for one hour. After sampling, tubes were sealed and kept at 4 °C prior to be analyzed. Chlorobenzene (Sigma Aldrich) was spiked in the sampling tubes as internal standard (35ng) before to be injected.

Gas chromatography analyses

The separation of sampled compounds was performed on a gas chromatograph (QP 2020 NX, Shimadzu, Kyoto, Japan). Prior to be separated, compounds were thermodesorbed at 280 °C for eight minutes. All compounds were cryofocused in a glass tube (cooling trap) set at -20°C by Peltier effect. The trap was then heated up to 280°C to inject compounds. A split ratio of 20 was applied. Compounds were separated on a HP-5ms column (30 m x 0.25 mm x 0.5 µm; Agilent technology). The temperature program started at 40°C held for two minutes. Temperature was first increased to 90 with a ramp of 2 °C/min, then to 300 °C at 10 °C/min. This final temperature was held for three minutes. Chemicals were detected on a quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) set at 1.1 kV. The ion source temperature was set at 200°C. All m/z ration from 30 to 500 were acquired each 0.2 s. The detected compounds were identified by interpretation of their mass spectra and comparison with spectral libraries (NIST version 2017 and FFNSC version 3). All compounds identified in the control conditions were removed from the data as well as compounds which are not considered as VOCs as defined by EU directive (2004/42/CE). While the mean profile was obtained, a single ion monitoring method (SIM) were applied for each compound on each chromatogram to identify them at a trace level.

Statistics

Statistical analyses were performed using R version 3.4.2. (R Foundation for statistical computing). To compare the duration of each decaying stage in the different water conditions, a generalized linear mixed model (GLMM) was performed. The volatile profiles comparison was conducted with a permutational multivariate analyses of variance (i.e., perMANOVA) using a Euclidean distance matrix and 999 permutations (“adonis” command, R-package vegan, (Oksanen et al., 2020)). This allowed to assess the potential impact of the decaying stage, the type of water and the interaction of these parameters. As the experiment was split into two blocks (March and April), the month of the experiment was placed in stratum using the "strata"

function in adonis. It allows to perform intra-month permutation in the data frame instead of inter-month permutations. In addition, it was considered in the model which allowed to include the random factor in the residue calculation. We adjusted *p*-values using Bonferroni's correction to avoid increases in type I error due to multiple testing. When a *p*-value was significant, pairwise comparisons were performed. The *p*-values of theses comparisons underwent Bonferroni's adjustment to avoid type I errors due to multiple analyses. Differences are illustrated with a Linear discriminant analysis (LDA) used for pattern recognition between groups. This method allows to determine which of the independent variables contributed the most to the differences in the average score profiles of the different condition of immerged decaying rats. A generalized linear mixed model (GLMM) was used on variables to highlight candidate VOCs driving the difference when observed.

Results

Decomposition

All rat cadavers went through 3 stages of decomposition in both sea and soft water, with the exception of two rats from the first batch which did not reach the floating stage (Figure 16). The floating stage duration was therefore not compared with the two first stages as the experiment was stopped at this stage. The salinity level of the water had no impact on the decaying process duration ($Z = -0.96$, p-value = 0.34) : sea water = 9.7 ± 0.6 days ; soft water = 9.7 ± 2 days). However, the salinity level impacted the duration of the first stage (p-value = 0.026) but not the second one (p-value = 0.77). Indeed, rats stayed longuer in the fresh stage when their were placed in soft water compared to sea water (Figure 16).

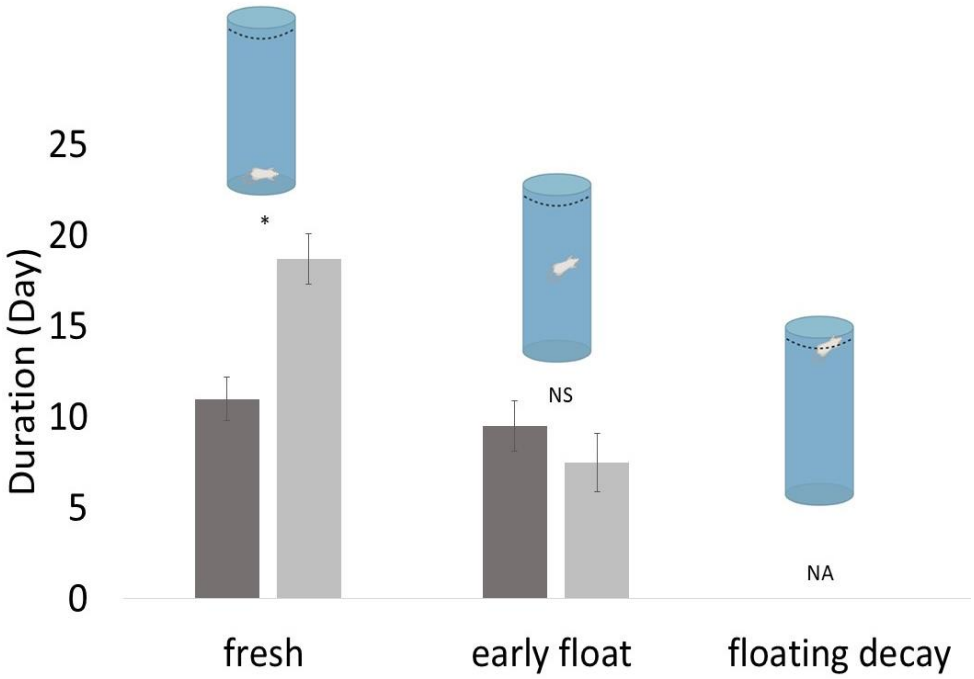

Figure 16 Stage duration in days in two water salinity conditions (dark grey: sea water; light grey: soft water) for each stage of decomposition, except for the floating decay stage, as we stopped the experiment at this last stage. (NS: no significant; NA: not applicable)

Volatile collection

In the present study a total of 33 cadaveric VOCs were identified in the headspace of soft and sea water. Eight chemical classes were represented (Table 10). The most represented class is the sulfur containing compounds. Only three compounds represent half the cadaveric volatilome released through the entire process in both types of water : dimethyl disulfide (DMDS), nonane and toluene. DMDS alone represents more than 30% of the cadaveric VOCs profile. Relative abondances are presented in supplemental data (sup Table 1). The majority of the compounds was already associated with the decomposition of vertebrate remains (Table 10). Only two compounds were only released in sea water condition : dimethyl sulfoxide and 2,4-diathiapentane. All other compounds were at least released in one of the three investigated stages of decomposition of each water type.

Table 10 List of volatile organic compounds released by rat remains decaying either in soft or sea water, according to the three decomposition stages

	Soft water			Sea water			
	fresh	**early floating**	**floating decay**	**fresh**	**early floating**	**floating decay**	**References**
sulfur compounds							
dimethyl disulfide	Δ	Δ	Δ	Δ	Δ	Δ	(Armstrong et al., 2016; Cablk et al., 2012; Dekeirsschieter et al., 2009; Forbes & Perrault, 2014; Martin et al., 2019; Perrault et al., 2014; Perrault, Stefanuto, et al., 2015; Rosier et al., 2016; Vass, 2012)
dimethyl trisulfide	Δ	Δ	Δ	Δ	Δ	Δ	(Armstrong et al., 2016; Dekeirsschieter et al., 2009; Forbes & Perrault, 2014; Martin et al., 2019; Perrault et al., 2014; Perrault, Stefanuto, et al., 2015; Rosier et al., 2016; Stefanuto et al., 2016; Vass, 2012)
dimethyl tetrasulfide	Δ			Δ	Δ	Δ	(Martin et al., 2019; Stefanuto et al., 2016)
dimethyl sulfoxide				Δ	Δ	Δ	(Armstrong et al., 2016; Martin et al., 2019)
methyl ethyl disulfide		Δ	Δ	Δ	Δ	Δ	(Armstrong et al., 2016; Martin et al., 2019)
methyl butanethioate	Δ		Δ	Δ	Δ	Δ	
methyl (methylthio)methyl disulfide			Δ	Δ		Δ	(Armstrong et al., 2016)
bis(1,1,3,3-tetramethylbutyl) disulfide	Δ	Δ	Δ	Δ	Δ	Δ	(Stefanuto et al., 2016)
dihydro-2(3H)-thiophenone	Δ	Δ	Δ	Δ	Δ	Δ	
2,4-dithiapentane				Δ	Δ	Δ	(Armstrong et al., 2016; Forbes & Perrault, 2014; Perrault et al., 2014)
1,1-bis(methylthio)ethane	Δ	Δ	Δ	Δ	Δ	Δ	
hexathiane			Δ	Δ	Δ	Δ	

Aldehydes							
decanal	Δ	Δ	Δ	Δ	Δ	Δ	(Cablk et al., 2012; Martin et al., 2019; Nizio et al., 2017; Perrault, Stefanuto, et al., 2015; Vass, 2012)
benzaldehyde	Δ	Δ	Δ	Δ	Δ	Δ	(Cablk et al., 2012; Dekeirsschieter et al., 2009; Martin et al., 2019; Perrault et al., 2014; Perrault, Stefanuto, et al., 2015)
cyclic compounds							
toluene	Δ	Δ	Δ	Δ	Δ	Δ	(Cablk et al., 2012; Forbes, Rust, et al., 2014; Martin et al., 2019; Perrault, Stefanuto, et al., 2015)
styrene	Δ	Δ	Δ	Δ	Δ	Δ	(Forbes, Rust, et al., 2014; Perrault, Stefanuto, et al., 2015)
3-carene		Δ	Δ	Δ	Δ	Δ	
7-methylbicyclo[4.4.1]undeca-2.4.8-triene	Δ	Δ	Δ	Δ	Δ	Δ	
phenylmaleic anhydride	Δ	Δ	Δ	Δ	Δ		
ketones							
3-hexanone	Δ	Δ	Δ	Δ	Δ	Δ	(Forbes, Rust, et al., 2014)
2-hexanone	Δ	Δ	Δ	Δ	Δ	Δ	(Perrault et al., 2014)
acetophenone	Δ	Δ	Δ	Δ	Δ	Δ	(Martin et al., 2019; Perrault, Stefanuto, et al., 2015)
6.10-dimethyl-5.9-undecadien-2-one	Δ	Δ	Δ	Δ	Δ	Δ	
2-butyl-1.1.3-trimethylcyclohexane	Δ	Δ	Δ	Δ	Δ	Δ	
alkanes							
nonane	Δ	Δ	Δ	Δ	Δ	Δ	(Martin et al., 2019; Nizio et al., 2017; Vass, 2012)
dodecane	Δ	Δ	Δ	Δ	Δ		(Cablk et al., 2012; Forbes, Rust, et al., 2014; Nizio et al., 2017)

tridecane	Δ	Δ	Δ	Δ	Δ	Δ	(Forbes, Rust, et al., 2014; Forbes & Perrault, 2014; Martin et al., 2019)
tetradecane	Δ	Δ	Δ	Δ	Δ	Δ	(Martin et al., 2019)
carboxilic acids							
benzoic acid	Δ	Δ	Δ	Δ	Δ	Δ	(Dekeirsschieter et al., 2009; Forbes & Perrault, 2014; Perrault, Stefanuto, et al., 2015)
pentanoic acid	Δ	Δ	Δ	Δ	Δ	Δ	(Armstrong et al., 2016; Cablk et al., 2012; Dekeirsschieter et al., 2009; Forbes & Perrault, 2014; Perrault, Stefanuto, et al., 2015)
alcohols							
2-ethyl-1-hexanol	Δ	Δ	Δ	Δ	Δ		(Cablk et al., 2012; Forbes, Rust, et al., 2014)
[1.1':3'.1"-Terphenyl]-2'-ol	Δ	Δ		Δ	Δ		
nitrogen compounds							
3-methyl-indole		Δ	Δ		Δ	Δ	(Nizio et al., 2017)

Stages and water salinity

The water salinity did not impact the cadaveric volatilome released at the water surface ($F_{1,95} = 2.39$; *p*-value = 0.124). However, a pattern of VOCs can be identified regarding each stage of decomposition ($F_{2,95} = 13.65$; *p*-value = 0.001). No differences were identified when water salinity and decaying stage interaction was investigated ($F_{2,95} = 0.39$; *p*-value = 0.717). The LDA was therefore only performed by considering the decaying stage (Figure 18). Regarding the effect of the stage, differences were observed between the fresh and the floating decay stages (*p*-value = 0.003) and between the early floating and the floating decay stages (*p*-value = 0.012). No differences can be demonstrated between the fresh and early floating stages (*p*-value = 0.129).

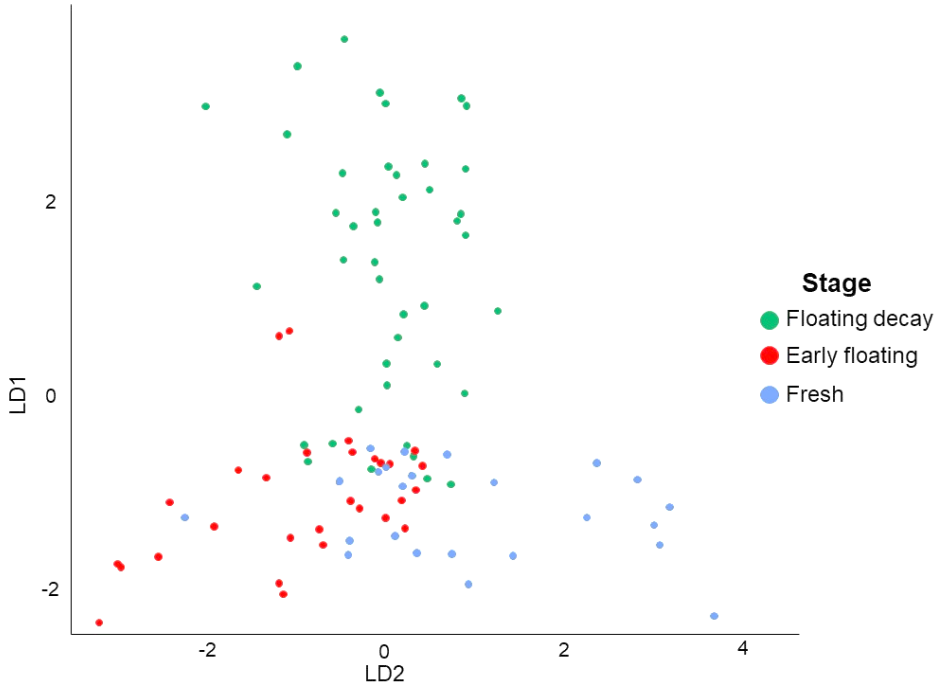

Figure 17 Linear discriminative analyses (LDA). Visualization of the VOCs profile during the decaying process of immerged rats remains, depending on the decomposition stage. Each sampling is represented in a two-dimensional plan constituted by the two first axes of the LDA.

Starting by the effect of the stage, the two first discriminant functions expressed 100% (LD1: 73.6%, LD2: 26.4%) of the observed variability. LD1 was mainly correlated with: terphenyl-2-ol, tetramethylbutyl disulfide; 6,10-dimethylundecadien-2-one; scatol; phenylmaleic anhydride. When performing a GLMM on these factors

to compare the decaying stages, all highlighted significative differences (p-value $_{6,10\text{-dimethyl-5,9-undecadien-2-one}}$ = 0.036, p-value $_{terphenyl\text{-}2\text{-}ol}$= 0.048, p-value $_{tetramethylbutyl\ disulfide}$ = 0.162, p-value $_{scatol}$ = 0.002, *p*-value $_{phenylmaleic\ anhydride}$ < 0.001). LD2 was correlated with tetramethylbutyl disulfide; 2,4-dithiapentane; dimethyl sulfoxide; 6,10-dimethyl-5,9-undecadien-2-one, 2-hexanone. When performing a GLMM with regards to the stage of decomposition, only tetramethylbutyl disulfide and dimethyl sulfoxide were not significantly different across them (p-value $_{tetramethylbutyl\ disulfide}$ = 0.162, p-value $_{2,4\text{-dithiapentane}}$ = 0.007, p-value $_{dimethyl\ sulfoxide}$ = 0.058, p-value $_{6,10\text{-dimethyl-5,9-undecadien-2-one}}$ = 0.036, *p*-value $_{2\text{-hexanone}}$ = 0.01);

Discussion

We confirm previous observations of a delay in the decaying process when the corpse is immerged, compared with a typical terrestrial decomposition (Dalal et al., 2020). During our experiment, the duration of the fresh stage was even longer than the one already reported (Dalal et al., 2020). In our study, a net prevented the insects colonization which are known to hasten the decaying process (Bugelli et al., 2018). Even if during the fresh stage the carcass is completely immerged, aquatic insects larvae are able to feed on the remains (Dalal et al., 2020). The duration of the early floating stage was close to the one already reported in the literature during the same year period (Dalal et al., 2020). Regarding the salinity level, no differences were observed in terms of the decomposition duration. Data on marine immerged cadaver are scarce. However, the decomposition does not occur the same way in fresh and marine water (Anderson & Hobischak, 2004). These differences seem not to be linked with water salinity level but rather with environmental conditions, that are different between freshwater and marine environment (*e.g.*, sediment, scavengers, depth, water movement) (Anderson & Hobischak, 2004).

More than 30 volatile compounds have been identified from the immerged rats decaying in the two salinity conditions. Two-third of those compounds were already reported in both surface and underground decomposition processes (Armstrong et al., 2016; Dekeirsschieter et al., 2009; Forbes & Perrault, 2014; Martin et al., 2019; Perrault et al., 2014; Perrault, Stefanuto, et al., 2015; Rosier et al., 2016; Stefanuto et al., 2016; Vass, 2012). The sulphur containing compounds were the most represented chemical class, participating to one-third of the identified VOCs profile. These compounds are cues during decomposition with DMDS being one of the most cited sulfur containing chemical (Dekeirsschieter et al., 2009; Dent et al., 2004; Vass et al., 2004; Verheggen et al., 2017). They are by-products of sulfur-containing amino acids (*e.g.*, cysteine, methionine) which undergo desulfhydralation (Janaway et al., 2009a). This high diversity of sulfur containing compounds was already reported in an adipocere dedicated study (Dubois et al., 2018) which is known to be formed under

moist conditions (Moses, 2012). Irish et al. (2019) also highlighted similar compounds with a higher diversity of nitrogen containing compounds and a smaller diversity of sulfur containing compounds. They also identified more molecules probably because they reached the advanced deterioration stage (Irish et al., 2019).

As during terrestrial decomposition, the cadaveric volatilome of immerged remains varies between decaying stages (Dekeirsschieter et al., 2009). The floating decay stage was the only stage for which a difference was shown. However, a trend can be observed on the LDA regarding the differentiation between fresh and early floating stages. Differences are probably more difficult to highlight as the remains stay below the water surface during these stages (Dalal et al., 2020). The difference is clear when the remains completely reached the surface. Differences are driven based on the compounds constituting the first axes of the LDA. Statistics performed on the compounds the most correlated with this axe highlighted the role of skatol, phenylmaleic anhydride and 6,10-dimethyl-5,9-undecadien-2-one. Skatol is released in higher concentration during the floating decay stage while the two others were more present during the two first decaying stages. As they have never been reported as cadaveric VOCs, they could be contaminants. However, skatol is known to originate from the breakdown of amino acids as phelalanine, tryptophan and tyrozine (Paczkowski & Schutz, 2011).

Conclusions

Further studies on cadaveric VOCs released during aquatic decomposition should be conducted. They would allow to provide an olfactive library of this particular decaying process. In the present study, we prevented insects to colonize the remains, but their impact could be investigated, as they are known to be important actors of terrestrial decomposition (Bugelli et al., 2018). In addition, we recommend investigating the volatile compounds that are not released at the water surface but remain dissolved in the water.

Potential of direct immersion solid-phase microextraction to characterize dissolved volatile organic compounds released by immerged decaying rat cadavers

This section is an adapted version of the article:

Malevic M, Verheggen F and Martin C (2023) Potential of direct immersion solid-phase microextraction to characterize dissolved volatile organic compounds released by submerged decaying rat cadavers. Forensic chemistry, DOI: 10.1016/j.forc.2023.100488

Abstract

The decomposition process involves the degradation of carbohydrates, nucleic acids, proteins and fats, and leads to the release of volatile organic compounds (VOCs) among many other decomposition by-products. Despite the extensive literature on the VOCs emitted in the air from vertebrate corpses, there is a lack of research dedicated to aquatic decomposition. In this study, we aimed to evaluate the potential of direct immersion solid-phase microextraction gas chromatography coupled with mass spectrometry (DI-SPME/GC/MS) to characterize dissolved cadaveric VOCs. Dimethyl disulphide and indole -two compounds commonly released during decomposition- were selected to evaluate and set the optimal methodological parameters, which were found to be 10 minutes of collection performed under 27.5 °C and a stirring rate of 250 rpm. Using responsive surface methodology, the obtained curves highlighted the appropriate conditions for the dissolved cadaveric volatilome analysis. The method allows to trap 17 dissolved cadaveric VOCs, including commonly encountered compounds such as dimethyl disulfide, 9-hexanoic acid, dimethyl trisulfide and indole. DI-SPME/GC/MS has therefore potential for the identification of dissolved cadaveric VOCs, pending further tests are performed to optimize the method and make it capable of detecting all dissolved VOCs, through all stages of decomposition.

Introduction

The decomposition process of vertebrates involves the degradation of macromolecules (carbohydrates, nucleic acids, proteins and lipids), that leads to the release of volatile organic compounds (VOCs) among many other decomposition by-products (Dekeirsschieter et al., 2012; Janaway et al., 2009a; Paczkowski & Schutz, 2011; Verheggen et al., 2017). Most previous studies have focused on cadavers placed in terrestrial environment, i.e. open-air or underground (Degreeff & Furton, 2011; Dekeirsschieter et al., 2012; Martin, Maesen, et al., 2020; Statheropoulos et al., 2005), and very few contributions have been made on submerged decomposition (Haefner et al., 2004; Heaton et al., 2010; Hobischak & Anderson, 2002; Keiper & Casamatta, 2015). Even though the decomposition rate differs (Giertsen, Johan Christopher Morild, 1989), underwater body decomposition follows a succession of stages similar to terrestrial ones (Hobischak & Anderson, 2002; Megyesi et al., 2005): the cadaver first experiences the submerged fresh stage, which starts in most cases upon its full submersion and lasts until the first signs of bloating appear. During the bloating stage, the corpse floats to the surface and a pronounced and evident decaying odour is released. The third stage is characterised by a green discoloration around the abdomen and skin sloughing due to gas and fluid pressure. In the fourth stage, the body starts to deflate and releases a much less pronounced odour. The body sinks during the fifth stage, leaving some dry skin remains floating at the surface (Dalal et al., 2020).

Hundreds of cadaveric VOCs have been reported by studies dedicated to open-air or below ground decomposition, and the most complete lists of VOCs were obtained from dynamic air samplings coupled with bidimentsional gas chromatography (Dekeirsschieter et al., 2012; Dubois et al., 2018). The molecules detected during open-air or underground decomposition belong to almost all chemical families including ketones, nitrogen based molecules, sulphur based molecules and carboxylic acids, just to name a few (Dekeirsschieter et al., 2009; Martin & Verheggen, 2018b; Stefanuto & Focant, 2016). These molecules are produced under aerobic and anaerobic conditions (Dent et al., 2004). One study has been dedicated to the characterisation of cadaveric VOCs profile released at the water surface by immerged cadavers (Irish et al., 2019), and 41 VOCs have been identified by headspace solid-phase-microextraction (SPME). The volatile profile released by immersed bodies is less diversified than open-air decomposition probably because (1) most of the process takes place under anaerobic conditions and (2) because some compounds remain dissolved in water, therefore could not reach the headspace and be adsorbed on the SPME fiber.

The present study aims to evaluate the potential of direct immersion solid-phase microextraction, coupled with gas chromatography and mass spectrometry (DI-

SPME/GC/MS), to characterize dissolved cadaveric VOCs. We performed volatile collection on water samples having contained submerged rat cadavers -used as surrogates for human cadavers- (Martin et al., 2019), and we have selected improved the sampling conditions using a response surface methodology (RSM).

Materials & methods

Rat decomposition and water sampling

Two male laboratory rats (244.5 ± 16.5 g) (Rattus norvegicus, Berkenhout, 1769) were reared and euthanized at the Faculty of Veterinary Medicine of the University of Liège (ethic agreement n°18- 2021). None of the rats was killed for the present experiments; they had been used in a previous one (which could not be communicated to the authors of the present research). Rats had been kept frozen prior the experiment. Despite the potential impact of freezing on VOC emissions [20], we believe that this procedure does not impact the objective of this work (i.e. evaluate the potential of DI-SPME to characterize dissolved cadaveric VOCs). Each rat was defrosted in a hot water bath (≈ 40 °C) and left to decompose inside 15L open glass cylindrical tanks filled to the top with distilled water. A third tank, that contained no rat but filled with distilled water, was used as a control. All three tanks were placed in a room set at 18°C. A sample of 40ml of water was collected in each tank including the "control tank" after 30 days to maximize the quantity of cadaveric VOCs in the water. The samples were kept in 50ml Falcon tubes in a freezer at -20°C prior to analyses.

Initial DI-SPME sampling

To target compounds which could be used for the optimization of the sampling, DI-SPME samples were performed using polydimethylsiloxane SPME fiber coating (PDMS, Supelco, Bellefonte, PA, USA). This coating is recommended by the supplier for the analyses of chemicals in water. After a 30 min conditioning at 300°C in the GC injector, the fiber was immersed in the water sample for 45 min. The samples were kept in a water bath set at 40°C and stirred at 500 rpm. After sampling, the fiber was dried under a nitrogen flow for one minute at room temperature (22°C) to avoid water reaching the mass spectrometer. The fiber was then introduced in the GC injector, and VOCs were desorbed at 275°C under a flow of 1 ml/min of helium in split mode (split ratio 1:5). The collected compounds were separated on a gas chromatograph (QP 2020 NX, Shimadzu, Kyoto, Japan) and detected by a quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). The electron impact mode was set at 0.1 kV (source at 200 °C, transfer line at 250 °C, scanned mass range: 35 to 500 m/z). The temperature program used was very slow to decrease the risk of coelution as this matrix is expected to be rich in compounds: 30°C held for 3 min, 5°C/min until 350 °C held for 5 min. The detected chemicals were identified by interpretation of their

mass spectra and comparison with spectral libraries (NIST version 2017 and FFNSC version 3).

Chemicals' preparation

Two compounds, namely dimethyl disulphide (99.8% purity, Sigma Aldrich) and indole (99% purity, Sigma Aldrich), were selected to assess the optimal values of the main experimental parameters. These chemicals are commonly reported as cadaveric compounds and were identified during the previous step. A stock solution containing the two compounds was prepared at a concentration of 100 ng/µl, in miliQ water. That concentration was chosen based on the literature (Clases et al., 2021) and after performing pre-test SPME analyses of two stock solutions (100ng/µL and 1000ng/µL). The testing conditions are provided in Suplemental table B1. Twenty-four glass vials (V=20 mL) were filled to the top with the stock solution (100 ng/µl) previously sealed with a cap in preparation for SPME analysis.

Data analysis

To optimize the sampling method, a response surface method (RSM) was performed. This method is based on a collection of mathematical and statistical techniques that are used to model and analyse engineering applications (Zou, Cai, & Shu, 2012). It aims at optimizing several experimental parameters (namely sample temperature, extraction duration and stirring speed) to increase the response of interest allowing a better sensitivity (i.e., the number of detected volatile compounds). RSM have already been applied in several industrial fields as well as in forensic science (Hou et al., 2007; Zou, Cai, & Shu, 2012; Zou, Cai, Du, et al., 2012). However, RSM has never been applied to improve the analysis of cadaveric VOCs. RSM is usually performed following four steps: designing a series of experiments, developing mathematical models, identifying optimal combination of parameters and representing the predictive model with 2D or 3D plots. The plot can be represented by an equation: $Y= f(x1, \dots xn)$, with RSM aiming at maximizing Y. To obtain RSM curves, an initial testing was conducted on 24 samples differing in conditions of stirring rate (between 200 and 400rpm), temperature (between 23 and 33 °C) and sampling duration (between 5 and 15min). The different tested points of the RSM are detailed in the supplementary material (Supplemental table B1). Each of the tested point was associated with a GC/MS chromatograph. The GC method used to separate the compounds was shortened (Table A1).

Testing the optimization

Following the result obtained with the RSM, a second sampling was performed on the water where a rat was left to decompose during one month and analysed based on

the previously described method (Table A1). The method was shortened to improve peak quality. A qualitative comparison was performed to determine if the optimized method allows to detect more compounds, in higher quantities.

Results & discussion

DI-SPME sampling

The preliminary analyses performed on the dissolved volatilome of thirty days submerged decaying rats allowed the identify seven compounds (Table 11). Among these, five have never been identified in the headspace of submerged bodies nor in terrestrial decaying bodies (Agapiou et al., 2015; Forbes, Rust, et al., 2014; Irish et al., 2019; Martin & Verheggen, 2018b; Rosier et al., 2014). The analyses reveal that tetrahydro-indazol-4-one, DMDS, lactic acid and indole are the major compounds. After six days, the rats switch from the first stage of decomposition to the second one. The analyses were performed when rats reached the third stages to maximize the quantity of expected compounds (thirty days after the begin of the decomposition). However, a small number of compounds was identify despite that the samples were taken at a late stage of decomposition (Dekeirsschieter et al., 2012). A too high stirring rate, temperature and/or sampling time could have removed compounds from the fibre resulting in the reduced number of detected compounds (Valor et al., 1997).

Table 11 Peak areas of the organic compounds collected from submerged rats before and after the optimisation of the sampling method (refers to compounds that were identified from submerged bodies by Irish et al 2019)*

		Pre-optimized samplings	Optimized samplings
Aldehydes	tetradecanal	46.505	0
Acids	lactic acid	281.577	0
	dodecanoïc acid	28.912	133.450
	formic acid	0	140.072
	tetradecanoic acid *	0	165.901
	9-hexanoic acid *	0	2,402.985
	9-octadecenoic acid *	0	405.476
Hydrocarbons	3-methyl-pentane,	0	185.999
	2-methyl-hexane	0	145.201
	4-(1,1-dimethylpropyl)-phenol	94.504	0
	methyl-cyclopentane,	0	654.939
Ketones	6,10 dimethyl-5,9-undecadien-2-one	0	138.665
	non-identified ketone	0	159.532
Nitrogen containing compounds	indole*	246.535	2,225.704
	3,6,6-trimethyl-1-O-tolyl-1,5,6,7-tetrahydro-indazol-4-one	377.576	0
Sulphur containing compounds	dimethyl disulfide *	311.075	4,010.741
	dimethyl trisulfide *	0	195.825

Response surface methodology

RSM curves have been generated using different sampling durations, sampling temperatures and stirring rates. Our data show that the stirring rate has a low impact on the adsorption of the compounds in the tested conditions. Also, they suggest temperature and collection duration to be the main parameters to focus on when using DI-SPME for forensic applications. Indeed, the adsorption of indole on the fiber follows a quadratic correlation with time and temperature, as highlighted by a single maximum on the curve. However, the curves could not highlight a single optimal combination of parameters in the case of DMDS, for which the adsorption increased with the sampling time and decreased with with the raise of temperature. The RSM curves highlight a stationary point that represent a good compromise for the dissolved cadaveric volatilome analysis: duration of the sample collection = 10 min; temperature = 27.5 °C; stirring rate = 250 rpm. These parameters are in the positive slope of each DMDS RSM cuve and meet the maximum observed on indole RSM curve (Time vs temperature).

In the case of the cadaveric volatilome, the diversity of molecules and chemical families is so important that our analysis could not guarantee an optimal result for all compounds (Dekeirsschieter et al., 2009; Forbes, Perrault, et al., 2014; Martin & Verheggen, 2018b). The SPME is mainly used as a qualitative method to characterize the compounds that are present in a volatilome and for cross-sample comparison. Quantification of complex samples requires laborious operations and is not often performed (Tholl et al., 2006). The stationary point observed on the RSM curve highlights appropriate conditions to sample dissolved cadaveric VOCs in water.

Testing the DI-SPME sampling

Under the selected conditions (time = 10 min; temperature = 27.5 °C; stirring rate = 250 rpm), the number of compounds trapped on the fiber almost doubled. The quantity of both indole and DMDS also increased with the new methodology. In total, 13 cadaveric compounds were identified after optimization instead of seven during pretests. The dissolved VOC profile is poor, when compared with the large lists of VOC collected in the headspace of drowned bodies (Irish et al., 2019) Among the possible explanations: the present experiment was performed with small amounts of water, under controlled environement, and rats were used as surrogate human cadavers. Unlike pigs, rats ar not the most appropriate to predict human decomposition (Rosier et al., 2015, 2016). Further tests performed outside laboratory settings should be carried out.

Conclusions and recommandations

In this study, we have shown that DI-SPME can be applied to characterize dissolved cadaveric compounds. After some optimizations, we managed to increase the number of VOCs directly collected from water, even though their concentrations in water were very low. This method should be optimised using a larger diversity of cadaveric compounds and a larger number of replicates. Other vertebrate species should be considered, since each species release specific compounds during their decomposition (Rosier et al., 2015, 2016). We are convinced that some cadaveric VOC remain dissolved in water and are therefore not released at the water surface, their identification remains to be done. Also, the method remains to be tested during all decomposition stages. Finally, water composition (saline water, river water, lake water) is expected to impact the decomposition and the released VOCs.

Chapter 4

Human smell of death

Introduction

Despite progress in the analysis of cadaveric VOCs from vertebrate remains, studies addressing the volatilome of human cadavers remain scarce. Among the limitations frequently encountered in the existing literature are the small sample size and the high variability in terms of investigated stages of decomposition. This explains the contrasting result observed in the literature investigating cadaveric VOCs released by human remains. In addition, most of them investigated the cadaveric profile released by body parts or gravesoil (Dubois et al., 2018; Dubois, Hugues, et al., 2019; Rosier et al., 2015, 2016; Vass et al., 2004). Finally, freshly deceased people has received less attention (Statheropoulos et al., 2007). During this stage, bidimensional gas chromatography has been recommended to highlight compounds that are released at a trace level (Dekeirsschieter et al., 2012). Indeed, this technic allow to decrease the baseline effect (Perrault, Nizio, et al., 2015).

This chapter investigates the potential of classical gas chromatography to decipher early cadaveric VOCs, by using a target ion methodology.

All equal in the face of death! – Volatile cadaveric compounds of human corpses in fresh stage.

This section is an adapted version of the article:

Martin C and Verheggen F (under review) All equal in the face of death! – Volatile cadaveric compounds of human corpses in fresh stage. Forensic Chemistry

Abstract

The smell associated with the decomposition of a human body has been the subject of a limited number of studies, most using body parts or other vertebrates as surrogate human models. Among the limitations frequently encountered in the existing literature are the small sample size and the high variability in terms of stages of decomposition. In the present study, we collected, identified and quantified the volatile organic compounds released by 20 human corpses at the fresh stage, using dynamic headspace collection. We also assessed the impact of some parameters on the volatilome: skin temperature, gender, age, size, postmortem interval, presence of lividities or rigidities. We found 2-heptanone to account for nearly half the scent of fresh human cadavers. Four additional compounds were also repeatedly identified: dimethyl disulfide, ethyl acetate, limonene and 3-methyl-1-butanol. The use of target ion method has allowed to increase the diversity of collected postmortem molecules, compared to previous works. However, no human specific markers were found, either because they do not exist at the fresh stage, or because they are released at trace level. Finally, none of the tested parameter impacted the volatile profile of human corpses. We recommend performing similar assays on more advanced stages to assess this target methodology.

Introduction

After death, a corpse undergoes the combined action of autolysis and putrefaction, leading to drastic physical and chemical changes. Macromolecules are degraded, resulting in the emission of hundreds of different volatile organic compounds (VOCs) (Martin and Verheggen 2018). The characterization of the smell released by decaying vertebrate is a recent research topic (Agapiou et al., 2015; Armstrong et al., 2016; Dekeirsschieter et al., 2009; Forbes, Perrault, et al., 2014; Irish et al., 2019; Martin et al., 2019; Nizio et al., 2017; Perrault et al., 2014). But we now have a good understanding of the biotic and abiotic factors impacting cadaveric volatilome, which include weather conditions, presence of necrophagous insects, soil types and decomposition stages (Dekeirsschieter et al., 2009; Forbes, Perrault, et al., 2014; Forbes, Rust, et al., 2014; Martin et al., 2019; Martin, Maesen, et al., 2020; Rosier et al., 2015). Pigs have mainly been used as surrogate human models, while human cadavers were only sampled in a handful of studies (Degreeff & Furton, 2011; Deo et al., 2020; Dubois et al., 2018; Dubois, Stefanuto, et al., 2019; Forbes, Perrault, et al., 2014; Hoffman et al., 2009; Perrault et al., 2017; Rosier et al., 2014, 2015, 2016; Statheropoulos et al., 2005, 2007; Ueland et al., 2021; Vass, 2012; Vass et al., 2004). Moreover, most of these studies include limited sample size, and deal with body parts or gravesoil, instead of complete human corpses.

Based on physical changes, five stages of decomposition have been defined. The first one is named ‘fresh stage’: it starts immediately after death and ends with the first signs of body inflation (Dekeirsschieter et al., 2009; Janaway et al., 2009b). VOCs released during the fresh stage have received limited attention (Degreeff & Furton, 2011; Statheropoulos et al., 2007). That initial stage is renown to be the poorest in terms of quantity and diversity of VOCs (Dekeirsschieter et al., 2009; Forbes, Perrault, et al., 2014; Iqbal et al., 2017; Perrault, Nizio, et al., 2015; Verheggen et al., 2017). However, the characterization of cadaveric VOCs released by recently deceased individuals will find applications during search and rescue operations (Statheropoulos et al., 2007). It would also help improving our understanding of cadaveric VOC genesis.

This study highlights the potential of classical gas chromatography to investigate early cadaveric VOCs, by using a target ion methodology. Skin temperature, *postmortem* interval (PMI) and the presence of lividities and rigidities were considered to identify their impact on the emission of specific VOCs at the very beginning of the decomposition process. Some intrinsic parameters (*e.g.*, age, size and gender) were also assessed.

Material and methods

Location and presampling procedure

All samplings were conducted in a morgue located in Gilly (Belgium), under the authority of the local medical examiner (Dr. Duverger). Between November 2019 and February 2020, we were informed about the presence of at least one new corpse. Upon our arrival, the corpse -laying on a metal trolley- was taken out of the cold room (4°C) it was kept in and placed in a room at 19°C for exactly 2 hours. Then, it was introduced in the autopsy room (previously cleaned, aerated, set at 19°C) where the sampling took place. The cadaver was provided to us inside a white plastic body bag (230 x 80 cm).

Volatile compounds sampling

To perform the VOCs sampling, we opened the zipper of the body bag a few centimeters and introduced a hydrophobic TenaxTa/Carbograph thermodesorption tube (Markes International, Llantrisant, UK). We sampled the headspace air directly inside the body bag using an air flow set at 200 mL/min (GilAir1 plus pump, Sensidyne1, St. Petersburg, Florida, USA). After 10 min of sampling, we sealed the tubes and stored them in a fridge at 4 °C to avoid desorption of the compounds. Clean empty body bag sampling was also performed each day as a control.

While we had no access to the person's identity, we were then allowed to collect the following descriptive data from the corpse: gender, age, size, skin temperature, decomposition stage, presence of rigidities and lividities, and PMI (all data were collected from the corpse after VOC sampling). Skin temperature was measured with an infrared thermometer (Etekcity®) close to the waist. Rigidity was estimated by moving the wrist join: in case no movement was allowed, the rigidity was considered present; if the join could move, then we estimated if the rigidity has ended or has not yet started based on the PMI (Janaway et al., 2009b). PMI was provided by the morgue manager.

Only corpses in the fresh stage were considered for the present study, for a total of 20 cadavers (Table 12). All 20 persons deceased inside a building. No consent was needed since our sampling was non-invasive and was exclusively performed on the VOCs leaving the corps.

Table 12 Cadavres information

Cadaver	Temperature	Rigidity	Lividity	Size (cm)	Age (year)	Sex	PMI (day)*
C1	18.5	A	A	186	60	M	0
C2	8.9	C	A	180	62	F	36
C3	9.5	A	A	174	64	F	0
C4	22.8	A	A	177	46	M	0
C5	24.5	B	B	177	71	M	0
C6	12.5	B	A	180	44	M	2
C7	29.1	B	A	1.8	60	M	0
C8	19	A	B	158	92	F	0
C9	13.1	B	B	175	na	M	1
C10	12.2	B	A	187	45	M	1
C11	7.2	B	A	160	76	F	11
C12	14.6	B	A	1.89	70	M	1
C13	6.3	C	A	1.84	58	M	5
C14	4.5	C	A	180	82	M	10
C15	3.8	C	A	1.84	47	M	15
C16	4.1	C	A	1.95	na	M	340
C17	na	A	A	167	33	M	9
C18	12.6	B	A	167	33	F	1
C19	8.8	A	A	90	63	M	10
C20	3.8	B	A	184	35	M	11

*Rigidity (before: A, present: B, after: C), Lividity (present: A, Absence: B); postmortem interval (PMI); na value are unknown. *All corpses deceased for more than 1 day were kept in a cold room since death.*

Gas chromatography analyses

Sample analyses were carried out using a gas chromatograph coupled with a mass spectrometer (QP 2020 NX, Shimadzu, Kyoto, Japan) and a thermodesorber (TD30R, Shimadzu, Kyoto, Japan). Compounds were first desorbed at 280°C for eight minutes prior to be cryofocused by Peltier effect in a glass tube set à -20°C. The trap was then warmed up to 270°C and injected on the column head with a split ratio of 20. Compounds separation was performed on a Rtx-5/ms capillary column (30 m x 0.25 mm x 0.25 mm; Agilent technology; Santa Clara, California, USA). The oven was set to an initial temperature of 40°C. The temperature increased to 100°C with a ramp of 2°C/min and then ramped to a final temperature of 300°C at a rate of 10°C/min. Helium was used as carrier gas at a constant flow rate of 1.9 $mL.min^{-1}$. The mass spectrometer interface temperature was held at 230°C and the ion source was set at 200°C. Mass spectra were collected from 35 to 500 m/z with a data acquisition time of 0.3 s. The detector voltage was set during the tuning of the mass spectrometer at 1.1 kV.

Data processing

To highlight cadaveric compounds, a post run single ion monitoring (SIM) was performed on each chromatogram. When a targeted compound was found, a specific integration method was created. We therefore obtained as many methods as targeted compounds. These methods were then applied on all chromatograms to identify the targeted compounds. These methods focused on four criteria: retention time and relative abundance of three ions. The target compounds were those gathered in Martin & Verheggen (2018). For each compound, the higher fragment of their mass spectrum was targeted using "fragment search table" in the Shimadzu postrun software.

All statistical analyses were performed using R version 3.4.2. (R Foundation for statistical computing). To evaluate the impact of the studied factors (Table 12), we conducted a permutational multivariate analyses of variance (i.e., perMANOVA) using an Euclidian distance matrix and 999 permutations ("adonis" command, R-package vegan, (J. F. Oksanen et al., 2020)). We adjusted *p*-values using Bonferroni's correction to avoid increases in type I error due to multiple testing. Homoscedasticity and normality were checked on each parameter prior the application of the permMANOVA. Regarding cadaver size, skin temperature, PMI and age, two classes were compared by using the median of the sample, since the median is not impacted by extreme values.

Results and discussion

A total of 23 cadaveric compounds were identified after removing background compounds (Table 13). The early fresh stage is usually assimilated with no cadaveric odor, especially when analyzed with a single dimension gas chromatography (Dekeirsschieter et al., 2009). Bidimensional gas chromatography allowed the identification of 20 compounds released from one-day postmortem piglet carcasses (Dekeirsschieter et al., 2012). The use of targeted methods in one-dimension gas chromatography, therefore, helps to reach the sensitivity of the bidimensional one on cadaver experiencing the fresh stage.

A single volatile compound, 2-heptanone, accounted for nearly half the volatilome of fresh human cadavers (Table 13). This molecules has been identified previously from fresh vertebrate remains (Cablk et al., 2012) and has been associated with the decomposition of several human tissues (*e.g.* muscle and skin) (Hoffman et al., 2009; Statheropoulos et al., 2005). Ethyl acetate is the second most abundant volatile (accounting for 16% of the collected blend) and was previously identified on putrefied human bodies (Degreeff & Furton, 2011; Rosier et al., 2014; Statheropoulos et al., 2005) as well as on surrogate human models (Dekeirsschieter et al., 2012; Perrault et al., 2014). The other volatile compounds are released in much smaller quantities (<1%

of the blend).

Only five of the 23 identified compounds were found in more than half of the sampled corpses (Table 13): dimethyl disulfide; 2-heptanone; ethyl acetate; limonene; 3-methyl-1-butanol. None of these compounds were identified during a previous assay performed in a morgue (Degreeff & Furton, 2011). However, a scent transfer unit was used in that previous study and, according to the authors, this sampling device led to high variability across replicates. Also, the sorbent material used as a trap has specific affinities with some cadaveric molecules (according to molecular weight and polarity), as previously demonstrated with solid phase microextraction (Perrault et al., 2017). We have probably collected and identified more compounds than previous works thank to the use of thermodesorption tubes, which were already validated on vertebrate remains (Rosier et al., 2014). Unfortunately, none of the compounds we identified in the present study are human specific markers. These markers have been highlighted later during the decomposition process (Rosier et al., 2015, 2016). If any of these markers are released during this early stage of decomposition, they were released at the trace level, below our limit of detection.

Table 13 List of identified cadaveric compounds from 20 human corpses experiencing the fresh decomposition stage.

Chemical name	Occurrence (# corpses)	Mean	±	SD	Percentage
Sulfide					
dimethyl disulfide	18	99125.6	±	53434.9	4.4%
dimethyl trisulfide	4	547.4	±	278.7	<1%
Alkanes					
unidentified C7 alkane	8	79206.3	±	62893.9	3.5%
2.4-dimethylheptane	1	126.0	±	126.1	<1%
Ketones					
2-pentatone	3	9313.4	±	7100.6	<1%
2-hexanone	1	129282.9	±	129282.9	5.8%
2-heptanone	18	1063418.6	±	175965.0	47.9%
Aldehydes					
hexanal	3	21488.0	±	21032.2	1.0%
heptanal	4	5187.6	±	4623.5	<1%
octanal	2	385.9	±	278.0	<1%
nonanal	1	20637.1	±	20637.1	1.0%
benzaldehyde	1	4830.9	±	4830.9	<1%

Alcohols					
2-methyl-1-propanol	7	14719.5	±	7474.6	<1%
3-methyl-1-butanol	16	27113.4	±	13785.4	1.2%
benzyl alcohol	1	62003.5	±	62003.5	2.8%
Aromatics					
p-xylene	3	8058.1	±	4831.9	<1%
phenol	2	95867.0	±	78724.3	4.3%
toluene	7	174245.4	±	118602.1	7.8%
limonene	12	27717.0	±	21312.0	1.2%
styrene	3	2321.8	±	1762.9	<1%
Furans					
2-pentyl-furan	4	4526.3	±	3870.6	<1%
Esters					
ethyl acetate	10	360281.2	±	155838.3	16.2%
butyl butyrate	9	7910.6	±	4704.7	<1%

None of the studied factors had a significant impact on the human cadaveric volatilome (Table 14). We expected the presence of lividity as well as occurrence of rigidity to modify the volatile emissions of a corpse. Indeed, these first postmortem modifications highlight the evolution of the decay. Lividities result from the discoloration of blood due to a depletion of oxygen. Rigidities are caused by the breakdown of adenosine triphosphate and lead the muscle to reach an irreversible state of contraction. The muscle relaxation occurs when alkaline liquid from putrefaction are released (Janaway et al., 2009b). These two postmortem changes were thus expected to have an impact on the cadaveric volatilome. We raise the hypothesis that the abundance of sulfur compounds should quickly change over time after death. Sulfur compounds are byproducts of amino acids (main components of both blood and muscle) degradation (methionine, cysteine) (Paczkowski & Schutz, 2011).

Surprisingly, we could not show the impact of PMI on the emission of VOCs. This is probably because corpses with higher PMI were directly stored after the deceased. Hence, all corpses stood at the early fresh stage. The storing sufficiently slowed the decomposition process, to avoid the release of additional cadaveric compounds, as already highlighted in previous studies conducted on human blood (Forbes, Rust, et al., 2014). In addition, the diversity of samples probably explains the absence of statistical difference: very few corpses had a long PMI. The same argumentation applies for the temperature of the skin.

Regarding intrinsic parameters, no differences were highlighted. We expected an

impact of the age of the deceased person. Indeed, elderlies release specific compounds, including dimethylsulphone, benzothiazole and nonanal (Gallagher et al., 2008). Here, only nonanal was detected, and only in one corpse. No differences were shown among male and female cadavers. Our sample included only six women, all being over 50 years old, while male cadavers belonged to more varied age groups. More standardized samples could help to confirm the absence of effect. Regarding the impact of the size of the cadaver, we recommend evaluating body mass index (BMI) instead of body length. BMI gives a good indication of the ratio of macromolecules in a body, including proteins, lipids, carbohydrates. BMI could therefore be a good candidate to sort out the available corpses, especially because each tissue/organ releases its own VOCs profile while decomposing (Dubois et al., 2018).

Table 14 Statistical values for studied factors

	lividity	**rigidity**	**PMI**	**skin T°**	**sex**	**age**	**size**
classes limit	na	na	2 days	9.5 °C	na	61 years	167 cm
F value	1.75	0.699	0.992	1.201	0.466	0.883	0.614
Df	1,19	3,19	1,19	1,18	1,19	1,17	1,19
p-value	0.101	0.797	0.44	0.278	0.978	0.551	0.869

Conclusions

Our study has been conducted on a large sample size, with standardized decomposition stage and sampling methodology. Targeted ion methodology coupled with a single dimension GC-MS separation allowed the identification of 23 cadaveric volatile compounds. We found that neither age, size, gender, PMI impact the volatilome at the fresh stage. We recommend using standardized method and homogenous samples to investigate the impact of specific parameters. Performing a similar assay on more advanced stages of decomposition would be extremely informative but remains challenging in terms of sample access and size.

Chapter 5

Human remains detection dogs: from performance to perception

Introduction

Human remains detection dogs are powerful assets to locate decaying human bodies. They should be able to signal entire bodies as well as body parts or fluids (Rebmann & David, 2000). Based on the selection performed by humans, breeds used for detection tasks show accurate, sensitive and reliable olfactory abilities (Riezzo et al., 2014). Despite the importance of such detection dogs, poor information is available in the literature that focus more on the characterization of cadaveric odor profile in different conditions than on detection dogs' work (Cablk et al., 2012; Hoffman et al., 2009; Knobel et al., 2018; Rosier et al., 2016). Even though gathering information on the volatiles released by cadavers is crucial to improve detection dogs' efficiency, their detection abilities should receive more attention. Up to now only applied detection dogs' interventions had been studied (*e.g.*, cancer, covid, explosive, endangered species' detections) (Cablk & Heaton, 2006; Concha et al., 2014; Hackner & Pleil, 2017; Hag-Ali et al., 2021). Both scientists in the field and detection dogs' handlers claim to better understand what makes an efficient detection dog.

In this chapter, we assess the human remains detection dogs' abilities. First of all, we aimed to define what an efficient detection dog is, and to develop a quantitative method to assess their performance. Finally, we investigated the perception of human remain detection dogs by highlighting cadaveric olfactory cues inducing the positive response of dogs during location tasks.

What a good boy! Deciphering the efficiency of detection dogs

This section is an adapted version of the article:

Martin C, Willem N, Desablens S, Menard V, Tajri S, Blanchard S, Brostaux Y, Verheggen F and Diederich C (2022) What a good boy! Deciphering the efficiency of detection dogs. Frontiers in analytical science, DOI: 10.3389/frans.2022.932857

Abstract

Dogs have a powerful olfactory system, which is used in many areas of the police and military to detect drugs, human remains, and explosives, among other items. Despite these powerful detection abilities, methods assessing the performance (MAP) of dogs remain scarce, and have never been validated. In particular, scientific knowledge on post-training performance assessments is scarce. To validate a quantitative MAP, an efficient detection dog (DD) must first be defined. Here, we aimed to define what an efficient DD is, and to develop a quantitative MAP. Specifically, we conducted (i) an international survey sent to professional DD practitioners (n = 50), and (ii) an experimental assay on human remains and drug DDs (n = 20). Based on the survey, efficient DDs were defined as confident animals, making few mistakes, alerting to the presence of target odors as close as possible, able to strategically screen the search area effectively, independent and not easily distracted. The developed quantitative MAP was based on video tracking DDs in a circular behavioral arena, in which the error rate of DD was recorded, including accuracy and the strategy level. Previous studies have already demonstrated that DDs are usually confidant. Guidance was not assessed during MAP development; however, handlers could not guide DDs during the search session. Based on this method, future studies should evaluate DD efficiency throughout the entire training process. Such monitoring would allow thresholds to be determined, allowing efficient DDs to be identified, along with the effect of certain factors on performance (*e.g.*, dogs breed, gender, and training aids used during DD conditioning).

Introduction

Dogs are efficient scent detection animals. Their powerful olfactory system and their ability to be trained explain why they are used in so many police and military applications as bio-detectors (*e.g.* drug, human remains, explosive) (Hackner & Pleil, 2017; R. J. Harper & Furton, 2007; Jezierski et al., 2014; Martin, Diederich, et al., 2020; Polgár et al., 2016; Quignon et al., 2012). Despite their high potential, low performance is sometimes observed in detection dogs (DDs), which might be due to a lack of knowledge on their neurophysiology and related behaviors (Beebe et al., 2016; Jamieson et al., 2017; Lazarowski et al., 2020). An operational DD is the result of several factors: (i) selecting a promising puppy, (ii) conducting efficient training, and (iii) continuously assessing the dog's working performance (Beebe et al., 2016; Martin, Diederich, et al., 2020; Polgár et al., 2016; Thrailkill et al., 2018). However, current methods used to assess the performance (MAP) of dogs are limited, and require validation. Here, we aimed to fill this knowledge gap.

Puppies are generally selected based on both anatomical and behavioral traits. Cranial anatomy and ability to thermoregulate are good biological indicators for selecting breeds to optimize odor perception by avoiding panting behavior which divert the inhaled air to the respiratory tract instead of the nose. Individual dogs are selected based on temperament. For instance, a social, cooperative, and confident puppy with a particular motivation to be trained (usually observed by a high reward drive) is preferred by handlers (Beebe et al., 2016; Gazit & Terkel, 2003; Hussein et al., 2012; Jamieson et al., 2017; Martin, Diederich, et al., 2020).

The second factor for producing an operational DD is the type of training that is implemented, particularly the olfactory stimuli used (termed training aids). Training aids must be representative of the target odor to avoid false identification (Martin, Diederich, et al., 2020; Porritt et al., 2015). While training methods are typically based on positive reinforcement (operant conditioning), training aids are diverse, with limited investigations on how they impact DD performance. Three types of olfactory training aids are commonly used: genuine materials, pseudo-scents, and non-pseudo alternatives. Genuine material is the target odor itself, and is the recommended aid (Simon et al., 2020). Pseudo-scents are synthetic aids that, ideally, mimic the smell of the genuine material (Stadler et al., 2012; Tipple et al., 2014). Non-pseudo alternatives include dilution, encapsulation, ad/absorption, or extraction of the targeted odor (Simon et al., 2020). The representability of pseudo-scents had been questioned (Stadler et al., 2012), while non-pseudo scent alternatives require validation (Simon et al., 2020).

Assessing the performance of working dogs allows the evolution of their field

efficiency to be followed, and to correct for errors. However, scientific knowledge on post-training performance assessment remains scarce (Brady et al., 2018; Lazarowski et al., 2020). Most studies assessed DD performance by measuring the duration (speed) and precision (number of false positives) of a task (Bennett et al., 2020; Jamieson et al., 2017; Jezierski et al., 2014). However, these approaches are not based on any definition of what is considered as an efficient DD. This raises questions about the validity of these measures to assess DD performance. Hence, there is a need to define what an efficient DD is, to allow the development of a validated quantitative MAP (Johnen et al., 2013).

Thus, here, we aimed to define what an efficient DD is, and to develop a quantitative MAP. To accomplish this, we conducted (i) an international survey sent to professional DD practitioners (n = 50), and (ii) an experimental assay performed on human remains and drug DDs (n = 20). Our results are expected to help define what an efficient DD is, and generate a baseline quantitative MAP.

Material and methods

Survey

A survey (LimeSurvey®) containing six questions based on empirical information (DD handlers in Belgium) and scientific literature (Table 15) was compiled. This survey was sent to the international police network for law enforcement dog professionals (Kynopol). The agency then forwarded the survey to all affiliated DD brigades globally. Twenty-five handlers and 25 brigade supervisors from Europe answered the survey between March 30 and April 27, 2021.

Quantitative MAP

Four human remains DD-handler teams (sex of dogs: male; breed: Springer spaniel) and 16 drug DD-handler teams (sex of dogs: nine males and seven females; breed: six Springer spaniels, eight Belgian shepherds, and two German shepherds) were used to validate a new quantitative method to assess performance (MAP) (Figure 18). All teams belonged to the Belgian federal police DACH (canine support direction). DDs belonged to operational police forces, and had between 2 and 6 years of experience, with the exception of four dogs (one year of experience). All dogs received training three times a week. Except for the one-year-operational DDs (for which no statistical analyses were available), drug DDs had performed 165 ± 35 police operations per year, while human remains DDs had performed 13 ± 3 police operations per year.

Each dog-handler team participated to three sessions, separated at one-month intervals. These sessions were considered by handlers as one of their dog's weekly

training sessions. Each session included 10 trials of target odor tasks in an arena (6 m × 8 m) kept at 20 °C. One session lasted about 2 h per dog. Between sessions, the room was naturally ventilated for 1 h, and the floor was cleaned with soap (Dettol®) to remove all odors from previous sessions. Twenty-five cinderblocks were placed in half a circle (d = 6 m, Figure 18). An empty plastic cup was placed in each cinderblock. In eight of the 10 trials, four distractive odors and one target odor (see odor section) were randomly placed in some of the 10 cups using "alea" function in excel. All other cups were filled with blank gauzes. Two control trials were included in each session: distractive odors only were dispatched in the blocks for Trial 1, and at random in one of the other nine trials. Handlers were advised that the first trial was a control. This first control was performed to confirm that the arena was not contaminated by previous assays. Regarding the second control, we advise handlers that some control searches were implemented through the session. However, they were not aware of the number of controls per trial neither when the control was performed to have a double-blind assay. To avoid cross contamination, the experimenter changed gloves between the placement of distractive odors and target odor in the arena. To prevent the experimenter smell providing a clue for the dog to reach the target odor, all the blocks were touched with one hand without gloves before the beginning of the trial.

Table 15 Survey sent to DD handlers belonging to the Kynopol network. For each question, a grade from 1 (strongly disagree) to 10 (totally agree) had to be given.

QUESTIONS
Q1 The faster a detection dog finds its target, the better it performs.
Q2 The more strategically a detection dog looks for its target, the better it performs. (The dog always proceeds in the same way; it does not look for the target randomly, but seems to follow a structured search procedure. For example, the dog always screens the area from the left to the right.)
Q3 The more errors in detection (*i.e.,* false negatives) a detection dog provides, the less successful it is. (False negatives = the dog does not mark the target, even though it is present).
Q4 The more errors in detection (*i.e.,* false positives) a detection dog provides, the less successful it is. (False positives = the dog marks when no target is present or at a distance higher than 15 cm from the target when present).
Q5 The more accurate a detection dog is, the better it performs. (The dog's accuracy is defined as the ability of the dog to mark as close as possible to the target as possible).
Q6 The more a detection dog needs to be guided, the less successful it is. ("to be guided" means that the handler has to indicate the direction to the dog by verbal and/or physical encouragement. In this case, the dog does not screen the area by itself, and its handler has to do a systematic search to be certain that the entire area is correctly checked).
Q7 The more confident a detection dog is, the better he performs. (The dog's confidence is defined as its ability to mark the target on the first pass without having to repeatedly pass it).

Each trial was initiated by placing the dog at an equal distance from each odor source (Figure 18). A Go-Pro camera, hanging from the ceiling (h = 3 m) was used to record how dogs worked. The Go-Pro was directly connected to a video monitor outside the arena (hidden experimenter), allowing live observation. To avoid the "clever Hans"

effect" (Sebeok & Rosenthal, 1981), the handler did not know whether the test was a control or target search. The handler was allowed to stay next to their dog to give commands, but was not allowed to guide the dog by pointing out any area of the arena. The signal to end the trial was given by the handler, by orally indicating the presence or absence of a target odor to the experimenter, as well as its position, if applicable. If the position was correct, the handler stopped the search and rewarded the dogs, if not, the search was stopped without rewarding dogs.

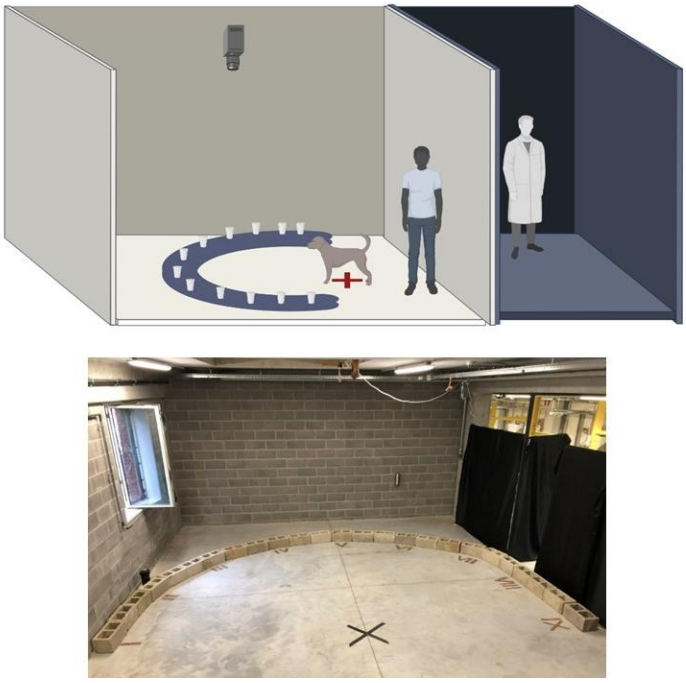

Figure 18 Behavioral arena formed of half circle cinderblocks, in which plastic cups were placed containing distractive and target odors. The dog started the odor search trial at an equal distance from each cinderblock. The handler stayed next to the detection dog (DD) to provide comments (motivation and starting), but was not allowed to guide the dog by pointing out any blocks. The experimenter stayed outside the arena to avoid the "clever Hans" effect, and followed the dog's behavior on a screen linked to the camera placed above the arena.

Odor

Four distractive odors were used for the quantitative MAP; namely, chocolate, toilet spray (Air Wick® aerosol, pure spring dew), fresh basil, and coffee. A week before a session, 20 sterile gauzes per odor were placed in a 2 L glass bottles containing 20 g chocolate, 20 g fresh basil, 20 g ground coffee, or was sprayed for 20 s with an odor spray. The bottles were then kept sealed at ambient temperature. The target odor for

human remains DDs, provided by DD handlers, was made of paperclips that had been placed 1 month before each session in a plastic container with decaying human tissue. Paperclips were used as odor carriers for decaying human smell because it allows to trap a small among of odor to avoid to saturate the arena with the decaying smell. The target odor for drug DDs was 1 g of pure heroine, provided by the drug detection canine brigade supervisor placed in a plastic zipper bag.

Data collection

Video recordings were analyzed with tracking software (Ethovision®, Noldus®, Bejing China). Collected variables included: search duration, target frequency, zero angle, target angle, eye contact, previous angle, previous target angle, number of false negatives, number of false positives, distance of search (see Table 16 for descriptions of the variables). We hypothesized angles could be indicators of strategy specially if dogs always use same angles through searches.

*Table 16 Descriptions of the variables collected per trial from video recordings of the arena. Ten trials of odor search were conducted across sessions. (Parameters identified by * are presented in Supplementary figure 1).*

Measured parameters	Definitions
Target frequency	Number of times the dog walked next to the target (max. 50 cm)
Search duration	Time between the handler search command and detection of the target by the dog
Distance of search	Distance the dog roamed between handler search command and detection of the target
Number of false positives	Number of trials (including control) the dog responded to an untargeted odor or at a distance >15 cm around the target.
Number of false negatives	Number of trials in which the dog did not signal a target odor while present.
Zero angle*	Angle between the starting direction of the dog's body and the extreme left of the arena
Target angle*	Angle between the starting direction of the dog's body and the position of the target
Previous angle*	Angle between the starting point of the trial and the starting point of the previous trial
Previous target angle*	Angle between the starting point of the trial and the target angle of the previous trial
Eye contact	Number of times the dog looked to its handler per trial

Qualitative MAP by handlers and brigade supervisors

Information on dogs was collected through interviews with handlers and two brigade supervisors (one supervisor for human remains DDs and one for drug DDs) prior the quantitative MAP took place. The supervisors were asked to grade the performance of their dogs during police operations (Table 17).

Table 17 Evaluation of detection dog (DD) operational performance by handlers and brigade supervisors. For each question, a graduation from 0 (no) to 10 (absolutely) was used.

Criteria	QUESTIONS of the survey
Strategy	Q1 Does the dog follow a particular strategy when performing a search?
Efficiency of the strategy	Q2 Does the strategy seem efficient?
Guidance	Q3 Does the dog need to be guided by the handler during the search?
False negative level	Q4 Is the dog predisposed to produce false negative answers?
False positive level	Q5 Is the dog predisposed to produce false positive answers?
Sensitivity to low concentration	Q7 Is the dog able to find low concentrated target odors?
Sensitivity to high concentration	Q8 Is the dog able to find high concentrated target odors?
Rapidity	Q9 Does the dog quickly find the target when it is present?
Search efficiency	Q10 Does the dog have a good search level?
Ranking	Q11 What is the rank of the dog's performance among DDs?

Statistics

(i) Survey and Qualitative MAP: Unpaired Student's t-tests ["t.test" command, R-package: stats, R studio® (ver. 1.0.3)] were used to compare the responses of surveyed international handlers and brigade supervisors. In addition to ranking different criteria in terms of importance, ANOVA was performed. (ii) Quantitative MAP: To test for consistency between the three sessions of each DD, a PermMANOVA was performed on behavioral measures, including a type-one Bonferroni correction and 999 permutations ("adonis" command, R-package vegan, (J. Oksanen et al., 2017), R studio). The results of the PermMANOVA were illustrated using principal compound analysis (PCA) ["PCA" command, R-package: FactoMineR, R studio® (ver. 1.0.3)]. If no differences between sessions were detected, the mean and variance of each variable were calculated, except for false positive and false negative numbers, which were added together to obtain a global error rate. (iii) Co-inertia analysis (COIA) ["coinertia" command, R-package: ade4, R studio (ver. 1.0.3)] was selected to enhance correlations among qualitative and quantitative MAPs (Dolédec & Chessel, 1994). To perform the COIA, a standard multivariate analysis as PCA was first computed on each MAP data set: qualitative MAP ($PCA_{handlers\ MAP}$ and $PCA_{supervisors\ MAP}$) and quantitative MAP ($PCA_{quantitative\ MAP}$) ["dudipca" command, R-package: ade4, R studio® (ver. 1.0.3)]. This method provided three pairs of axes: $PCA\ (D_1, D_2)_{handlers\ MAP;}$ $PCA\ (D_1, D_2)_{supervisors\ MAP}$ and $PCA\ (D_1, D_2)_{quantitative\ MAP}$. The COIA uses the first plan of two PCAs to identify common trends in both datasets (by selecting axes that maximized the square covariance). Hence, three COIAs were performed allowing pairwise comparisons between each PCA: (COIA 1: $PCA_{handlers\ MAP}$ *vs.* $PCA_{supervisors\ MAP}$, COIA 2: $PCA_{handlers\ MAP}$ *vs.* $PCA_{quantitative\ MAP}$; COIA 3: $PCA_{supervisors\ MAP}$ *vs.* $PCA_{quantitative\ MAP}$). The global correlation or co-structure of each COIA was measured by the RV coefficient (multivariate extension of the Pearson correlation coefficient), which ranged from 0 to 1, whereby a high RV-coefficient indicated a high degree of

co-structure (Robert & Escoufier, 1976). COIA 1 allowed us to compare the evaluation of dogs by handlers versus the brigade supervisors. COIA 2 and 3 allowed us to compare the handlers and brigade supervisors qualitative MAP using the developed quantitative MAP. When a trend was highlighted between two axes (PCA dimensions), the axes correlations (behavioral parameters of $PCA_{handlers}$ and $PCA_{supervisors}$, and measured data for $PCA_{quantitative\ MAP}$) were confirmed using a correlation test ["cor.test" command, R-package: stats, R studio® (ver. 1.0.3)] on variables explaining each axis.

Results

Survey

Twenty-five international DD handlers and 25 international DD brigade supervisors responded the survey about efficient DDs. The t-tests performed on each question (parameter) showed no difference in the responses of handlers versus supervisors: rapidity ($t_{47,205}$ = -1.121; *p*-value = 0.268), strategy ($t_{47,699}$ = -1.614; *p*-value = 0.113), false negative ($t_{41,979}$ = -1.786; *p*-value = 0.081), false positive ($t_{47,582}$ = -0.907; *p*-value = 0.369), accuracy ($t_{44,35}$ = -0.583; *p*-value = 0.563), confidence ($t_{43,912}$ = -1.729; *p*-value = 0.091), and guidance ($t_{47,258}$ = -0.096; *p*-value = 0.924). The survey parameters to assess performance were grouped according to their importance (F_6 = 91.04; *p*-value < 0.001; Figure 19): major importance (confidence, false positive, and false negative), medium importance (accuracy, strategy, and guidance) and minor importance (rapidity).

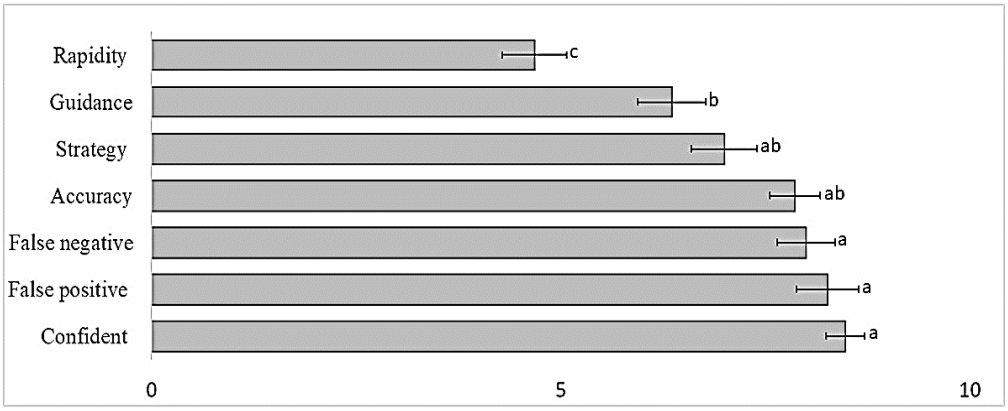

Figure 19 Importance of different criteria on the performance of detection dogs (DDs). These criteria were based on surveys completed by 25 DD brigade supervisors and 25 DD handlers. For a description of the criteria, see Table 1. More important criteria had higher ratings (modalities sharing the same letters are not different p-value > 0.05).

Quantitative methods assessing the performance accuracy

The means and standard deviations of each variable studied during the 490 trials were calculated and are presented in Table 18. When comparing recorded false positives and false negatives, DDs did not miss a target so often but could sometime mark area of the arena where the target is not present. Regarding the distance, dogs mostly focused their search around the cinderblock circle explaining the small distance covered in the arena during trials. DDs usually need to pass next to the target several time before to advise its presence to the handlers. During trials, the eye contacts with handlers are mainly observed when dogs mark the target instead of during the search time. Finally, no particular pattern was observed regarding the different studied angles. The PermMANOVA highlighted no difference in behavioral responses across the three sessions ($F_{2,57} = 1.44$; *p*-value = 0.122). Therefore, we can confirm that the accuracy of the quantitative MAP is respected.

Table 18 Means and standard deviations of each studied quantitative MAP variable

	S 1	***S2***	***S3***	***All sessions***
Search duration (s)	17.7 ± 1.1	18.1 ± 1.1	19.45± 1.5	18.42 ± 0.7
Distance of search (m)	21.1 ± 1.1	24 ± 1.36	23.6 ± 1.6	22.9 ± 0.8
Number of false positives	8	14	8	30
Number of false negatives	2	1	3	6
Eye contact	10.1 ± 0.1	12 ± 0.1	8.4 ± 0.2	10.14 ± 0.1
Target frequency	1.83 ± 0.1	2 ± 0.1	1.2 ± 0.1	1.9 ± 0.05
Zero angle (°)	66 ± 5	55 ± 3.8	44 ± 4	55.02 ± 2
Target angle (°)	25.6 ± 1.9	29 ± 4.2		26.51 ± 1.6
Previous angle (°)	48 ± 3.8	64.12 ± 4.1	57.1 ± 4	56.43 ± 2.3
Previous target angle (°)	69.5 ± 4	73.8 ± 4.3	75.1 ± 4.3	72.81 ± 2.38

Principal compound analysis analyses

To interpret the COIAs, each PCA was first described ($PCA_{\text{handlers MAP}}$, $PCA_{\text{supervisors MAP}}$ and $PCA_{\text{quantitative MAP}}$) (Figure 20). **The first factorial plane of the $PCA_{\text{handlers MAP}}$** explained 52.2 % of variation in the handler's evaluation. The five variables loaded on the first dimension (D1) were: Search efficiency, Sensibility to low concentration, False negative level, Ranking given by handlers, and Presence of a strategy (Table 18). The second dimension (D2) was explained by: Strategy, Sensitivity to high concentration, Efficacy of the strategy, and Rapidity (Table 18). **The first factorial plane $PCA_{\text{supervisor MAP}}$** explained 71.3 % of variation in the supervisor's evaluation (Figure 20). The five variables loaded on the first dimension were: Efficacy of the strategy, Strategy level, Search efficacy, Rapidity, and Level of

false positives. The second dimension was explained by: Guidance, Ranking, Sensibility to high concentration, Search efficacy, and Level of false positives. **The PCA $_{\text{quantitative MAP}}$** explained 56.7% of variability in dog behavioral parameters (Figure 20). The criteria loaded on the first dimension were: Variance of frequency, Mean distance, Variance of distance, Variance of search time, Mean of frequency, Mean of time, and Variance in eye contact number. The second dimension was explained by: Variance of starting point angle, Mean of previous angle, Variance of targeted angle, Number of false negatives, Variance of previous angle, Mean starting angle, Number of false positives, and Mean of search time.

Comparison of both qualitative methods assessing the performance (handlers and supervisors)

The COIA comparing the evaluation of dog performance by handlers versus brigade supervisors covered 86 % of the observed variability in both qualitative MAPs. Despite, similarities between both qualitative MAPs (RV = 0.31) no agreement between the two groups when assessing the performance of the same DD was observed. In fact, none of the qualitative criteria were correlated;(R^2= -0.47, $D1_{handlers}$ *vs.* $D1_{supervisor}$: t_{18} = 1.982; *p*-value = 0.063, $D1_{handlers}$ *vs.* $D2_{supervisor}$: t_{18} = -0.926; *p*-value = 0.366 and $D2_{handlers}$ *vs.* $D1_{supervisor}$: t_{18} = 0.565; *p*-value = 0.579) except one ($D2_{handlers}$ *vs.* $D2_{supervisor:}$ t_{18} = -2.220; *p*-value = 0.039). However, when investigating the variables explaining both second dimensions for handlers and supervisor qualitative MAP, no agreement was found for the same variable. Indeed, in a qualitative MAP comparison, the aim is to observe agreement for the same criterion of evaluation. In other word, handlers and supervisor have to be agree for the same questions ask during the qualitative MAP (Table17).

Quantitative MAP validation

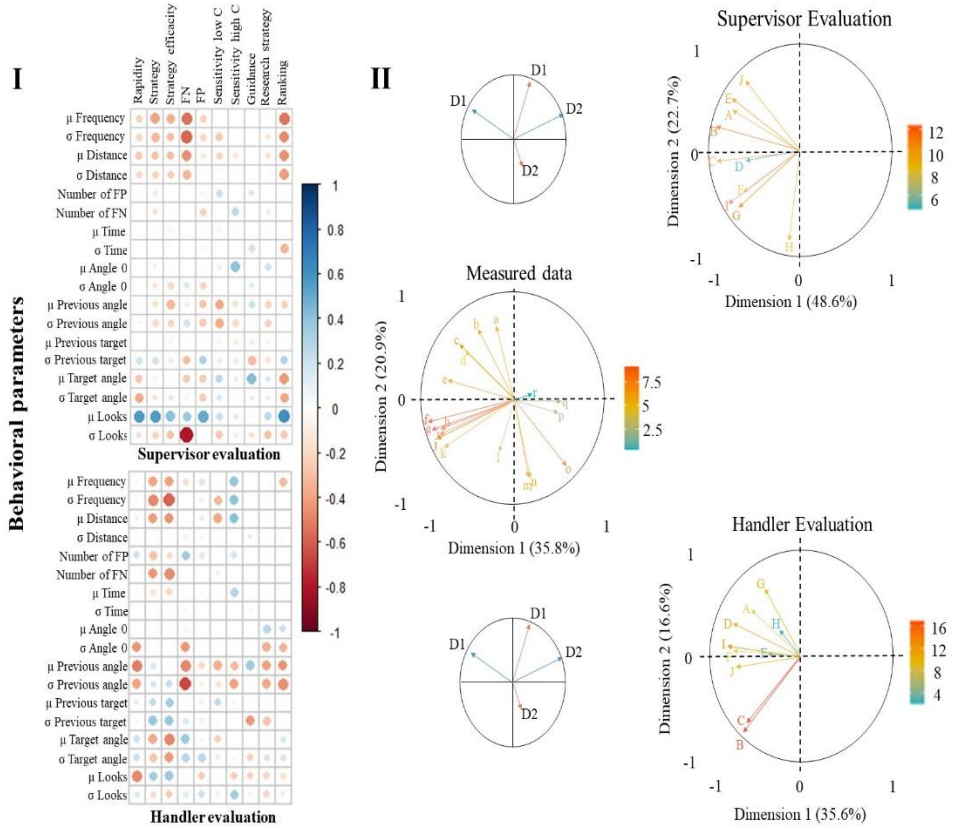

Figure 20 Coinertia analyses (I) including the correlation (II) between the evaluation of the handlers or supervisor (top right correlation circle). A: Rapidity; B: Strategy level; C: Strategy efficacity; D: False negative; E: False positive; F: Sensitivity to low concentration; G: Sensitivity to high concentration; H: Guidance; I: Search efficacity; J: Ranking. The data were measured during the behavioral tests (top left correlation circle). a: variance of angle between the start point and the target; b: number of false negative; c: number of false positive; d: mean of angle between the start point and the target; e: variance in eye contact; f: variance of frequency target zone visit; g: mean of distance; h: mean of frequency target zone visit; i: variance of search time; j: mean of search time; k: variance distance ; l: mean starting point angle ; m: mean precedent angle; n: variance starting point; o: variance previous angle, p: mean angle previous target; q: mean eye contact; r:variance previous target). The correlation between the dimensions of each correlation circle is represented in the two circles. Red: PCA dimension of the experiment; blue: PCA dimension of the evaluation of handler and supervisor, respectively. (The contribution of each variable is provided on the correlation circle)

The **COIA** **quantitative MAP *vs.* supervisors MAP** explained 79.5% of total variability observed in both MAPs. The similarities between both MAPs identified a low level of concordance (RV= 0.23), which was confirmed by the absence of correlation between variables of the two first dimensions of both PCAs (quantitative MAP and Supervisor

MAP). DD performance could not be assessed in the quantitative MAP, as supervisors assessed their performance during the qualitative MAP ($D1_{PCA\ supervisors\ MAP}$ *vs.* $D1_{PCA\ quantitative\ MAP}$: t_{18} = -1.10; *p*-value = 0.287, $D1_{PCA\ supervisors\ MAP}$ *vs.* $D2_{PCA\ quantitative\ MAP}$: t_{18} = -0.55; *p*-value = 0.589, $D2_{PCA\ supervisors\ MAP}$ *vs.* $D2_{PCA\ quantitative\ MAP}$: t_{18} = -0.27; *p*-value = 0.792 and $D2_{PCA\ supervisors\ MAP}$ *vs.* $D1_{PCA\ quantitative\ MAP}$: t_{18} = 1.07; *p*-value = 0.297). The **COIA** $_{\textbf{quantitative MAP VS handlers MAP}}$ explained 81.3% of total variability observed in both MAPs. The similarities between MAPs were slightly higher than those for $COIA_{quantitative\ MAP\ vs.\ Handlers\ MAP}$ (RV = 0.34; Figure 20). A correlation test was performed on both dimensions from the first plan of the $PCA_{quantitative\ MAP}$ and $PCA_{handlers\ MAP.}$ Only the second dimension of the qualitative MAP was correlated with the first dimension of the quantitative MAP ($D1_{PCA\ handlers\ MAP}$ *vs.* $D1_{PCA\ quantitative\ MAP}$: t_{18} = -0.57; *p*-value = 0.577, $D1_{PCA\ handlers\ MAP}$ *vs.* $D2_{PCA\ quantitative\ MAP}$: t_{18} = -1.22; *p*-value = 0.238, $D2_{PCA\ handlers\ MAP}$ *vs.* $D2_{PCA\ quantitative\ MAP}$: t_{18} = 1.20; *p*-value = 0.246 and $D2_{PCA\ handlers\ MAP}$ *vs.* $D1_{PCA\ quantitative\ MAP}$: t_{18} = -3.59; *p*-value = 0.002). Some criteria of the handler evaluation were correlated with some measures in the quantitative MAP. One such correlation was detected in $COIA_{quantitative\ MAP\ VS\ handlers\ MAP}$: $D2_{PCA\ handlers\ MAP}$ VS $D1_{PCA\ quantitative\ MAP}$ (t_{18} = -3.59; *p*-value = 0.002). To confirm this result, each variable from the first dimension of $PCA_{quantitative\ MAP}$ was tested with each criterion of evaluation from the second dimension of $PCA_{handlers\ MAP}$ using a correlation test. Six correlations were recorded: strategy level versus variance frequency (t_{18} = -2.32; *p*-value = 0.033; R^2 = -0.48), strategy level versus variance in the distance (t_{18} = -2.55; *p*-value = 0.020; R^2 = -0.51), efficacy of the strategy versus variance frequency (t_{18} = -2.94; *p*-value = 0.009; R^2 = -0.57); efficacy of the strategy versus mean distance (t_{18} = -2.32; *p*-value = 0.033; R^2 = -0.44); efficacy of the strategy versus variance in distance (t_{18} = -2.98; *p*-value = 0.008; R^2 = -0.57), and efficacy of the strategy versus variance in time (t_{18} = -2.46; *p*-value = 0.024; R^2 = -0.50). Thus, certain qualitative criteria in the evaluation used by handlers could be linked with quantitative measures in the quantitative MAP.

Discussion

This study identified and validated criteria for selecting appropriate detection dogs. Through our international survey, the defining of the performance of DDs were delineated in order of importance: major (confidence, false positives, and false negatives), moderate (accuracy, strategy, and guidance), minor (rapidity). As such, efficient detection dogs were defined as confident animals, making few mistakes, alerting to the presence of target odors as close as possible, able to strategically screen the search area effectively, independent and not easily distracted. Team supervisors and dog handlers had different opinions regarding the qualitative MAPs of tested DDs. Only the qualitative MAP of handlers was correlated with quantitative measures,

indicating that handlers were more reliable than supervisors in assessing DD performance. This difference was attributed to handlers knowing the strengths and weaknesses of their dogs better. This study provides new insights on how to define efficient DDs, and the reliability of the identified criteria as good predictors of performance.

The three criteria of **major importance** (confidence, false positives, false negatives) supported the published literature (Alexander et al., 2015; Cablk & Sagebiel, 2011; De Meester et al., 2008; Martin, Diederich, et al., 2020; Sinn et al., 2010). A dog's confidence is typically measured as a fear response to a stimulus; specifically, the more confident the dog, the less fearful (Beebe et al., 2016; De Meester et al., 2008; Fratkin et al., 2015). Hence, an efficient DD should express no fear (*e.g.*, shaking, salivation) when performing searches. Fearful DDs should be scarce, as dogs should trained based on a positive reinforcement method, which reduces undesirable behaviors, including fear (E. J. Blackwell et al., 2008; Hiby et al., 2004). A good indicator of fear is the eye contact avoidance even if no correlation was observed on our data (N. Rooney et al., 2009a). Eye contact can also be a good indicator of the intensity of the relation between the dog and the handler (Hare & Ferrans, 2021). Reduced number of errors (including false positives and false negatives) is also a major criterion (Alexander et al., 2015; Cablk & Sagebiel, 2011). Errors may result from unintentional handler cues promoting DD responses (Curran et al., 2010; Greatbatch et al., 2015), or inappropriate training aids (Hayes et al., 2018; Rice & Koziel, 2015; Tipple et al., 2014), inadequate and variable training and working methods (Lazarowski et al., 2019; Lit & Crawford, 2006). Such errors should be identified during training or operation, and should be corrected by handlers to avoid recurrence. Previous studies highlighted that sniffing duration represents a good way of identifying errors; the shorter the duration, the more correct the response (Concha et al., 2014). False positives and false negatives rates were calculated during the MAP validation. However, comparison of error rates to the level of error (false positives and negatives) identified by handlers and supervisors during MAP validation in our study showed no relationship; thus, it was not possible to delineate an acceptance threshold for errors to rank DDs. The mean error rates (false positives and false negatives rates) measured in this study could be used as tentative thresholds: 5% false positives and 1% false negatives. Other studies investigating the performance of DDs recorded success rate ranging of 83–100% (Angle et al., 2016; Dickey & Junqueira, 2021; Gazit & Terkel, 2003; Porritt et al., 2015), with our results falling within this range.

Accuracy, as well as the strategy of the dog and guidance by the handler were **of moderate importance** when assessing DD performance. In our study, accuracy was defined as the ability of the dog to detect the targeted odor source, based on Cablk and

Sagebiel (2011). Hence, accuracy was closely correlated with the level of error. For instance, if the dog marked the target odor too far away (that is more than 15 cm), it was considered as a false positive. Before the current study, there is no precedent on acceptable target-marks distance. Yet, this criterion is highly important, especially during police operations when odor sources must be detected with high precision, particularly if dangerous (*e.g.*, explosive). Existing studies on DD search strategies were also lacking in the published literature. Our quantitative MAP showed that search strategies could be defined as the search behavior in a known environment. Because variance is a measure of data dispersion, the search strategy level was mainly explained by the variance of certain parameters (frequency of visiting the target zone, search time, search distance), rather than the mean value. As all observed correlations were negative, the closer to the mean a that dog performs its search, the more strategic it is. In a known environment, then search behavior should be consistent, when defining a good dog strategy. This criterion could be assessed using the duration and distance covered by the search, including the number of times the dog passes at predefined distance thresholds of the target before reporting it. These parameters should be similar every time the dog repeats the search in the same environment (*e.g.*, arena). Finally, professionals suggest that handlers should need to minimally guide efficient DDs. This criterion was directly correlated with dog independence. Thus, surveyed professionals considered an independent DD as performant. This criterion has been highlighted in the published literature as important, especially during DD selection (Martin, Diederich, et al., 2020). The more you guide a DD, the more distracted the dog becomes (Lazarowski et al., 2020). During the quantitative MAP in our study, handlers were not allowed to give any instruction to their dogs that were searching independently. Dependent dogs were expected to make more mistakes during the quantitative MAP. Ultimately, a clear definition and measure of DD independence would help enhance the proposed method.

Finally, the rapidity of searches was ranked as a **minor important criterion**, as the main goal of DDs is to locate the target. Whether the dog is fast or not, is not relevant to the quality of the dog's work.

Conclusions

This study provided the first definition of performance in the framework of DD work by professionals, and validated it through objective trials. Future studies should implement DD performance evaluation throughout the entire training process. This way of monitoring performance would allow thresholds to be delineated to identify efficient DDs, as well as to identify factors affecting performance during conditioning (*e.g.*, dog breed, gender, and training aids).

Copycatting the smell of death – Deciphering the role of cadaveric scent components used by detection dogs to locate human remains

This section is an adapted version of the article:

Martin C, Malevic M, Diederich C, and Verheggen F (under review) Copycatting the smell of death – Deciphering the role of cadaveric scent components used by detection dogs to locate human remains. Journal of forensic science

Abstract

Human remains detection dogs (HRDDs) are commonly used by law enforcement agencies to search for cadavers. Biological material is typically used as a training stimulus, also called aids, to train dogs to recognize the smell of cadavers. While HRDD training approaches have received extensive attention, information remains limited on the olfactory cues used to train them. Here, we aimed to decipher the chemical basis of detection dog olfaction. Five specific objectives were explored to precise whether the composition or the concentration of the training aids drives the HRDDs responses. We recorded the behavioral responses of four HRDDs exposed to different cadaveric-like smells. We found that HRDDs recognized a simplified synthetic aid composed of cadaveric compounds. The lowest concentration at which HRDDs continued to perceive the cadaveric smell was determined. HRDDs were not impacted by slight modifications to the chemical composition of a blend of odors that they have been trained with. HRDDs associated sulfur and nitrogen compounds as human cadaver. Our findings highlight a lack of specificity of HRDDs to cadaveric compounds, which could lead to error of detection. Moreover, all dogs did not positively respond to the same blends, despite being trained with the same aids and procedure. However, we confirmed that dogs could be trained with a simplified blend of molecules. The chemical composition of a training aid has therefore high consequences on the performance of the trained animal, and this conclusion opens additional questions regarding olfaction-based detection animals.

Introduction

In 1985, the German author Patrick Süskind wrote a novel featuring a young man obsessed by the idea of creating a perfume to bewitch humans. He murdered young women and extracted the smell from their cadavers (Süskind, 1985). Who would have thought that, 40-years later, Süskind's idea would be put in action to constitute perfumes mimicking the smell of death and used to train human remains detection dogs (HRDDs)?

HRDDs are commonly used by law enforcement agencies to search for cadavers, body parts, or fluids during criminal investigations, or after a suspicious disappearance (Komar, 1999; Lorenzo et al., 2003; Nizio et al., 2017). HRDD handlers rely on operant conditioning to train their dogs, by positively rewarding a desired behavior (sitting or barking) when their dog approaches the olfactory stimulus (Martin, Diederich, et al., 2020; Webb et al., 2020). This stimulus is called a training aid, and typically releases a smell mimicking that of a cadaver. This smell can be natural (*e.g.*, cadaveric fluids, tomb soil) (Martin, Diederich, et al., 2020) or synthetic (*e.g.*, Pseudo corpse® Merck) (Deldalle and Gaunet, 2014). The chemical composition of these odors has been extensively studied for both natural (Cablk et al., 2012; Eckenrode et al., 2006; Nizio et al., 2017; Stadler et al., 2016) and synthetic aids (Stadler et al., 2012). The latter poorly mimic the smell of a cadaver, as they are not composed of cadaveric molecules. Consequently, they are not used by HRDD handlers. Instead, handlers preferentially use a wide variety of natural aids, including teeth, hair, and gauze soaked with cadaveric fluids from humans and other mammals (Martin, Diederich, et al., 2020).

To date, natural aids remain the most reliable odorant source to train HRDDs (Nizio et al., 2017; Oesterhelweg et al., 2008; B. L. Rust, 2018). Unexpectedly, exploratory assays implemented by our group showed that experienced HRDDs exhibited a positive behavioral response to both natural and synthetic training aids (Pseudo Corpse formulation one and two, Merck, unpublished data), despite no prior training with the molecules in the blend. These experiment were conducted following the protocol described in Martin et al, 2022 (Martin et al., 2022): two of the four tested HRDDs positively recognized the Pseudo corpse in the arena. These observations indicate that the performance of HRDDs might be more related to the chemical structure of the training aid, rather than to its components. This hypothesis was raised for explosive detection dogs that failed to recognize their training aid when a single odor was mixed with other odors (Lazarowski & Dorman, 2014). While HRDD training approaches have received extensive attention, knowledge remains limited on the relationship between the composition of training aids and HRDDs efficiency. In fact, the olfactory cues inducing a positive response in HRDDs have yet to be

identified (Martin, Diederich, et al., 2020).

Here, we aimed to decipher the chemical basis of detection dog olfaction. Specifically, we ask how the chemical composition of a simplified blend mimicking the odor of decaying remains impacts the responses of HRDDs. To accomplish this, we explored five objectives: (i) whether HRDDs can recognize a synthetic blend made of few cadaveric components; (ii) the role of the absolute concentration of chemicals making up the cue; (iii) how the chemical composition of an odorant allows HRDDs to associate it with a human cadaver; (iv) essential chemical functions (sulfur, nitrogen, aromatic) allowing HRDDs to associate the odorant cue with a human cadaver; and (v) how distracting odors impact the ability of HRDDs to associate a cadaveric odorant cue with a human cadaver. These questions would open a new field of investigation dedicated to the impact of the chemical composition of training aids on the perception of HRDDs, in addition to provide guidance which could be directly applied by the handlers.

Material and methods

Human remains detection dogs

Four HRDDs, all male springer spaniels, belonging to the canine support direction of the Belgian federal police (DACH) participated to this study. The dogs had at least one year of expertise in human remains detection (range: 1–5 years). All dogs were trained during their whole career using natural training aids made of gauze or paperclips impregnated with vertebrate cadaveric fluids.

Chemicals

The molecules selected for use were based on an extensive review of the literature on the volatilome of human cadavers (Martin & Verheggen, 2018b). Only chemicals that are released during the entire decomposition process were selected. Five representatives of the three major chemical families were identified: sulfur compounds (dimethyl-disulfide and diethyl-disulfide), nitrogen compounds (indole and pyridine), and aromatic compounds (p-xylene). Diethyl-disulfide and pyridine are supposed to be human specific (Rosier et al., 2015, 2016). DMDS and indole were chosen as they are the most common sulfur and nitrogen containing compounds released during the decomposition of vertebrate remains (Dekeirsschieter et al., 2011; Dent et al., 2004; Irish et al., 2019; Statheropoulos et al., 2005; Vass et al., 2004, 2008b). The absolute and relative concentrations of each compound were arbitrarily decided based on the published literature (Table 19). This blend (later referred to as ‘’initial blend”) was stored at -20 °C. It was used to answer Questions 1 and 2. We aimed to test the ability of HRDDs ability to associate this simplified blend with the

smell of a cadaver.

Tableau 19 Formulation of the initial blend made of cadaveric odorant cues.

Compound	Concentration (M)
Dimethyl-disulfide (DMDS)	1
Pyridine	0.57
p-xylene	0.42
Indole	0.25
Diethyl-disulfide (DEDS)	0.1

Distractive odor

In some of the following assays, distractive odors were included in the behavioral arena (described below). Four distractive odors were prepared: coffee (20g of ground coffee), basil (20g of freshly cut plant), chocolate (20g of crushed dark chocolate), and a synthetic indoor perfume. They were introduced in separate glass bottles with 20 sterile gauzes. Bottles were then sealed and left at room temperature for 24 hours. A single gauze of each distractive odor was then hidden in the arena before the beginning of the assay.

Q1 – Are HRDDs able to recognize a synthetic blend of few cadaveric components?

The initial blend was tested on three of the four HRDDs at the headquarters of the police brigade (Neerhespen, Belgium). In a separate room, sterile gauze was impregnated with 50 microliters of the initial blend, and was then hidden in a homemade wall. The gauze was changed for each search, as well as its position in the wall. A period of 15 min was applied before each test to ventilate the room. We recorded the ability of each HRDD to indicate the correct location of the impregnated gauze to their handler.

The same exercise was performed outdoors in a wood with the same three dogs. The initial blend (1 ml) was applied on a rock. During the session, one dog was placed 50 m away from the rock, and was asked to perform a search under real conditions. We recorded the ability of each HRDD to provide their handler with a positive response when facing the correctly marked rock. The behavioral response of each HRDD was recorded once.

Table 20 Composition of solutions used to answer Q3 to Q5.

Blends	Compounds	Research question
Removed blends		
Blend excluding DMDS	DEDS, pyridine, indole, p-xylene	Q3
Blend excluding DEDS	DMDS, pyridine, indole, p-xylene	Q3
Blend excluding pyridine	DMDS, DEDS, indole, p-xylene	Q3
Blend excluding indole	DMDS, DEDS, pyridine, p-xylene	Q3
Blend excluding p-xylene	DMDS, DEDS, pyridine, indole,	Q3/Q4
Blend excluding DMDS and DEDS	pyridine, indole, p-xylene	Q4
Blend excluding pyridine and indole	DMDS, DEDS, p-xylene	Q4
Copycat blends		
Blend sulfur replaced	B-mercaptoethanol, pyridine, indole, p-xylene	Q4
Blend nitrogen replaced	DMDS, DEDS, 4-benzoxyaniline, p-xylene	Q4
Blend aromatic replaced	DMDS, DEDS, pyridine, indole, cyclopropylbenzene	Q4
Blend all replace	B-mercaptoethanol, 4-benzoxyaniline, cyclopropylbenzene	Q4
Plant distractor + cadaveric compound		
Plant scent including DMDS	Limonène, β-Caryophyllène, α-Phellandrène, α-Pinène, DMDS	Q5
Plant scent including DEDS	Limonène, β-Caryophyllène, α-Phellandrène, α-Pinène, DEDS	Q5
Plant scent including DMDS and DEDS	Limonène, β-Caryophyllène, α-Phellandrène, α-Pinène, DMDS, DEDS	Q5
Plant scent including pyridine	Limonène, β-Caryophyllène, α-Phellandrène, α-Pinène, pyridine	Q5
Plant scent including indole	Limonène, β-Caryophyllène, α-Phellandrène, α-Pinène, indole	Q5
Plant scent including pyridine and indole	Limonène, β-Caryophyllène, α-Phellandrène, α-Pinène, pyridine and indole	Q5
Plant scent including p-xylene	Limonène, β-Caryophyllène, α-Phellandrène, α-Pinène, p-xylene	Q5

Q2 – What is the role of the absolute concentration of the chemicals making up the cue?

Here, the initial blend was repeatedly diluted by a factor of 10 (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}). The ability of all four HRDDs to respond positively to each diluted blend, presented at random, was evaluated in a behavioral arena similar to that previously described by Martin et al. (2022). In brief, the arena consisted of an 8 x 8 m room containing 25 cinderblocks in a semi-circle (d = 6 m). An empty plastic cup was placed in each cinderblock. Before and after the behavioral assays, when no chemical cues were present, the handler allowed each dog to search the arena, to confirm that the arena did not induce any response. To avoid the "clever Hans effect" (Sebeok & Rosenthal, 1981), handlers were not informed about the location of the target odor. After the handler left the room, the experimenter introduced a piece of gauze impregnated with the initial blend at one of the tested concentrations, inside one of the plastic cup placed in cinderblocks. In addition to the blend, four distractive odors were placed in other plastic cups, to avoid dogs to positively respond to a strong of odor. The handler was then invited to enter the arena again with their HRDD and the test was initiated. The handler was allowed to stay close to their dog to give commands, but was not allowed to guide the dog by pointing out the cinderblock. To signal the end of the trial, the handler told the experimenter whether a target cue was present or absent in the arena, and to provide the position of the cue, if applicable. If the position was correct, the handler was invited to stop the search and was allowed to reward his dog. If an incorrect answer was given, the search was stopped without rewarding the dog. The behavioral response of each HRDD was recorded once.

Q3 – How does the chemical composition of an odorant cue affect the ability of HRDDs to associate it with a human cadaver?

The two previous experiments demonstrated the ability of HRDDs to respond to a simplified and diluted initial blend (Table 20). Here, we evaluated how each compound contributed to detection by HRDDs. We modified the initial blend by diluting it by a factor of 10^{-3}, and out of the mix one odor was removed each time, so four odors were always present (Table 20). We then exposed the HRDDs to these partial blends using the method described for Question 2, and recorded their ability to detect them. The behavioral response of each HRDD was recorded once

Q4 – Which chemical functions (sulfur, nitrogen, aromatic) are essential for HRDDs to associate the odorant cue with a human cadaver?

Here, the initial blend was adjusted in two ways. The same behavioral arena was used as in Q2 and 3. HRDDs were first exposed to "removed blends," in which the compounds belonging to the same chemical family were excluded from the initial

blend. Then, HRDDs were exposed to copycat blends, in which non-cadaveric compounds belonging to the same chemical family were added (Table 20). The behavioral response of each HRDD was recorded once.

Q5 – How does the presence of distracting odors impact the ability of HRDDs to associate a cadaveric odorant cue with a human cadaver?

Here, we formulated distracting floral blend, composed of four plant volatiles (limonene, β-caryophylene, α-phelandrene, and α-pinene) mixed to the same proportions. First, we validated that the floral blend did not induce a positive behavioral response in the HRDDs. Then, each compound from the initial blend was added to it, one at a time (Table 19). The behavioral response of each HRDD was recorded once.

Behavioral assay sequence

To avoid any learning from the dogs during the assays, twelve months separated the first exposure to the cadaveric blend (all assays from Q1) from the assays associated with the other questions (Q2-Q5). During that period, no dog was exposed to the blend. All assays performed to answer Q2 to Q5 were randomly organized. Each dogs performed 10 behavioral assays per day of experiment. Finally, each day of experiment were separated by a period of a month. This procedure was followed to minimize dogs learning during the tests.

Results

Q1 – Are HRDDs able to recognize a synthetic blend of few cadaveric components?

Two out of the three detection dogs recognized the initial blend, and marked it as they would a cadaver. The third dog expressed an interest in the smell by sniffing the gauze for 4 seconds, but did not mark it as a cadaver. In the outdoor test, the same two dogs responded positively to the odor, as in the indoor test. The third dog showed no interest in the marked rock.

Q2 – What is the role of the absolute concentration of the chemicals making up the cue?

All four HRDDs responded to the four highest concentrations of the initial blend (undiluted, and 10^{-1}, 10^{-2}, 10^{-3} dilutions). Three of the four HRDDs positively detected and respond to the 10^{-4} and 10^{-5} dilutions. The 10^{-5} dilution corresponded to 112.3 ng molecules on the gauze (47.00 ng DMDS, 22.51 ng pyridine, 22.1 ng p-xylene, 14.60 ng indole, 6.11 ng DEDS). No HRDD was able to detect the 10^{-6} dilution of the initial

blend.

Q3 – How does the chemical composition of an odorant cue affect the ability of HRDDs to associate it with a human cadaver?

Removing DEDS, pyridine, or p-xylene from the simplified blend did not prevent any dog from responding correctly. Removing DMDS or indole prevented one of the four dogs from responding positively.

Q4 – Which chemical functions (sulfur, nitrogen, aromatic) are essential for HRDDs to associate the odorant cue with a human cadaver?

The lowest rate of positive responses was observed when sulfur compounds were removed from the mixture (2 out of 4 dogs responded positively). However, when these compounds were replaced by a non-cadaveric sulfur compound, the rate of response returned to 100%. Removing nitrogen compounds did not affect the ability of dogs to respond positively. Replacing these compounds with a non-cadaveric nitrogen compound led to three out of four positive responses. The removal of the aromatic compound from the blend did not impact the behavioral response of HRDDs, nor its replacement with a non-cadaveric aromatic compound. The rate of response remained 100% when all compounds from the initial blend were replaced with non-cadaveric compounds belonging to the same chemical families.

Q5 – How does the presence of distracting odors impact the ability of HRDDs to associate a cadaveric odorant cue with a human cadaver?

None of the four HRDDs responded to the plant distractor blend. When DMDS or DMDS + DEDS was added to this blend, all dogs responded positively. The addition of DEDS or indole or pyridine + indole to the plant distractor blend resulted in three of the four dogs responding (the same dogs responded to the two latter blends). Two of the four dogs responded to the plant distractor blend supplemented with p-xylene. Just one dog responded to the plant distractor blend with pyridine.

Discussion

This study evaluated how the chemical composition of training aids affects the behavioral response of HRDD. While there are many reports on the chemical composition of training aids (including vertebrate cadavers), information remains limited on detection dog response (Alexander et al., 2016; Forbes, Rust, et al., 2014; Frederickx et al., 2011; Komar, 1999; Lit & Crawford, 2006; Martin et al., 2022; Martin, Diederich, et al., 2020; Migala & Brown, 2012; Oesterhelweg et al., 2008; Riezzo et al., 2014; Williams & Johnston, 2002). The chemical basis of HRDDs odor recognition remains largely unquantified (Oesterhelweg et al., 2008).

The dogs used in the present study were chosen because of their experience in necro-searches in Belgium (Van Denhouwe & Schotsmans, 2014). The sequence of the different blends was randomly assigned for each dog and divided in several days (separated by a month) to avoid any learning of the blend through the experiments. The results of the Q1 test showed that HRDDs originally trained with a natural aids (containing several hundred compounds; (Nizio et al., 2017)), are able to detect a simplified cadaveric blend composed of just five cadaveric molecules. It is unlikely that HRDDs are able to detect hundreds of individual compounds released by natural aids. For instance, previous studies showed that explosive detection dogs that trained daily with a mixture of compounds later respond to certain individual chemicals (Gazit et al., 2021). However, this previous study investigated a mixture of unnamed explosives, for which the chemical composition was not provided.

The present study reaffirmed that canine bio-detectors are very sensitive (Angle et al., 2016; Oesterhelweg et al., 2008; L. T. Rust et al., 2018). For example, dogs were shown to be more sensitive at detecting Covid-19 than PCR tests (Hag-Ali et al., 2021), differentiating virus-infected cells from healthy ones (Angle et al., 2016). HRDDs are able to detect the cadaveric residues on a piece of cloth, even after several washes (L. T. Rust et al., 2018). However, the current study provided the first limit of detection for cadaveric material. We showed that around 100 ng of cadaveric compounds could still elicit a positive response in experienced HRDDs.

Our results showed that the chemical composition of the blend did not impact the ability of HRDDs to identify a cadaveric scent. Even though the synthetic blend purchased from Sigma Aldrich did not contain a single cadaveric compound, it did elicit a response from most tested HRDDs. Because these dogs were trained using highly variable cadaveric aids, they probably learned to respond to a wide range of chemicals, belonging to a limited number of chemical families, allowing them to recognize several compounds within a blend (Gazit et al., 2021). The odors released by decaying remains are indeed highly variable (Dekeirsschieter et al., 2009; Dubois, Stefanuto, et al., 2019; Forbes et al., 2016; Forbes, Perrault, et al., 2014; Verheggen et al., 2017). Two of the four dogs did not recognize the blend when sulfur compounds were removed. Out of these sulfur compounds, DMDS was the key compound for cadaver recognition by HRDDs, as it was the only compound that induced 100% response when mixed with plant-derived distractive odors. Sulfur compounds, including DMDS, are commonly released during the entire decomposition process of vertebrate tissues (Dekeirsschieter et al., 2009; Forbes & Perrault, 2014; Hoffman et al., 2009; Martin & Verheggen, 2018b; Vass et al., 2004; Verheggen et al., 2017). DMDS are by-products of sulfur based amino acids, as methionine, degradation (Dekeirsschieter et al., 2012; Statheropoulos et al., 2011).

DMDS is a major compound that is released during the entire decaying process, and might explain why HRDDs are particularly responsive to this chemical (Verheggen et al., 2017). Nitrogen chemicals seem to have a secondary role in detection. For instance, indole is the only nitrogen compound detected through the entire decomposition process (Forbes & Perrault, 2014; Kasper et al., 2012). Like DMDS, indole is a typical cadaveric odor that originates from the decomposition of various aromatic amino acids, including phenylalanine, tyrosine and tryptophane (Dent et al., 2004; Kasper et al., 2012; Vass, 2001). Dogs focus on by-products released during protein degradation, probably because the natural training aids used by their handlers were mostly composed of proteins (*e.g.*, muscle, flesh, blood). Aromatic compounds seem to provide negligible cues for cadaver recognition by HRDDs. p-xylene is released throughout the entire decomposition process, but in much smaller amounts than the other compounds of the blend (Cablk et al., 2012; Hoffman et al., 2009). In addition, p-xylene is an ubiquitous atmospheric compound, limiting its reliability as a cadaver cue (Forbes, Rust, et al., 2014).

The results of our study showed that HRDDs do not target specific cadaver compounds. When all cadaveric compounds were replaced with non-cadaveric compounds from the same chemical family, HRDDs still positively recognized the blend mimicking a cadaver odor. This generalization ability of HRDDs was previously identified (Oldenburg et al., 2016) : animals trained to respond to a specific molecule are likely to respond to all compounds sharing similar chemical structure. However, the length of the carbon chain appears to contribute to the mechanism of generalization (Lazarowski et al., 2020). This could be a problem as dogs could positively recognize untargeted compounds, which could (at least partly) explain the lack of detection exhibited by some dogs during the experiment. Moreover, due to individual variability in HRDD response, more than one dog should be used in searches.

Conclusions

In conclusion, using a simplified aid that mimics the odor of a decaying corpse could help HRDDs to recognize all compounds from the blend, and ensure that all dogs respond to the same compounds. How HRDDs perceive cadaveric compounds requires further investigation, along with the mechanism of the odor learning process during HRDD training.

Chapter 6

General discussion

The complexity of the decaying process of vertebrate leads to the release of a complex fragrance that my supervisors and I were used to call “the smell of death”. The current PhD research has been designed along this intriguing scent. It has been investigated from two complementary standpoints: (i) the chemical composition of cadaveric volatile profile and (ii) the perception of this profile by police detection dogs. We first investigated the impact of the environmental conditions on the cadaveric volatilome by using surrogate human model (*i.e.*,, rat). We were able to identify environmental factors impacting the release and the composition of the cadaveric odor profile. Then, we collected, identified and quantified the volatile organic compounds (VOCs) released by human bodies at the fresh stage. Following these successful investigations and the previous work of our colleagues, we oriented our research to decipher the behavior and perception of detection dogs specialized in the location of the odor of decaying bodies (called human remains detection dogs or HRDDs). We realized that their efficiency has never been defined. We crossed the handlers’ opinion with the literature to suggest traits that define an efficient detection dog. Based on it and with the collaboration of the Belgian police handlers, a behavioral test/procedure was developed to assess the HRDDs’ efficiency. Finally, the recognition of the target odor by those dogs was studied. A simplified blend of odorous molecules was developed and validated as a copycat of the smell of decaying corpses. For the first time, a detection threshold was identified as well as the olfactory cues which drive the recognition. However, we also demonstrated that these dogs are not specific to cadaveric odor.

This dual way to investigate the cadaveric odor profile allowed me to stand back from both point of view. Hence, I have decided to discuss the two axes of my PhD research differently. The discussion of the chemical axe is trending towards an opinion on methodology to decipher the cadaveric volatilome used in the present work and by colleagues. It aims at highlighting the strength and weaknesses of current technics and to investigate new available one for future researches on this field. Regarding the second axe, I propose perspectives which could help to improve current knowledge on detection animals. Indeed, this research field is in its early stages and therefore has to be more investigated. The collection of more information on detection animal will reach wider fields of application than only to enhance HRDDs’ efficiency.

Despite the extensive literature dedicated to the analyze of the cadaveric volatilome, the majority focused on the development of analytical methods to sample and analyses VOCs released during decomposition (Clases et al., 2021; Degreeff & Furton, 2011; Dekeirsschieter et al., 2012; Dubois et al., 2017; Forbes et al., 2016; Perrault et al., 2017; Rosier et al., 2014). This work has not been an exception. A sampling method was developed to study the cadaveric VOCs dissolved for immerged decomposition cases. Moreover, we have investigated the possibility to use targeted ion method to

analyze the volatile profile of early deceased people. Regarding sampling methods, scientists mainly use sorbent that can be heated to desorbed trapped molecules as solid phase micro extraction (SPME) fibers and thermodesorption tubes (Dubois et al., 2017; Forbes, Rust, et al., 2014; Rosier et al., 2014). The use of thermodesorption technics allows to have a higher sensitivity which improve the characterization of such complex odor profile (M. Harper, 2000). We also noticed that sampling devices with multi coating materials are also preferred by scientists. The high diversity of the cadaveric volatilome probably explains the use of sorbent with different affinity to trapped all these different molecules (Cernosek et al., 2019; Statheropoulos et al., 2011; Stefanuto et al., 2015; Verheggen et al., 2017). Dynamic sampling through a tubes should be preferred to static one as it allows to perform quantitative analyses (Tholl et al., 2021). This trend is more and more followed to analyze the cadaveric volatilome (Deo et al., 2020; Dubois et al., 2021; Dubois, Stefanuto, et al., 2019). However, more attention should be given to the sorbent material used. Indeed, the degradation coating material during desorption produces by-products which in some cases could lead to an overestimation of some compounds. In fact, Tenax decomposition products benzaldehyde (M. Harper, 2000; Vercammen et al., 2000) which is often associated with the cadaveric VOCs profile (Agapiou et al., 2015; Brasseur et al., 2012; Cablk et al., 2012; Dekeirsschieter et al., 2009). Benzaldehyde was also detected several times through this work. Despite some focal point that scientist have to consider during the sampling of cadaveric VOCs (*e.g.*, coating remanence), we can assume that the technics is well mastered.

In the literature, it appears that bidimensional gas chromatography is the silver bullet to characterize decaying smell. This prevalent method in such analyses offers an increased peak capacity, sensitivity and selectivity allowing to better manage peak co-elution and chromatographic artefacts (Perrault, Nizio, et al., 2015; Stefanuto & Focant, 2016). Indeed, for the last decade, bidimensional gas chromatography overpasses classical gas chromatography to characterize the cadaveric VOCs profile (Deo et al., 2020; Dubois, Stefanuto, et al., 2019; Gruber et al., 2018; Knobel et al., 2018). In fact, the high complexity of the cadaveric samples makes this technic the more accurate one. This powerful technic is especially interesting in complex environment as building collapse (Ueland et al., 2021), soil (Dubois et al., 2018) or blaze (Sampat et al., 2018) to pull out target compounds from complex matrix. Thus, GC x GC is essential to determine relevant decaying associated compounds (Perrault, Nizio, et al., 2015). Moreover, it would be interesting to investigate the elution pattern of the cadaveric volatilome as it is already done for hydrocarbon analysis (Arey et al., 2022; Genuit & Chaabani, 2017). Indeed, this pattern is correlated with the compound structure which could ease the chromatogram interpretation and widen the access to this powerful tool (Skoczyńska et al., 2008). The wonderful perspective that offers

bidimensional gas chromatography should therefore be used to decipher the cadaveric volatilome despite the cost of such tool (Tranchida et al., 2011). However, the classical GC may still be used to track specific cadaveric compounds (Clases et al., 2021). We have successfully applied such target method on fresh cadaver and highlighted 30 cadaveric compounds. However, we would be careful in the application of this method on more advanced stages which are more abundant in terms of molecules (Dekeirsschieter et al., 2009). Even if it was the strategy during my PhD thesis, I would not recommend to use GC to investigate impact of some parameters on the VOCs released during the decomposition. Indeed, we were able to confirm in some cases the impact of parameters but without being able to highlight impacted compounds. As explained earlier, GC x GC would be more efficient. Finally, we have also investigated the cadaveric volatilome release at the water surface. In this particular case and because it seems that there is a partition of the compounds between water and air, simple GC could remain an interesting scanning technic.

The detection of the cadaveric volatile profile after separation relies mainly on a screening strategy rather than targeted one (Degreeff & Furton, 2011; Dekeirsschieter et al., 2009; Deo et al., 2020; Dubois, Stefanuto, et al., 2019; Nizio et al., 2017). Scan analyses decrease the bias introduced by arbitrary decision of the scientist. However, it also increases the variability of the data by considering outstanding values. It is therefore important to exclude non studied compounds (*e.g*, sorbent artefacts). In the literature two strategies are used to remove potential non cadaveric compounds from the data. First, volatiles identified in more than half of the samples are considered for statistical analyses (Degreeff & Furton, 2011; Dekeirsschieter et al., 2009). The other way is to use the Fisher ratio technic. This last one is mainly used with bidimensional gas chromatography to keep the most significant components (Deo et al., 2020; Dubois et al., 2018; Verheggen et al., 2017). In experiments performed in the present work the first strategy was used as classical gas chromatography was performed. If we investigate last decade research on the cadaveric volatilome, we can assume that compounds are well-known and could even be predicted. Based on these studies, scientists should find an agreement on a "cadaveric library" of compounds. It would help to perform reliable target analyses (Clases et al., 2021; Christian Von Hoermann et al., 2022). It would also decrease the bias of the scientist as compounds are integrated based on a target ion and a ratio between two ions of reference. Targeting compounds also increases the sensitivity of the analyses (Gómez et al., 2009). It is even better to combine both methods with the Scan/SIM mode which allows to investigate untargeted compounds as well as to confirm the presence of targeted one without losing sensitivity in the single ion monitoring mode (SIM). Moreover, such library would also offer the possibility to perform powerful meta-analyses of the literature. The monitoring of compounds would also be eased.

Analytical conditions are not the only ones to consider when analyzing the cadaveric volatilome. Indeed, the choice of the statistical analyses is crucial. Most of the studies compared the cadaveric volatile profile with principal component analyses (PCA) (Armstrong et al., 2016; Dekeirsschieter et al., 2009; Dubois et al., 2017; Forbes, Rust, et al., 2014; Kasper et al., 2012; Knobel et al., 2018; Rosier et al., 2016). Usually, these PCAs are never associated with a *p*-value allowing to confirm the repartition of the samples on the PCAs depending on the studied conditions. The PCA is a descriptive statistical method which allows to summarize complex dataset in a plan draw by principal components that are the independent combination of initial variables, in this case the cadaveric volatiles. This method should therefore always be associated with statistical values confirming the PCA illustrations. Multivariate analyses of the variance are good candidates to confirm PCA discriminations. Moreover, other technics than PCA could be used to better discriminate the studied classes as the linear discriminant analyses (LDA). If the condition of the experiment considers several classes, it would be better to use LDA as they take into account the information of class. A recent review compares the dimensionality reduction algorithms (Anowar et al., 2021). Both PCA and LDA should not be the last step of the analyses. Indeed, they allow to highlight differences in a plan created by to vector. If the multivariate analyses highlight differences among studied conditions, the variables that contribute to the axes of the PCA or LDA should be compared depending on these conditions to propose chemicals that would drive these differences. Finally, predictive model as partial least-squares discriminant analyses (PLSDA) could also be considered in the analyses of the cadaveric volatile profile. This test develops a model to discriminate the samples according to the volatile composition. It is a powerful machine learning tool. It is already used in the comparison of volatile profiles of other sources (Ledauphin et al., 2010; van Ruth et al., 2010). In addition, it outperformed PCA when the number of samples is low, which is usually the case in forensic science. Future analyses on the cadaveric VOCs profile should therefore better investigate statistical tools that are available to maximize the outcomes from their studies.

The use of powerful statistical analyses would allow to highlight compounds that are case-specific from compounds that are commonly found and steady during the decomposition process. These compounds would make good candidates to be used as olfactory stimulus during the training of human remains detection dogs (HRDDs, Rosier et al., 2015). However, the question of the aids that have to be used is still discussing. Indeed, some scientist argue that as the cadaveric VOCs profile is diversified, the aids used during training should also be diversified.(Dargan & Forbes, 2021). In my opinion, the answer to this interesting question relies on the perception spectrum of these dogs after the training as well as on the method to present and teach

the recognition of the target odor. It will be discussed in the second part of this discussion.

In the last part of chapter 5, we showed that professional HRDDs are not specific to cadaveric compounds. This conclusion confirms previous works that detection dogs generalize they response: they can mark a compound they were not trained with. However, this compound should be chemically close to the original one (Hall et al., 2016; Moser et al., 2019). The kind of similarity (*i.e.*, chemical structure, chemical class) that induces this generalization has not been identified yet. Physiological studies performed on mice highlighted that homologue molecules induce similar mapping of the activated zone in the olfactory bulb. The differentiation of the bulb map was even more difficult when the number of carbon constituting the molecules was close (Xu et al., 2003). Chemical structure as well as chemical classes are therefore good candidates to explain the detection dog's generalization. To finally characterize this phenomenon, I would suggest to train dogs or other detection animal as rats, with only one molecule. After training, the recognition of homologues (*i.e.*, same carbon length but different classes, same chemical classes but different carbon length) should be assessed. This experiment would allow to better predict on which odor sources detection animal would be able to generalize.

We also have shown that human remains detection dogs did not response to all compounds of the training aids they were trained with. Indeed, sulfur containing compounds were shown to be cues that drive the positive response of this detection dog. Sulfur compounds are continuously released through the decomposition and are among the most abundant molecules of the cadaveric VOCs (Verheggen et al., 2017). These dogs were trained with natural aids which are known to be diversified (Nizio et al., 2017). This information suggests that the use of such aids did not increase the adaptability neither the recognition spectrum of HRDDS as previously suggested (Dargan & Forbes, 2021). Therefore, it would maybe be better to train dogs with one odor at a time instead of with a complex mixture. Indeed, it was already shown that the learning of odor in sequence did not impact the recognition of odors (Waggoner et al., 2022). Both strategies should therefore be assessed to enhance the more appropriate one.

Furthermore, as mentioned HRDDs trained with a complex mixture only learn a small number of its constituent to target the odor. The choice of molecules to target in a complex blend could either be driven by the abundance of the molecules in the blend or the number of times the dogs was in contact with the molecule during the training. Indeed, tested dogs recognized sulfur containing compounds which are the more current as well as one of the most abundant compounds in the cadaveric volatilome (Clases et al., 2021; Dekeirsschieter et al., 2009; Verheggen et al., 2017). We therefore

suggest to train different groups of naïve dogs. Each group could be trained with olfactory stimulus which are different either in abundance or in frequency of compounds.

The challenge regarding detection dogs is the deciphering of the post-training perception depending on the olfactory stimulus used during the training. It could allow to better understand which characteristics of the aids influence the recognition of the target odor. Also, the strategy to teach the odor of detection dogs should be assessed. It would help to identify if a complex mixture is more efficient than a simplified one in addition to provide guidelines for training procedures.

In summary, the investigation of the cadaveric odor by considering both chemical deciphering and detection dogs' perception is promising for forensic science as well as for the application by law enforcement agency. It would provide insights that would benefit both point of view. Finally, the behavior of detection dogs should now than ever be more investigated. Indeed, since a couple of years detection dogs are more and more suggested in different application fields (Beebe et al., 2016; Dickey & Junqueira, 2021; Grandjean et al., 2020; Hackner & Pleil, 2017; Hag-Ali et al., 2021; Oldenburg et al., 2016). Any results on any detection dogs would benefit the whole community investigating detection dogs' behavior and efficiency.

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