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Characterisation of volatile organic compounds in dingo scat and a comparison with those of the domestic dog

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ABSTRACT

Olfaction is a widespread mode of communication in mammals. The volatile organic compounds (VOCs) in biological samples such as scat and urine give them either a repellent property to warn prey or an attractant property to communicate reproductive, social, and territorial status to conspecifics. The aim of this study was to determine whether there are VOC differences in the scat of a dingo compared to that of a domestic German Shepherd Dog (GSD). We standardised the diet for 10 days and then collected scat from 14 dingoes and 11 GSDs. Using headspace solid phase microextraction (HS-SPME) with gas chromatography–mass spectrometry (GC-MS), we characterised VOCs present in the dingo scat and compared the composition with those of GSDs. We identified 58 significant VOCs out of a total of 154 VOCs identified in both dingoes and GSDs. Of these, 12 were significantly higher in the scat of dingoes and 46 elevated in the GSD. Of the 12 elevated in the dingo, three were unique and present in high concentration (>10 ng/g), four were elevated and present in high concentration and five were present at low concentrations (<10 ng/g). We suggest that the detected differences show potential to be incorporated into dingo management strategies.

Keywords: canids, dingo, German Shepherd dog, HS-SPME-GC/MS, native animal management, odours, olfaction, scat, untargeted volatilomics, volatile organic compounds.

Introduction

Olfaction is a critical means of communication in the animal kingdom. Terrestrial animals often deposit scat, urine, and specialised gland secretions to mark their territories, locate prey and communicate with conspecifics (Bradbury 1998; Brennan and Kendrick 2006; Parsons *et al.* 2018). Predator odours lead to altered activity patterns, behavioural changes and habitat shifts in the prey species (Gittleman 1989; Ache and Young 2005; Apfelbach *et al.* 2005; Webster *et al.* 2018). In canids, odours are commonly used to communicate an individual's social and reproductive status, sex, and age, and inform their presence to the other competing predators (Vogt *et al.* 2016; McLean *et al.* 2021). The communication signals are conveyed by scent cues such as sulfur-containing volatiles, hydrocarbons, carboxylic acids, ketones, phenols, and esters released in the scat and urine (Soso and Koziel 2017; Noonan *et al.* 2019; Jones *et al.* 2021). A diverse range of these odoriferous compounds, such as aromatic organic compounds, aldehydes, fatty acids and alcohols, have been detected in the scat of wild Iberian wolves (*Canis lupus signatus*) (Martín *et al.* 2010). The volatiles (VOCs) identified from African wild dog (*Lycaon pictus*) urine, scat and gland secretion show a considerable overlap with compounds detected in other mammals. Nevertheless, the chemical profiles markedly vary from other canids (Apps *et al.* 2012), indicating the usage of some unique population-specific chemicals in olfaction. Understanding the composition of the volatilome in the biological samples is essential to provide insights into olfactory communication between and among species.

The Australian dingo (*Canis* (*familiaris*) *dingo*) went through a population bottleneck when it colonised Australia (Ballard and Wilson 2019). It is the continent's only indigenous canid and terrestrial top-order predator (Smith *et al.* 2015). Due to livestock predation, the dingo is subject to lethal control methods (Fleming *et al.* 2014). However, non-lethal

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approaches such as barrier fencing are also in use (Smith and Appleby 2018). Like other wild animals, dingoes use scent marking for social communication and territory defence and have been reported to increase scent marking prior to the mating season (Thomson 1992). Dingoes urinate and defaecate on particular sites to communicate with the members of their own and other packs (Thomson 1992). The dingo body odour and scat interfere with the acquisition of food and water by red foxes (*Vulpes vulpes*) more strongly than conspecific odour (Leo *et al.* 2015).

Similarly, dingo olfactory scents from urine and scat induce fear-based responses and reduce foraging in the macropod marsupials (Parsons *et al.* 2007; Parsons and Blumstein 2010). Dingoes are occasionally sympatric with feral dogs (*Canis familiaris*) and have been shown to respond to the olfactory cues of other canids. For instance, dingoes engage in olfactory communication with the livestock guardian dogs' (Maremmas') urine and might recognise territorial boundaries established by guardian dogs (van Bommel and Johnson 2017). The difference in the chemical profile of the predators' odour can generate a distinct response in the prey species. For instance, Eurasian beavers (*Castor fiber*) showed reduced foraging in the presence of odours from the wolf (*Canis lupus*) but not to those from domestic dogs (Rosell and Czech 2000). A comparison of the chemical profile of sympatric predators could provide insights into population-specific olfactory cues.

Wolves, dingoes, and modern-day dog breeds shared a common ancestor and inherited the ability to use olfaction for specific tasks. Domestication and artificial breeding, however, have mainly influenced the utilisation of olfaction in domestic dogs, predominantly in populations bred for enhanced olfactory abilities (Polgár *et al.* 2016; Jamieson *et al.* 2017). Domestication may also have reduced the need to efficiently metabolise or absorb nutrients as food is provided regularly. Relaxation of selective constraint since domestication has been found in domestic dogs (Bjornerfeldt *et al.* 2006; Cruz *et al.* 2008) and changes in intestinal structure have been observed in mutant mice fed a high fat diet (Lackey *et al.* 2020).

Along with artificial selection for olfactory traits, canine domestication has been associated with diet change, human-assisted mating, and relaxed environmental pressures. A previous study has shown a significant difference in the plasma metabolome between the dingo and domestic dog breeds and the dingo and dingo–dog hybrid, likely an outcome of the carbohydrate-rich diet in domestic dogs (Yadav *et al.* 2021). Diet and gut microbiome composition are associated with the faecal VOC composition, as shown in several human studies (De Angelis *et al.* 2015; Karu *et al.* 2018). Therefore, the domestic dog may show a distinct composition of the VOCs in the scat compared to the wild canids like dingoes. Nevertheless, studies comparing the VOCs in faeces and urine of the dingo and domestic dog are rare (Robley *et al.* 2015; Carthey *et al.* 2017). Such comparisons could detect dingo-specific scent markers with a potential application in the non-lethal management of human–dingo conflict.

Here we compare the dingo and German Shepherd Dog (GSD) volatilome profiles. The GSD was developed 200 years ago (Field *et al.* 2020) and is one domestic breed that has been extensively used for olfaction-based detection and scent discrimination tasks (Jezierski *et al.* 2014). The GSD's large body size translates into a sizeable olfactory epithelium and thus a larger area for sensory neurons (Jamieson *et al.* 2017), providing it with a benefit for scent detection tasks (Kokocińska-Kusiak *et al.* 2021). Further, the positive effects of nucleotide substitution in an olfactory receptor gene on the binding affinity of some odours in the sniffer dogs, including GSDs, suggests a genotypic basis of enhanced olfaction (Lesniak *et al.* 2008).

Bioanalytical techniques can help create volatilome profiles of the VOCs present in the biological sample, even if present in low concentrations. Headspace solid phase microextraction gas chromatography–mass spectrometry (HS-SPME-GC/MS) is currently among the most sensitive and accurate analysis methods (Vas and Vékey 2004). Using HS-SPME-GC/MS, we aim to characterise VOCs in the dingo scat and compare them with those in the domestic GSD. We predict that dingoes will show significant differences in the profile and quantities of the compounds compared with domestic dogs due to a difference in olfactory communication in the natural versus domestic settings and distinct selection pressures.

Data presented here suggest that population-specific chemical cues have the potential to be integrated into current integrated pest management strategies to minimise biodiversity loss and agricultural impact. We detected 12 VOCs that were significantly higher in the dingo scats and 46 in the GSD implying a relaxation of metabolic efficiency in the domestic breed. Of these 12, seven were present in high (>10 ng/g) and five in low concentrations (<10 ng/g). These VOCs have the potential to be ecologically important and may have translational importance in the management of pure dingoes.

Materials and methods

Sample collection

Scat samples were collected once from 14 pure dingoes (seven of each sex) from Dingo Sanctuary Bargo and 11 GSDs (eight females and three males) from Kingvale Kennels in December 2018 (Supplementary Table S1). All animals were healthy with no known signs of illness or disease. No females were in oestrus, and none came into season for at least five weeks after the study was completed.

The purity of all dingoes was tested using the microsatellite marker-based dingo purity genetic test (Wilton 2001). Sanctuary dingoes were either wild born and humanised before six weeks or were sanctuary born. The sanctuary is

rural, with *Eucalyptus* gum trees providing shade. GSDs were registered with the Australian Kennel Club and roamed open grassland with pine trees providing cover. The mean age of dingoes was 4.18 ± 0.61 (s.e.) years, and the mean age of GSDs was 3.7 ± 0.53 (s.e.) years (Table S1). If metabolic efficiency is equal, we predict similar numbers of VOCs will be elevated in the dingo and the GSD. Alternatively, if there has been a relaxation of selective constraint in the GSD, we predict an elevated number of VOCs in the domestic breed.

The individuals were fed with an antibiotic and then a probiotic at the commencement of the study to standardise the microbiome (Field *et al.* 2022). They were fed chicken, rice and Blackhawk kibble (Masterpet, NSW, Australia) to standardise their diet (Field *et al.* 2022). This diet was selected as it was the standard diet for Dingo Sanctuary Bargo canids. Fresh untreated rainwater was used in sanctuaries and kennels during the experiment. Scat samples were collected carefully to avoid contamination from soil and plant material. The samples were stored in sterile sealed plastic bags and were immediately stored at −80°C until assayed.

Reagents

High purity (>90%) sodium chloride, nitrobenzene (internal standard), 2-butanone, 2-heptanone, 2-nonanone, 1-octen-3-ol, 2-phenylethanol, benzyl alcohol, delta-valerolactam, methyl salicylate, quinaldine, octanal, acetophenone, benzaldehyde, hexanal, indole, nonyl aldehyde, phenylacetaldehyde, volatile free acids mix and 1000 μg/mL saturated *n*-alkane mix (C7–C30) were supplied by Sigma-Aldrich (Melbourne, Australia). GC/MS-grade methanol and dichloromethane were purchased from Thermo Fischer (Melbourne, Australia). Individual stock standard solutions (1 mg/mL) were prepared in methanol and stored in the amber glass vials at −20°C and diluted as required for quality control (QC) standard mix (20 mg/L). The *n*-alkane mix was diluted using dichloromethane (20 mg/L). The saturated sodium chloride solution was freshly prepared by diluting 8 g of NaCl in 20 mL of Milli-Q water.

HS-SPME-GC/MS analysis of all the samples was completed over six consecutive days of batch runs (for example, see Tables S1, S5). The total HS-SPME-GC/MS run time was 126.5 min for each HS vial and 15 vials were run each day. Each day, six selected scat samples were placed in a cooler of ice, homogenised in their bags and promptly weighed (300 mg) into headspace (HS) amber vials (20 mL). A total of 2 mL of the saturated NaCl solution was added to each vial before adding 10 μL of 20 mg/L nitrobenzene (internal standard) and immediately capped with magnetic crimped caps with silicone septa. NaCl solution was added to the samples to enhance their volatility (Peng and Wan 1998) and to instantly quench any enzymatic reactions that may cause changes to the volatilome should the scats have thawed. A bulk sample of dog scat was homogenised and divided into three groups: (1) untreated dog samples (n = 3), (2) treated dog samples with 100 μL of 5M sulfuric acid (Aggarwal *et al.* 2020) (pH = 2, n = 3), and (3) treated dog samples with 2 mL saturated NaCl solution (n = 3). They were spiked with 10 μL of 20 mg/L VOC standard mix, analysed by HS-SPME-GC/MS, and the resulting peak areas of the standards were compared. The saturated NaCl solution gave the most consistent and reliable results (data not shown). QC-spiked samples and QC-pooled samples were included to account for sample matrix effects and retention time shifts for each HS-SPME-GC/MS batch run. QC-spiked samples were prepared each day in two 20 mL HS vials containing 2 mL NaCl. From here, 50 mg of randomly selected scat from the same batch run was transferred into the designated QC vials and spiked with the QC standards (10 μL of nitrobenzene, *n*-alkanes, and VOC standard mix (20 mg/L)). QC-pooled samples were prepared using an additional 50 mg of scat from every biological replicate included in a batch run. For the QC-pooled samples, NaCl and nitrobenzene were added to two additional 20 mL HS vials containing the accumulated total of 300 mg of scat per batch run. During the study, we found that the *n*-alkanes added to the spiked samples were masking the detection of some of the VOCs. As additional controls, 50 mg of scat from all pooled samples were transferred to a separate HS vial containing 2 mL NaCl and stored at −20°C each day. These additional samples were not spiked and thus facilitated the analysis of VOCs masked by the alkanes in the QC-pooled sample sets. To enable background subtractions during data analyses two blanks and two QC laboratory blanks with 2 mL NaCl solution were left uncapped and exposed to the ambient laboratory environment during sample and standard preparation.

HS-SPME-GC/MS analysis

We used a Triplus RHS multipurpose sampler affixed to a Trace 1300 Series gas chromatograph (GC) with a TSQ 8000 Series triple quadrupole mass spectrometer (Thermo Fisher Scientific, Massachusetts, USA). The GC had a split/spitless injector, and the spitless mode was used with a 1.0 mm straight no-wool liner. Following the optimised HS-SPME-GC/MS methodology (from Rivers *et al.* 2019) with some modifications, scat samples, QC samples, laboratory blanks and standards were block randomised and incubated at 73°C for 15 min with continuous shaking at 350 rpm to release the VOCs into the headspace of the 20 mL HS vial. The VOCs were extracted and adsorbed onto a general-purpose SPME Arrow fibre (1.10 mm DVB/C-WR/PDMS 110 μm × 20 mm, Thermo Scientific) for 40 min at 73°C. Then the fibre was retracted from the vial and promptly inserted into the heated 260°C GC inlet for 10 min for VOC desorption. The SPME Arrow fibre was preconditioned and postconditioned for 10 min in the SPME fibre conditioning module at 230°C before sample extraction and after desorption to prevent carry-over contamination. Chromatographic separation of the VOC analytes was achieved using ultra-high

purity helium (Coregas, Australia) as the carrier gas at 1.0 mL/min along in a semistandard non-polar 30 m × 0.25 mm × 0.25 μm Agilent J&W VF-5 ms column, equipped with a 10 m EZ-Guard column. The oven temperature program was initially set at 35°C for 6 min, then ramped at 5°C/min to 150°C and held for 2 min, then ramped to 320°C at 20°C/min and held for 3 min. The total HS-SPME-GC/MS run time was 126.5 min. The GC/MS was operated in full MS scan acquisition using electron impact ionisation (EI) at 70 eV from m/z 35–450 with a solvent delay of 2 min. Temperatures for the aux transfer line was 320°C, the ion source 250°C and the quadrupoles 248°C.

Data analysis

The Gerstel Maestro software (ver. 1.5, Gerstel GmbH & Co. KG, Germany) controlled the Thermo Triplus RSH multipurpose sampler's operations, and the Thermo Fisher Scientific Xcalibur software (ver. 4.5.) was used for GC/MS data acquisition. Thermo TraceFinder software (ver. 5.1) and FreeStyle (ver. 1.8) were used for data analysis. Statistical analysis was performed using the free online MetaboAnalyst 5.0. The NIST/EPA/NIH Mass Spectral Library (ver. 2020) was used for mass spectral matching (≥70% confidence) and peak annotation. Kovats non-isothermal retention indices (RIs) were calculated for all identified peaks using *n*-alkanes (C7–C30) and compared against reference semistandard non-polar RIs from the National Institute of Standards and Technology (NIST) and PubChem databases.

For VOC concentration in scat sample relative to the nitrobenzene internal standard:

$$X = \frac{\text{VOC area}}{\text{Istd area}} \times \frac{v}{m} \times C$$

where X (ng/g) is the VOC concentration in the sample, VOC area is the peak area of the analyte in the sample, Istd area is the peak area of the internal standard in the sample, v is the volume of the HS vial (mL), m is the sample weight (g), and C is the concentration of the internal standard (ng/mL). Note: peak area is unitless.

Results

Untargeted volatilomics of scats from dingoes and German Shepherd dogs

In total, 159 VOCs, including *n*-alkanes, were detected by HS-SPME-GC/MS (Table S2). Following the set criteria set by the Metabolomics Standards Initiative (MSI), identification confidence levels of these VOCs were established as follows: (1) confirmed identification with standard, NIST library mass spectra and Kovats non-isothermal retention index (RI), (2) identification with NIST reference mass spectra and Kovats RI, or (3) putative NIST reference spectra match score (≥70% confidence). Therefore, QC standards were included in this study to confirm mass spectral matches of metabolic VOCs with similar characteristic mass spectra of mass-to-charge ion fragments. VOCs without authentic reference standards were matched to well-known NIST and PubChem mass spectral databases for identification (≥70% confidence) and peak annotation.

Following exclusion of controls, 154 VOCs were detected, identified, and quantified from the dingo and GSD scats (Table S3). Based on sample type, peak response and detection rate, the quality assurance/quality control (QA/QC) percentage relative standard deviation cut-off for VOCs detected in the pooled ($n = 8$) samples was ≤40%. Therefore, VOCs detected in these QC samples that were >40% were removed. VOCs in the pooled samples were used to normalise data across datasets for statistical analysis. Background subtractions were carried out on scat samples with VOCs matching those detected in the QC laboratory blanks to remove false positive metabolites. Samples, QC blanks, QC samples, QC standard spiked samples and QC standards were statistically block-randomised to account for possible systemic fluctuations during HS-SPME-GC/MS analysis.

Principal component analysis (PCA) illustrates the robustness of the HS-SPME-GCMS methodology (Fig. 1). The PCA scores plot shows Component 1 captured the most variation (19.8%), suggesting that the GSD highly influenced the metabolomic VOC profile.

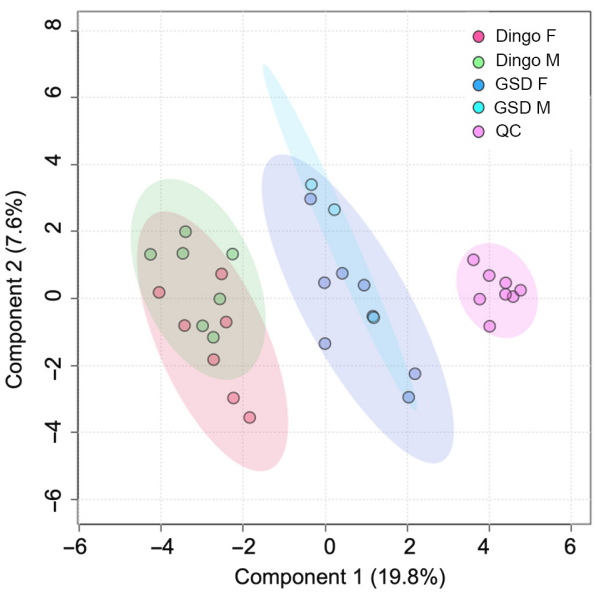

Fig. 1. Log-transformed principal component analysis of scat metabolome from female and male dingoes and German Shepherd dogs (GSD). Green and red data points represent the male and female dingoes, and purple and blue data points represent the female and male GSDs, respectively. Pink data points represent the QC samples (pooled).

Stack plots of the 154 VOC concentrations show the diverse profiles in the females (dingoes versus GSDs), and males (dingoes versus GSDs) (Fig. 2; Table S3). The VOCs were vastly different in their chemical structures and volatility: aldehydes, ketones, alcohols, hydrocarbons, carboxylic acids, esters, volatile phenols, benzenoids, terpenoids, sulfur-containing compounds and nitrogen-containing compounds, as well as miscellaneous (Table S3).

From the 154 VOCs, 58 were significant using univariate statistical analysis based on fold-changes (>1.5; also shown as log2(FC)) and *t*-tests between dingoes and GSDs. Corresponding false discovery rate (FDR) and *P*-values less than 0.5 and 0.05, respectively, were also determined (Table 1). Relative concentrations (ng/g) and the relative standard deviations of the VOCs are also shown in Table 1 and divided by sex in Table S4.

Looking at the fold changes (FC) of the 58 statistically significant VOCs, 12 were significantly higher in the dingoes and 46 in the GSDs (Table 1). We used a Chi-square test to test the hypothesis that the numbers of VOCs that were significantly higher were equal in the dingo and the GSD. This test shows that significantly fewer metabolites were found in the dingo than the GSD ($\chi^2 = 19.93$, $P < 0.0001$), suggesting a relaxation of selective constraint in the domestic breed.

We group the 12 VOCs that were significantly higher into three categories. Category 1 is present in high concentration (>10 ng/g) and unique to the dingo: 2,4-tridecanedione

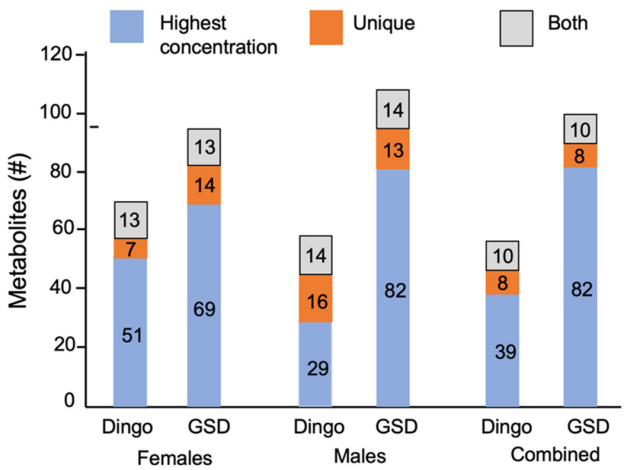

Fig. 2. Stack plot showing the number of metabolic VOCs detected in the males of dingoes ($n = 7$) versus German Shepherd dogs (GSDs $n = 3$); and females of dingoes ($n = 7$) versus GSDs ($n = 8$); both males and females equate to 154 VOCs detected (Note: 13, 14 and 10 VOCs were found in both dingoes and GSDs for females, males and combined, respectively so they are counted once). However, 147 VOCs were detected in the two collective groups, dingoes ($n = 14$) and domestic dogs ($n = 11$), where VOCs with missing values greater than 50% were removed. Blue represents the total number of VOCs showing the highest concentrations. Orange represents the number of unique VOCs detected for each group, and grey indicates the shared VOCs found in both female and male groups.

(FC 5.00), 3-undecanone (FC 4.08) and eucalyptol (FC 55.50). The elevation in eucalyptol levels is entirely due to a single individual (Pinchi) but is still considered biologically important as eucalyptol levels were detected only in the female dingoes. With Pinchi included = 41.21 ng/g ± 1.99; without Pinchi = 10.56 ng/g ± 1.23. Category 2 is present in high concentration and significantly elevated in the dingo: phenylethyl alcohol (FC 2.34), benzeneacetic acid (FC 3.07), benzeneacetaldehyde (FC 2.71), octen-2-one (FC 3.07). Category 3 is present in low concentrations (<10 ng/g): mequinol (FC 12.39), methyl 2-oxohexadecanoate (FC 12.68), butyl phenylacetate (FC 5.21), gamma-eudesmol (FC 4.67) and mesitaldehyde (FC 1.65).

For a quick visual overview, the top 48 of the 58 most significant VOCs were represented in the hierarchal cluster analysis (HCA) and heat maps (Fig. 3*a*, *b*) created by MetaboAnalyst 5.0. The dingo females and males clustered in the HCA dendrogram, shown as red and green respectively, illustrated a relatively strong discrimination of the dingo volatilome to the GSDs' females and males (dark blue and light blue clusters) (Fig. 3*a*). The heat maps show the VOCs for males and females of each population (Fig. 3*b*).

Discussion

The results from scat VOCs data support the hypothesis that there has been a relaxation of selective constraint in GSDs compared to dingoes. We found 12 statistically significant VOCs that were elevated in the dingo scats. In comparison we found 46 VOCs were elevated in the GSD, implying relaxation of metabolic efficiency in the domestic breed. Relaxation of selective constraint in domestic dogs has previously been reported. Bjornerfeldt *et al.* (2006) sequenced the complete mitochondrial DNA genome in 14 dogs, 6 wolves, and 3 coyotes. They show that domestic dogs have accumulated non-synonymous changes at a faster rate than wolves, leading to elevated levels of variation in their proteins. Cruz *et al.* (2008) compared variation in dog and wolf genes using whole-genome single nucleotide polymorphism data. The $d(N)/d(S)$ ratio (omega) was approximately 50% greater in dogs than in wolves, indicating that a higher proportion of non-synonymous alleles segregate in dogs, suggesting that slightly deleterious mutations are accumulating in the domesticated dog.

Accumulation of non-synonymous mutations in metabolic genes may influence absorption and digestion of food. Zhang *et al.* (2020) identified 50 positively selected genes associated with digestion and metabolism, suggesting dietary adaptation in dingoes. Lackey *et al.* (2020), studied fatty acid binding protein mutant mice and showed marked alterations in intestinal morphology and secretory cell abundance as well as malabsorption of a multitude of nutrients in addition to lipid. One alternate explanation is that differences in the

Table 1. Statistically significant metabolic VOCs (58) in the scats of the dingoes and German Shepherd dogs (GSD).

Metabolite	*P*-value (dingo/GSD)	FDR (dingo/GSD)	Fold change (FC) (dingo/GSD)	log2 (FC) (dingo/GSD)	GSD (ng/g, *n* = 11)	%RSD	Dingo (ng/g, *n* = 14)	%RSD
Methyl 2-oxohexadecanoate*	1.24E-06	2.74E-05	12.683	3.6648			2.36	66.62
2,4-Tridecanedione*	1.13E-05	1.34E-04	5.0029	2.3228			27.39	40.62
Benzeneacetic acid*	1.54E-03	1.13E-02	3.0696	1.6181	20.71	71.66	42.0	78.30
Mequinol*	2.63E-03	1.84E-02	12.386	3.6306			5.23	72.11
gamma-Eudesmol*	5.10E-03	3.14E-02	4.6683	2.2229			3.72	316.08
3-Octen-2-one*	6.28E-03	3.45E-02	1.5582	0.6398	8.01	63.44	12.47	31.94
3-Undecanone*	9.49E-03	5.04E-02	4.0761	2.0272			26.95	81.71
Eucalyptol*	1.51E-02	6.42E-02	55.5	5.7944			41.21	198.83
Mesitaldehyde*	1.86E-02	6.75E-02	1.6503	0.7227			2.92	62.70
Phenylethyl alcohol*	7.30E-04	4.99E-03	2.3402	0.3693	193.96	114.01	453.93	49.64
Benzeneacetaldehyde*	6.80E-05	5.40E-04	2.7123	0.4333	21.52	49.61	58.36	39.30
Butyl phenylacetate*	3.33E-02	1.05E-01	5.2111	2.3816			2.99	58.27
2,6-Di-tert-butylbenzoquinone	2.46E-11	2.71E-09	0.0034	−8.216	140.4	94.65		
2-[(2-Ethoxy-3,4-dimethyl-2-cyclohexen-1-ylidene)methyl]furan	3.52E-11	2.71E-09	0.0215	−5.5382	4.5	95.63		
Acetophenone	2.48E-08	9.66E-07	0.0648	−3.948	213.57	48.47	15.96	61.83
3,5-ditert-butyl-4-hydroxycyclohexa-2,4-dien-1-one	2.51E-08	9.66E-07	0.0718	−3.7994	13.94	53.57		
3-Phenylpropanol	8.20E-08	2.53E-06	0.0578	−4.1136	128.26	110.06	7.41	107.11
4-Formyl-2,6-di-tert-butylphenol	1.60E-07	4.11E-06	0.0036	−8.1153	15.36	95.84		
2-Methoxy-4-vinylphenol	2.43E-06	4.25E-05	0.3819	−1.3889	83.1	28.83	31.73	37.48
2-tert-Butyl-4-methoxyphenol	2.58E-06	4.25E-05	0.0148	−6.0817	2155.96	117.40	31.83	68.54
Benzenepropanal	2.76E-06	4.25E-05	0.1794	−2.4788	17.49	97.77	3.14	40.81
Khinaldin	7.19E-06	9.66E-05	0.1892	−2.4024	1400.92	38.51	305.79	63.93
Hydrocinnamic acid	7.53E-06	9.66E-05	0.088	−3.5061	583.41	69.61	55.23	76.29
Methyl 3-phenylpropanoate	1.87E-05	2.05E-04	0.1525	−2.7136	1048.73	60.21	159.88	125.73
2,6-Di-tert-butyl-p-cresol	2.09E-05	2.08E-04	0.0235	−5.4111	191.7	112.08	5.17	24.57
trans-beta-Ionone	2.16E-05	2.08E-04	0.3518	−1.5072	25.8	63.29	9.08	27.34
3-tert-Butyl-4-methoxyphenol	2.81E-05	2.54E-04	0.0004	−11.316	35.6	115.94		
Dihydroactinidolide	3.63E-05	3.11E-04	0.3067	−1.7051	11.39	61.73	3.49	30.20
Pentadecane	3.76E-04	3.05E-03	0.2647	−1.9178	16.61	53.49	5.08	68.01
2-Ethylhexanol	1.00E-03	7.70E-03	0.5857	−0.7717	49.88	27.97	29.22	42.49
1-Nonanol	4.07E-03	2.73E-02	0.5627	−0.8295	78.30	55.63	44.06	33.14
1-Heptanol	4.78E-03	3.07E-02	0.4893	−1.0312	23.02	46.67	12.91	34.06
3,3-Methylenebis-1H-indole	5.86E-03	3.37E-02	0.3251	−1.621	33.33	64.58	11.63	71.04
2-Pentadecanone	5.90E-03	3.37E-02	0.344	−1.5395	27.88	87.17	11.09	53.25
2-Thienaldehyde	1.13E-02	5.78E-02	0.6164	−0.698	0.8	16.03	0.56	23.35
Heptadecane	1.23E-02	6.10E-02	0.4828	−1.0505	16.27	57.44	9.04	42.55
Indole	1.35E-02	6.42E-02	0.493	−1.0205	22 957.11	41.64	13 155.1	65.31
Isocaproic acid	1.39E-02	6.42E-02	0.2805	−1.8338	29.4	37.62		
Octadecane	1.54E-02	6.42E-02	0.4452	−1.1675	5.39	68.90	2.77	67.66
2-Heptenal	1.55E-02	6.42E-02	0.5383	−0.8934	7.16	50.60	3.85	39.93

(*Continued on next page*)

Table 1. (*Continued*).

Metabolite	*P*-value (dingo/GSD)	FDR (dingo/GSD)	Fold change (FC) (dingo/GSD)	log2 (FC) (dingo/GSD)	GSD (ng/g, *n* = 11)	%RSD	Dingo (ng/g, *n* = 14)	%RSD
2-Nonylthiophene	4.32E-02	3.12E-01	0.5078	−0.97769			6.14	91.93
Tridecane	1.60E-02	6.42E-02	0.187	−2.4189	11.76	141.62	3.08	57.88
Hexadecane	1.63E-02	6.42E-02	0.4962	−1.0111	6.94	63.85	4.31	60.75
2-Tridecanone	1.63E-02	6.42E-02	1.5782	0.6583	29.16	33.72	26.60	46.29
2-Piperidinone	1.70E-02	6.42E-02	0.5419	−0.8839	1843.31	52.74	998.88	57.74
Methanethiol isovalerate	1.71E-02	6.42E-02	0.4191	−1.2545	23.14	103.31	14.89	114.88
Heptanal	1.89E-02	6.75E-02	0.6297	−0.6672	7.81	20.38	5.63	21.74
Methyl *cis*-9-tetradecenoate	1.93E-02	6.75E-02	0.1709	−2.5489	4.98	78.52		
Diethyltoluamide	2.39E-02	8.17E-02	3.1727	1.6657	1.65	53.72	5.23	82.35
5,6-beta-Ionone epoxide	2.49E-02	8.33E-02	0.2726	−1.8752	35.33	48.19	10.58	32.71
Phenol	2.60E-02	8.53E-02	0.4891	−1.0317	2166.57	82.75	1059.74	148.78
Tetradecane	3.27E-02	1.05E-01	0.1266	−2.9815	4.5	140.73	0.80	71.57
Ethylmethylmaleimide	3.47E-02	1.05E-01	0.488	−1.0351	91.23	69.11	44.52	51.01
1-Hexanol	3.47E-02	1.05E-01	0.6177	−0.695	77.36	46.18	54.71	53.47
Geranylacetone	3.60E-02	1.07E-01	0.6372	−0.6501	42.45	62.76	27.05	26.26
2-Decanol	3.88E-02	1.13E-01	0.5259	−0.9273	21.97	62.28	11.55	112.86
Nonanal	4.09E-02	1.17E-01	0.618	−0.6944	18.49	35.50	13.23	36.98
p-Cymene	4.57E-02	1.28E-01	8.9411	3.1604	10.04	239.6	66.66	215.16

Fold change (FC), log2(FC), false discovery rate (FDR) and *P*-values and relative concentrations (ng/g) with relative standard deviations (%) are shown for only the 58 statistically significant VOCs. Asterisks denotes VOCs significantly higher in dingoes.

population history of dingoes and GSDs has affected their metabolic efficiency. Dingoes went through a population bottleneck when they arrived in Australia some 5000 year ago whereas GSDs were developed just 200 years ago (Ballard and Wilson 2019; Field *et al.* 2020).

Of the 12 VOCs that were elevated in the dingo, three were present in high concentrations and were unique to the dingo: 2,4-tridecanedione, 3-undecanone and eucalyptol. Four VOCs were present in high concentrations and upregulated in the dingo compared to the GSD: phenylethyl alcohol, benzeneacetic acid, benzeneacetaldehyde, and octen-2-one. Five VOCs were present in low concentrations (<10 ng/g): mequinol, methyl 2-oxohexadecanoate, gamma-eudesmol, mesitaldehyde, and butyl phenylacetate (Table 1). VOCs are diverse in their chemical structures and volatility and, therefore, are ubiquitously present in mammals. This result suggests that the uniqueness of the scent for olfaction communication is not likely determined by their diet alone. Notable, however, is that multiple VOCs are expected to have antioxidant activity (Gibka *et al.* 2009; Tanapichatsakul *et al.* 2018).

As mammals share standard metabolic processes, it is unsurprising that the dingo's volatile compounds overlapped with other canines' chemical profiles. The similar chemical composition of the dingo and domestic dog observed in our study is consistent with the observations made in a previous study on five domestic dingoes, two sanctuary dingoes and six domestic dogs (Carthey *et al.* 2017). The study suggested that native prey can recognise predation risk from domestic dogs due to a similar chemical profile with the dingo, despite only ~150 years of exposure (Frank *et al.* 2016). A study with dingo urine detected 10 functional groups, including indole, alkenes, phenols, ketones, epoxides, amines, alcohols, alkanes, disulfides, and aldehydes. Of these 10, seven were shared by domestic dogs (Robley *et al.* 2015). Previous studies comparing the faecal chemical composition of the wild Iberian wolf and domestic dog faeces have also reported a considerable overlap in the chemicals (Arnould *et al.* 1998; Martín *et al.* 2010).

There is considerable overlap in the functional groups of compounds present in the scent markings of different canid species and breeds. The majority of the functional groups detected here, such as indole, phenol, ketone, amine, carboxylic acid, alcohols and aldehydes, are commonly present in the urine or scat of other canines such as wolf (Martín *et al.* 2010), African wild dog (Apps *et al.* 2012), coyote (Schultz *et al.* 1988), domestic dog (Schultz *et al.* 1985; Dzięcioł *et al.* 2018) and the red fox (McLean *et al.* 2021). We hypothesise that the relative proportion of biological material within these functional groups confers each species its unique olfactory signals, as shown in other mammal species such as spotted hyenas (Burgener *et al.* 2009; Liberles 2014). Notably, most

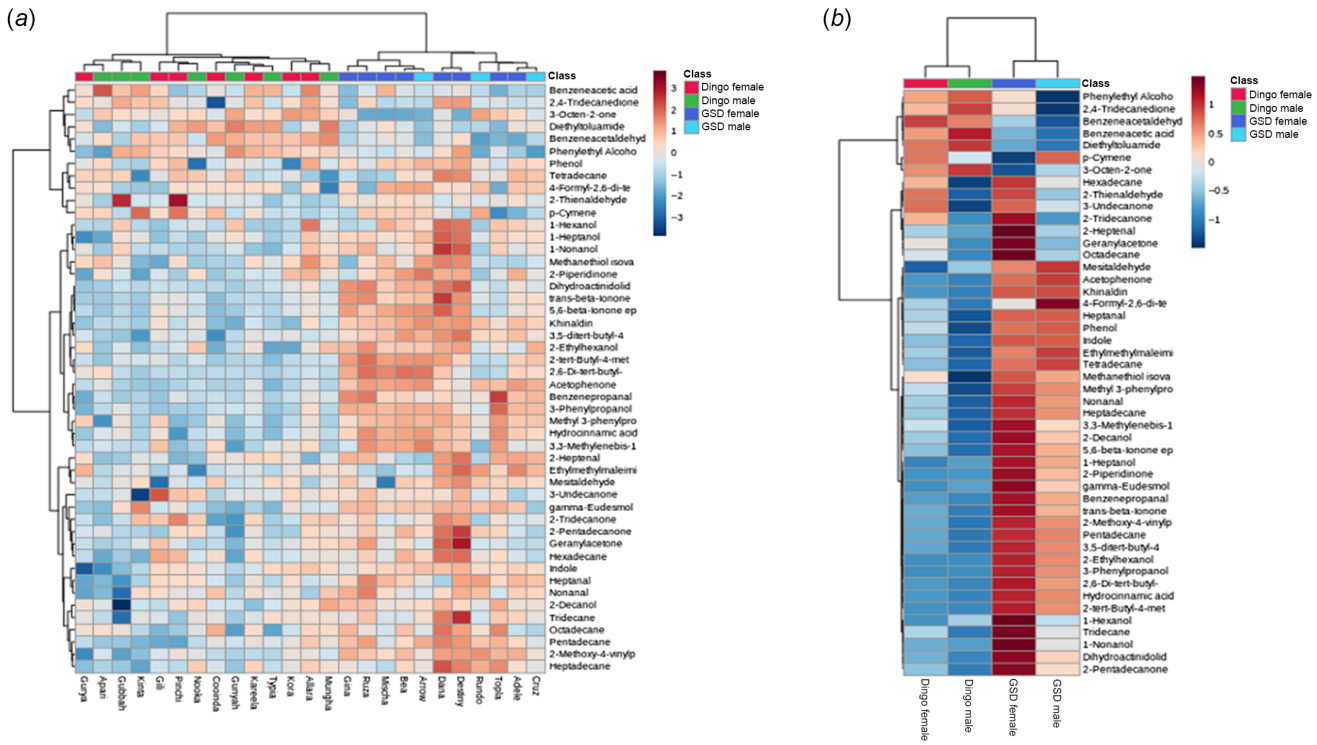

Fig. 3. Log transformed two-way hierarchal cluster analysis (HCA) and heat maps of the metabolic Volatile Organic Compound differences in all the female and male dingo and German Shephard Dog (GSD) samples. The red and blue colour spectrum scale (3 to −3 for the overview heat map and 1 to −1 for the averaged heat map) denotes the intensity of the VOC concentrations, with dark red (crimson) indicating concentrated to dark blue as being non-detected in the sample(s). (*a*) Overview heat map of the 48 most concentrated VOCs in all samples tested for dingoes (female and male) and GSDs (female and male), and (*b*) heat map showing the average linkage in the clustering of the mean concentrations of all the female and male dingo samples observed, respectively, likewise for the GSD females and males. For both (*a*) and (*b*) heat maps: green and red cluster points represent the male and female dingoes, and purple and blue cluster points represent the female and male GSDs. The dendrograms of hierarchal clustering used the Pearson correlation for the distance measured and the clustering Ward algorithm statistics.

canine studies have reported the sulfide group and its derivatives as a dominant functional group. For instance, one-third of the urine VOCs in the red fox (*Vulpes vulpes*) was odiferous sulfur compounds (McLean *et al.* 2021). Similarly, the disulfide group was the most abundant compound in the dingo urine (Robley *et al.* 2015). However, this was not the case in the scat, where most VOCs were aliphatic, consisting of diverse groups of hydrocarbons, ketones, aldehydes, esters, alcohols, and acids.

The VOCs 2,4-tridecanedione, 3-undecanone and eucalyptol were present in high concentrations and unique to the dingo. 2,4-tridecanedione is structurally like 3-undecanone with an additional carbonyl functional group. Thus far, no literature has been reported on 2,4-tridecanedione. Structurally, it is a diketone variant of 2-tridecanone, a plant metabolite with antibacterial (López-Lara *et al.* 2018) and insecticidal properties (Kimps *et al.* 2011; Gu *et al.* 2022). 3-undecanone is a terpene with antimicrobial function (Gibka *et al.* 2009). It has been determined as an attraction pheromone of the ant *Oecophylla longinoda* (Francke and Schulz 1999) and the butterfly *Heliconius erato* (Ehlers *et al.* 2021). Plausibly, elevated levels of 3-undecanone could be due to the diet, as it is often added to canine kibble (Chen *et al.* 2017). Eucalyptol, a monoterpenoid, was present in all dingo females and in high concentrations in a single individual. Eucalyptol was first identified in the oils of *Eucalyptus globulus* by French chemist François Stanislas Cloez in 1870. It constitutes 90% of *eucalyptus* oil, has been found to have insecticidal and insect-repellent properties, and has also been used in food flavourings, including meat products and baked goods (Boland *et al.* 1991). Plausibly, elevated levels of eucalyptol in dingo females are due to them chewing gum tree branches in the sanctuary.

Four VOCs were upregulated in the dingo compared to the GSD. Phenylethyl alcohol is an uncommon compound in other canids except for its detection in dog urine and faeces of the African wild dog and the anal gland and faeces of the black-backed jackal (Apps *et al.* 2012). It is, however, commonly found in the marking fluid of large cats such as the Bengal tiger (Burger *et al.* 2008), the Siberian tiger (Soso and Koziel 2016) and the African lion (Soso and Koziel 2017). The VOC is an aromatic alcohol and is known to have antimicrobial

activity and antibacterial activity (Corre *et al.* 1990), particularly inhibiting the growth of gram-negative bacteria (Lilley and Brewer 1953). Phenylethyl alcohol is significantly correlated with metabolically active bacterial OTUs (family and genus levels) such as Clostridiaceae and Lactobacillaceae (De Angelis *et al.* 2015). Phenylethyl alcohol is a by-product of the incomplete microbial degradation of phenylalanine (Dickinson *et al.* 2003). Due to the involvement of the microbial communities in the production of phenylethyl alcohol, the difference in the gut microbial composition in the dingo and the domestic dog may change the faecal compound composition (Deschamps *et al.* 2022).

Three additional VOCs were upregulated in the dingo compared with the GSD. Benzeneacetic acid has been found in the anal excretions of the African wild dog (Apps *et al.* 2012), the anal sacs of wolverines (Wood *et al.* 2005) and the red fox (McLean *et al.* 2021). Benzeneacetaldehyde can be derived from phenylalanine and has been found as communication pheromones in insects (El-Sayed 2017). Octen-2-one is one of the many scents added to dry dog kibble (Chen *et al.* 2017), which could be due to the diet fed.

Five VOCs were present in low concentrations (<10 ng/g) but higher in the dingo than the GSD (Table 1). Mequinol was only found in dingoes and was previously identified in dingo urine (Robley *et al.* 2015). Gamma-eudesmol was detected in a male GSD and has been found to have bactericidal activity from the essential oil extract of the baby sage *Salvia microphylla* (Lima *et al.* 2012). Methyl 2-oxohexadecanoate and butyl phenylacetate are found in dingoes but not GSDs. Mesitaldehyde was also detected in female GSDs.

An additional chemical of interest is khinaldin. It was significantly lower in dingo scat compared to GSDs but has been previously reported in a SPME-GC/MS of the dingo urine under the synonym quinoline, 2-methyl (Robley *et al.* 2015). It has a distinct odour and is an electron carrier in several pathways. This VOC is also found in the urine of ferrets (*Mustela furo*) and the anal sac of four skunk species, suggesting its central role in social behaviour (Jorgenson *et al.* 1978; Apps 2013; McLean *et al.* 2021). We detected other quinoline derivatives at high levels in dingo and GSD scats. Bacteria can produce quinoline derivatives, and several dihydroxyquinolines have been identified in human faeces (Hubbard *et al.* 2019). The potential role of bacteria in scent marking cannot be overlooked, as compounds produced in the anal gland and then deposited in the scat would have utility in territorial marking.

Integrated pest management, including all available control measures implemented in a coordinated manner at a landscape scale, is a recommended control method for wild dogs in Australia. These include baiting, trapping, shooting, exclusion fencing and guardian animals. These strategies can adversely affect the regulating impact of herbivory in an ecosystem (Morris and Letnic 2017; Fisher *et al.* 2021) or production system (Choquenot and Forsyth 2013). This study takes the first step in identifying potential population-specific chemical cues in scat as a step towards supplementing existing strategies with chemical approaches aimed at excluding pure dingoes from agricultural lands.

Supplementary material

Supplementary material is available online.

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Data availability. The data that support this study will be shared upon reasonable request to the corresponding author.

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Author contributions. JWOB collected the scat samples and TL prepared samples for VOC analysis. TL and TT carried out solid phase microextraction (SPME) gas chromatography–mass spectrometry (GC-MS). TL and TT carried out the GC/MS data analysis. TL conducted the statistical analyses and wrote the initial draft of the manuscript. SY conducted a preliminary analysis that aided this study. All authors read and approved the final version of the manuscript.

Ethics approval. Samples were collected under the ASAB/ABS guidelines and were approved by the Institutional Animal Care and Use Committee of the University of New South Wales (18/148B).

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