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Putative Chemosignals of the Ferret (*Mustela furo*) Associated with Individual and Gender Recognition

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Abstract

Quantitative stir bar sorptive extraction methods, both in the aqueous and headspace modes, followed by thermal desorption gas chromatography–mass spectrometry were used to investigate individual variations in the volatile components of male and female ferret (*Mustela furo*) urine. The urinary profiles were further compared with volatile profiles of anal gland secretions of breeding male and female ferrets. Thirty volatile compounds were quantified in male and female urine. Among them, 2-methylquinoline was unique to male urine. Four ketones (4-heptanone, 2-heptanone, *o*-aminoacetophenone, and a dimethoxyacetophenone) and several nitrogen compounds (e.g., 2,5-dimethylpyrazine, quinoline, 4-methylquinazoline) and low levels of three unidentified nonsulfur compounds were significantly more abundant in males than in females. Quantitative comparison of 30 volatile urinary compounds showed several statistically significant differences between the sexes and individuals of the same sex. These findings suggest that ferrets may use urine marking for sex and individual recognitions. Ten of the 26 compounds identified in anal gland secretions from females and males were also found in urine. However, most of the major compounds (thietanes, dithiolanes, and indole) in anal glands were not present in urine. This suggests that urine may convey specific signals that differ from those of anal glands. Additionally, 10 volatiles (two aldehydes, five ketones, benzothiazole, 2-methylquinoline, and 4-methylquinazoline), not previously identified, were found in ferret anal gland secretions. Among the new compounds, *o*-aminoacetophenone was found only in males, while only traces of this compound were found in females. Similar results were previously obtained in anal glands of three other *Mustela* species. These findings provide new information about the constituents of urine and volatile components of anal gland secretions in ferrets.

Key words: anal gland secretion, chemical signals, GC-MS, *Mustela furo*, stir bar sorptive extraction, urine

Introduction

Chemical signals in carnivores are both diverse and redundant. Multiple scent sources such as urine, feces, and anal gland secretions are widely involved in recognition of sex, individuality, reproductive condition, and social status (Macdonald, 1985). The genus *Mustela* is known to emit odors from the anal glands, and chemical profiles of anal gland secretions from seven *Mustela* species have been reported (Brinck *et al.*, 1983; Zhang *et al.*, 2002, 2003). Sex and individual recognitions by anal gland secretions have been reported in ferrets (*Mustela furo*) and stoats (*Mustela erminea*) by several investigators (Erlinge *et al.*, 1982; Clapperton *et al.*, 1988, 1989; Kelliher and Baum, 2001; Woodley and Baum, 2003, 2004). There have also been reports on the contribution of urinary scents to social communication in *Mustela*. Urine-mediated behavioral effects have been demonstrated in mink (*Mustela vison*) (Gerell, 1970; Birks, 1981), European polecat (*Mustela putorius*) (Sokolov and Rozhnov, 1983), and ferret (*M. furo*) (Clapperton, 1987). However, urinary compounds were not characterized in these studies. In one study, which included only one male and one female ferret, urine was chemically characterized using a labor-intensive fractionation by a series

of liquid–liquid extractions or distillation of large urine volumes, followed by gas chromatography (GC) mass spectrometry (MS) analysis (Schildknecht *et al.*, 1986).

Mammalian chemical signals are characteristically complex. Chemical analysis is thus particularly important, being often the first step in deciphering a complex chemical signal (Sun and Müller-Schwarze, 1998). The ferret has been among the most extensively studied models of chemosensory communication in carnivores (Crump, 1980b; Clapperton, 1987; Clapperton *et al.*, 1988, 1989; Weiler *et al.*, 1999; Kelliher *et al.*, 2001; Kelliher and Baum, 2002; Woodley and Baum, 2003).

Malodorous carnivore secretions are thought to originate from sulfur-containing compounds derived from high-protein diets (Brinck *et al.*, 1983; Mason *et al.*, 1994; Wood *et al.*, 2002). However, chemical analysis has revealed multiple cases in which sulfur-containing compounds of certain carnivore secretions and excretions were not present or occurred only at low levels. This was found with both urine and subcaudal gland secretions of badgers (*Meles meles*) (Service *et al.*, 2001; Buesching *et al.*, 2002a,b) and anal gland secretions of *Martes* (Schildknecht and Birkner, 1983). Anal gland secretions of *Canis* and *Vulpes* genera (Preti *et al.*, 1976; Raymer, 1984; Raymer *et al.*, 1985) did not contain sulfur compounds. However, their urine was found to be an abundant source of such substances (Jorgenson *et al.*, 1978; Raymer, 1984; Raymer *et al.*, 1986; Schultz *et al.*, 1988). The majority of identified anal gland volatiles of seven species of the genus *Mustela* were sulfur-containing compounds that are unique to this genus (Brinck *et al.*, 1983; Crump and Moors, 1985; Zhang *et al.*, 2002, 2003). Only one study (Schildknecht *et al.*, 1986) has reported the presence of sulfur compounds in ferret urine, albeit not quantitatively.

Mammalian pheromones, by definition, compounds affecting physiology and/or behavior within the same species (Johnston, 2003) in voided urine, may originate from general metabolism (bladder urine) or from excretions of specialized urethral glands (Harvey *et al.*, 1989; Novotny *et al.*, 1990). The origin of urinary pheromones can be clarified by either a link to a particular anatomical structure (Brahmachary and Dutta, 1987; Clapperton, 1987) or through chemical analysis (Harvey *et al.*, 1989; Novotny *et al.*, 1990).

Eighteen volatiles have been identified in the anal gland secretions from seven species of the genus *Mustela*, including the American mink (*M. vison*) (Brinck *et al.*, 1978; Sokolov *et al.*, 1980), the stoat and the ferret (Crump, 1980a,b; Crump and Moore, 1985), the mountain weasel (*Mustela nivalis*) and the European polecats (*M. putorius*) (Brinck *et al.*, 1983), the steppe polecat (*Mustela eversmanni*), and the Siberian weasel (*Mustela sibirica*) (Zhang *et al.*, 2002, 2003). These volatiles include 2 nitrogen-containing compounds (indole and *o*-aminoacetophenone) and 16 sulfur-containing compounds. A more recent investigation of anal gland secretions of the steppe polecat (*M. eversmanni*) and the Siberian weasel (*M. sibirica*) found *o*-aminoacetophenone in both sexes and both species (Zhang *et al.*, 2003). Previously, the presence of *o*-aminoacetophenone has been confirmed only in male stoat (Crump, 1980a; Brinck *et al.*, 1983; Zhang *et al.*, 2002). Use of different analytical methods may have contributed to the reported differences in composition of volatile compounds in complex biological samples. It is known that different sample preparation procedures can greatly influence recoveries of the compounds of interest (Soini *et al.*, 2005). Knowing the nature of ferrets' chemical signaling mechanisms in a more quantitative manner could elucidate the nature of possible prey–predator cues. This could potentially lead to identification of substances that are useful for either predator or prey (rodent) population management.

In the present study, we have employed a relatively new and quantitative stir bar sorptive extraction (SBSE) method in both the aqueous and headspace sorptive extraction (HSSE) modes to analyze different ferret specimens. The methodology (Baltussen *et al.*, 1999, 2002; Soini *et al.*, 2005) involves the quantitative isolation and precise comparison of the levels of a wide range of volatile and semivolatile compounds in odorant-containing body fluids. We have, subsequently, measured the individual variation in volatile compounds of male and female ferret urine during the breeding season. Moreover, to determine possible anal gland metabolic contributions to the urinary scents, we have compared the composition of anal gland secretions from male and female ferrets to the urinary component profiles.

Materials and methods

Animals

For urine collection, four adult male and five adult female ferrets (*M. furo*) in breeding condition (about 1 year of age) were purchased from Marshall Farms (North Rose, NY). Upon arrival at the Boston University Animal Facility, they were individually housed in modified rabbit cages under a long-day (16:8, light:dark) photoperiod. The ferrets were fed moistened Purina ferret chow once a day, while water was available *ad libitum*.

Urine and anal gland collections

To collect urine, ferrets were placed individually in a clean cage with a grid floor and a clean collecting pan underneath. As soon as an animal urinated, its urine was collected and stored on ice. Urine that was deposited with or next to feces was not collected. Urine of each of four males and three females was collected on two different occasions, separated by 1–2 weeks, from late August to mid-September 2002. Urine of two females was collected once in late July 2002. All urine samples were frozen at −20°C until being analyzed.

Veterinarians at Marshall Farms removed anal sacs from three anesthetized mature males (more than 1 year old) and from females (two were 6 months old and one was more than

1 year old), while they were in breeding condition, during August 2000 and 2003. Anal glands were frozen immediately at −20°C and kept frozen until being analyzed. Frozen urine and anal gland samples were shipped overnight on dry ice to Indiana University where the analyses were conducted. Samples were thawed just prior to the sample preparation for analysis.

SBSE of urine samples

All glassware was washed first with distilled water, followed by acetone, and dried at 80°C for 1 h. Urine samples were diluted with water (0.5 ml urine and 0.5-ml high-purity OmniSolv water, EM Science, Gibbstown, NJ). As an internal standard (IS), 16 ng of 7-tridecanone (Aldrich Chemical Company, Milwaukee, WI) was added in 20 µl of ethanol. Volatile and semivolatile compounds were extracted from diluted urine in a sealed 20-ml glass vial by sorptive extraction with a Twister polydimethylsiloxane (PDMS) polymer–coated stir bar (10 mm, 0.5 mm film thickness, 24 µl PDMS volume, Gerstel GmbH, Mülheim an der Ruhr, Germany) for 60 min. Stirring speed was >800 rpm on the Variomag Multipoint HP 15 stir plate (H+P Labortechnic, Oberschleissheim, Germany). Extraction temperature was 20°C.

After extraction, a stir bar was rinsed with a small volume of high-purity water, dried gently on paper tissue, and placed in the glass injector liner for thermal desorption and MS measurements. After the analysis, stir bars were reconditioned in a stream of helium (60 ml/min, 250°C) for about 30 min. Our aqueous sampling procedure is illustrated in Figure 1A.

HSSE of anal gland secretion

Approximately 5 mg of anal gland secretion was placed on the weigh paper and absorbed from there into a piece of Kimax-51 glass micropipette (Kimble Products, Vineland, NJ). The tube was placed on aluminum foil in a 20-ml glass vial (similar vial as used for the urine analysis). The headspace sampling procedure is shown in Figure 1B. Before sealing the vial, a stir bar was positioned (hanging on the vial air space above the sample) using a stainless steel wire (Tienpont *et al.*, 2001, 2002; Bicchi *et al.*, 2002). The vial was kept at 50°C in a water bath for 60 min, while trapping the volatiles into the PDMS phase on the stir bar. After sampling, the stir bar was moved into a clean glass injector liner for thermal desorption and GC-MS or GC analysis using atomic emission detection (AED).

GC-MS analysis

A Finnigan MAT Magnum ion trap GC-MS system was used for compound identification and quantification(Finnigan MAT, San Jose, CA). The system had a DB-5 capillary column [30 m × 0.25 mm, internal diameter (i.d.), 0.25 µm film thickness, J&W Scientific, Folsom, CA]. The helium carrier gas head pressure was 12 psi. At the beginning of the column,

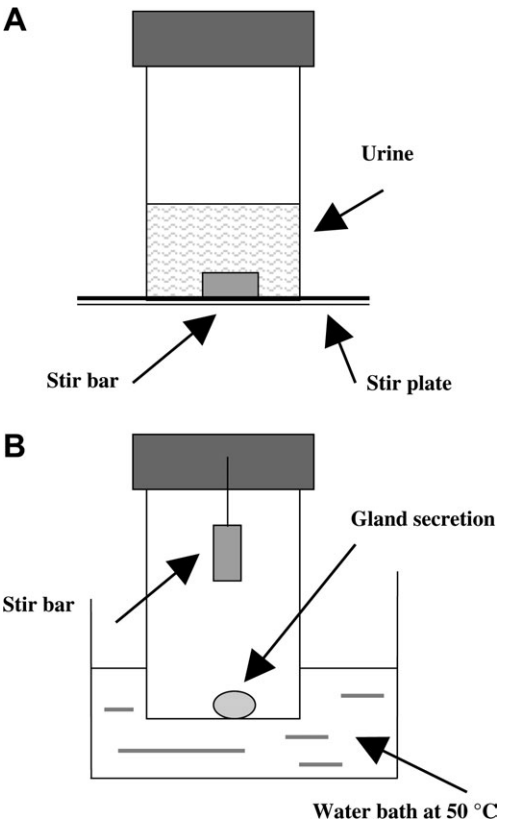

Figure 1 Sampling by the stir bar–extraction methodology. **(A)** Aqueous stir bar extraction and **(B)** headspace stir bar extraction.

a loop of uncoated deactivated silica tubing (30 cm × 0.25 mm, i.d.) was attached using a universal Press-Tight Connector (Restek Corporation, Bellefonte, PA) as described earlier (Ma *et al.*, 1999). The loop was cooled by liquid nitrogen, while the Twister stir bar was held in the injector liner for 15 min at 250°C for thermal desorption of the analytes. Subsequently, the desorbed compounds were cryotrapped in the liquid nitrogen–cooled loop. After discontinuing the cooling step, the GC oven temperature was held at 40°C for 5 min and increased to 200°C at the rate of 2°C/min. The final temperature was held for 10 min. The manifold and transfer line temperatures were 220°C and 300°C, respectively. The ion trap was operating in the positive electron ionization mode (70 eV). Spectra were scanned from 40 to 350 m/z (1 scan/s).

Sulfur-selective detection by GC-AED

Gas-chromatographic equipment used for quantitative analysis consisted of an Agilent GC Model 6890 system with Agilent ChemStation software and an AED Model G2350A (Agilent Technologies, Inc., Wilmington, DE) and a thermal desorption autosampler (TDSA, Gerstel GmbH). The separation capillary was DB-5 (30 m × 0.25 mm, i.d., 0.25 µm film thickness from J&W Scientific). Samples were thermally

Table 1 Sexual differences in urine volatile compounds

Peak number	Compounds	MW	Formula	Normalized GC-MS TIC PA (PAs divided by the ISA)		Mann–Whitney *U*-test–Wilcoxon Rank Sum *W*-test		
				Male	Female	*U*	*W*	*P* (n = 4–5)
1	Acetic acid	60	$C_4H_4O_2$	7.09 ± 9.94	19.01 ± 5.31	26.0	58.0	0.8665
3	Hexanal	100	$C_6H_{12}O$	3.45 ± 2.37	7.21 ± 5.93	15.0	36.0	0.2824
7	4-Heptanone	114	$C_7H_{14}O$	1766.63 ± 0.86	51.84 ± 33.35	0.0	100.0	0.0002
9	2-Heptanone	114	$C_7H_{14}O$	111.51 ± 67.84	15.86 ± 13.50	2.0	98.0	0.0006
11	Heptanal	114	$C_7H_{14}O$	2.53 ± 0.80	4.81 ± 3.22	17.0	53.0	0.1304
12	2,5-Dimethylpyrazine	108	$C_6H_8N_2$	1974.26 ± 1292.21	70.91 ± 65.05	0.0	100.0	0.0002
15	3-Ethylcyclopentanone	112	$C_7H_{12}O$	21.68 ± 15.34	54.87 ± 39.56	18.0	54.0	0.1605
16	Benzaldehyde	106	C_7H_6O	10.11 ± 10.10	20.94 ± 16.61	20.0	56.0	0.2345
17	6-Methyl-5-hepten-2-one	126	$C_8H_{14}O$	9.67 ± 10.81	6.61 ± 6.19	25.0	75.0	0.5054
18	6-Methyl-6-heptenone	126	$C_8H_{14}O$	1.00 ± 1.18	3.08 ± 3.36	12.0	33.0	0.3939
20	Unidentified			13.25 ± 17.45	31.60 ± 24.97	10.0	31.0	0.1375
21	Octanal	128	$C_8H_{16}O$	12.00 ± 12.19	5.52 ± 2.07	11.0	26.0	0.3434
24	Unidentified			3.70 ± 1.41	3.80 ± 2.24	30.0	70.0	0.8785
25	Acetophenone	120	C_8H_8O	41.60 ± 34.64	21.25 ± 31.52	17.0	45.0	0.2319
27	Unidentified			24.00 ± 17.70	1.59 ± 2.38	1.0	29.0	0.0006
28	Unidentified			1.12 ± 0.97	0.31 ± 0.59	12.5	87.5	0.0379
29	Nonanal	142	$C_9H_{18}O$	36.41 ± 10.50	27.61 ± 17.48	21.0	79.0	0.2768
31	Unidentified			1.50 ± 2.62	4.42 ± 3.84	3.0	13.0	0.1111
33	Unidentified			3.65 ± 2.50	1.36 ± 1.32	3.5	22.5	0.2000
34	2-Decanone	156	$C_{10}H_{20}O$	12.10 ± 6.23	16.94 ± 4.63	17.0	53.0	0.1304
35	Decanal	156	$C_{10}H_{20}O$	5.64 ± 4.76	8.81 ± 9.27	28.0	64.0	0.7209
36	Benzothiazole	135	C_7H_5 NS	6.46 ± 3.53	5.07 ± 5.83	12.0	27.0	0.6623
38	Quinoline	129	C_9H_7 N	3073.95 ± 2069.78	321.55 ± 231.28	10.0	90.0	0.0207
40	*o*-Aminoacetophenone	135	C_8H_9NO	58.66 ± 48.87	1.12 ± 2.95	2.0	82.0	0.0012
41	2-Methylquinoline	143	$C_{10}H_9N$	32.98 ± 27.08	0.00 ± 0.00	8.0	61.0	0.0426
42	4-Methylquinazoline	144	$C_9H_8N_2$	501.29 ± 378.72	6.40 ± 11.32	0.0	100.0	0.0002
43	1-Undecanol	172	$C_{11}H_{24}O$	22.45 ± 2.83	19.93 ± 7.61	19.0	81.0	0.1949
45	Unidentified			35.66 ± 27.90	0.36 ± 0.87	2.0	23.0	0.0027
46	Geranylacetone	194	$C_{13}H_{22}O$	10.19 ± 5.75	11.30 ± 10.18	27.0	57.0	0.9551
47	IS (7-tridecanone)	198	$C_{13}H_{26}O$	100.00 ± 0.00	100.00 ± 0.00	32.0	68.0	1.0000
48	Dimethoxyacetophenone	180	$C_{10}H_{12}O_3$	82.74 ± 60.68	22.54 ± 39.84	12.0	88.0	0.0379

MW, molecular weight; TIC, total ion chromatogram.

desorbed in a TDSA-automated system, followed by injection into the column with a cooled injection system CIS-4. The TDSA operated in the splitless mode. The temperature program for desorption was 20°C (0.5 min) and then 60°C/min to 280°C (10 min). The temperature of the transfer line was set at 280°C. The CIS was cooled with liquid nitrogen to −60°C. After desorption and cryotrapping, the CIS was heated at 12°C/s to 280°C with a hold time of 10 min. The temperature program in the GC oven was 40°C for 5 min and then increased to 200°C at the rate of 2°C/min. The final temperature was held for 10 min. The carrier gas head pressure was 14 psi (flow rate, 1.2 ml/min). The

GC unit was operated in the constant-flow mode. The emission lines for carbon (193 nm), sulfur (181 nm), and nitrogen (174 nm) were monitored during AED.

Identification of volatile compounds in urine and anal gland secretions

Compounds in urine and anal gland secretions were identified by comparing their GC-retention indices and mass spectra to reference compounds and literature citations. Standard compounds 4-heptanone, 2-heptanone, 6-methyl-5-hepten-2-one, nonanal, 2,5-dimethylpyrazine, indole, benzothiazole, quinoline, *o*-aminoacetophenone, 2-methylquinoline, and geranylacetone were obtained from Aldrich Chemical Company. The compounds identified in urine and anal glands are shown in Tables 1 and 2, respectively.

Quantitative analytical data

Peak areas (PAs) were integrated using Magnum GC-MS software (Finnigan MAT). PAs were normalized by dividing a respective PA by the PA of an IS (ISA) (i.e., PA×100/ISA). Statistical comparisons of concentrations between males and females were performed using a nonparametric Mann–Whitney *U*-test. Significance was accepted at $P < 0.05$.

Hierarchical cluster analysis is a statistical method for finding relatively homogenous clusters of cases, as based on measured characteristics. We used hierarchical cluster analysis (average linkage), with Pearson correlation coefficients being used to test the similarity of inter- and intraindividual urine volatiles in the samples collected at 1- or 2-week intervals.

Results

Volatile constituents in urine and anal gland secretion

In the urine of four male and five female ferrets, 30 volatile compounds were selected for quantitative comparisons among the GC-MS–separated compounds. Selection criteria were based on sufficient spectral intensities and adequate peak purity. The respective compounds and their normalized PAs (PAs divided by the ISA) and standard deviations are shown in Table 1. Acetic acid, 6 aldehydes, 10 ketones, 2 quinolines, 4-methylquinazoline, 2,5-dimethylpyrazine, benzothiazole, and 1-undecanol were tentatively identified. Seven compounds remained unidentified, with the presence of sulfur in these molecules being ruled out through the element-specific AED.

Six heterocyclic nitrogen compounds were also identified. A representative urinary total ion chromatogram profile of the male ferret is shown in Figure 2. Chromatogram blanks also revealed several compounds originating from environmental material sources, such as siloxanes, phthalates, antioxidants, and plastic softeners (data not shown).

In anal glands of three females and three males, 26 volatile compounds were selected for quantitative comparisons among the detected compounds using the same selection criteria as with the 30 compounds selected in urine volatile profiles (Table 2 and Figure 3).

Table 2 Volatile compounds in anal gland samples from ferrets (*Mustela furo*)

Peak number	MW and formula	Identification
2	102, $C_5H_{10}S$	2,2-Dimethylthietane
4		*Z*- or *E*-2,4-Dimethylthietane
5		*E*-2,3-Dimethylthietane
6		2-Ethylthietane
8	116, $C_6H_{12}S$	*E*-2-Ethyl-3-methylthietane
10		2-Isopropylthietane
13		*Z*-2-Ethyl-3-methylthietane
14		2-Propylthietane
16	106, C_7H_6O	Benzaldehyde
17	126, $C_8H_{14}O$	6-Methyl-5-hepten-2-one
19	128, $C_8H_{16}O$	2-Octanone
22	134, $C_5H_{10}S_2$	3-Ethyl-1,2-dithiolane (only females)
23		3,3-Dimethyl-1,2-dithiolane
25	120, C_8H_8O	Acetophenone
26	134, $C_5H_{10}S_2$	*E*-3,4-Dimethyl-1,2-dithiolane
29	142, $C_9H_{18}O$	Nonanal
30	134, $C_5H_{10}S_2$	*Z*-3,4-Dimethyl-1,2-dithiolane
32	144, $C_8H_{16}S_2$	2-Pentylthietane
36	135, C_7H_5 NS	Benzothiazole
37	148, $C_6H_{12}S_2$	3-Propyl-1,2-dithiolane
38	129, C_9H_7 N	Quinoline
39	117, C_8H_7N	Indole
40	135, C_8H_9NO	*o*-Aminoacetophenone
41	143, $C_{10}H_9N$	2-Methylquinoline
42	144, $C_9H_8N_2$	4-Methylquinazoline
46	194, $C_{13}H_{22}O$	Geranylacetone

MW, molecular weight.

Nine thietanes, five dithiolanes, two aldehydes, five ketones, two quinolines, indole, benzothiazole, and a methylquinazoline were identified. Fourteen sulfur-containing compounds represented the majority of the anal gland molecules detected. A comparison of the sulfur compound profiles in male ferret anal gland and male urine (measured by the element-specific GC-AED sulfur line) is shown in Figure 4 which demonstrates an overwhelming abundance of sulfur compounds in anal gland secretion over urine. Ten volatile compounds were found both in ferret urine and anal gland samples: two aldehydes (peak 16, benzaldehyde; peak 29, nonanal), four ketones (peak 17, 6-methyl-5-hepten-2-one;

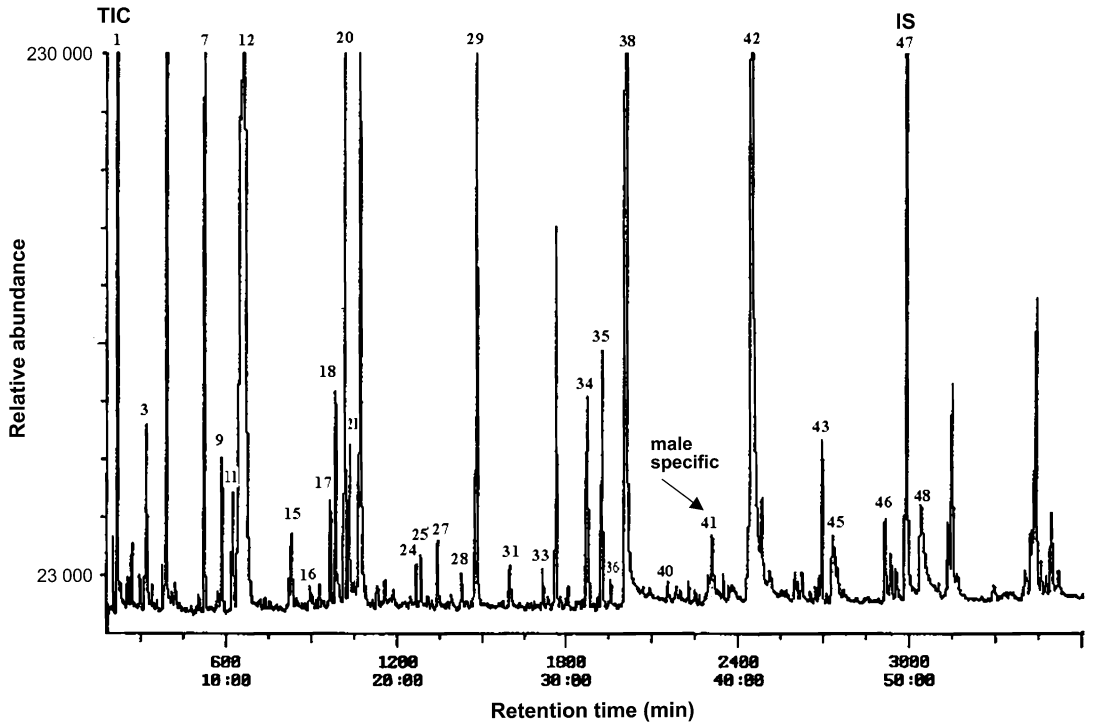

Figure 2 Representative total ion chromatogram (TIC) of volatile compounds from male ferret (*Mustela furo*) urine as measured by GC-MS. Number for each peak corresponds to peak number given in Table 1.

peak 25, acetophenone; peak 40, *o*-aminoacetophenone; peak 46, geranylacetone), two quinolines (peak 38, quinoline; peak 41, 2-methylquinoline), benzothiazole (peak 36), and 4-methylquinazoline (peak 42). The compound numbers are listed in Tables 1 and 2.

Sex differences in volatile urinary and anal gland secretions

Analysis of 16 samples from male (n = 4) and female (n = 5) ferrets revealed the presence of a male-specific compound, 2-methylquinoline (peak 41), in urine. In addition, several compounds were significantly more abundant in male than in female urine. The numbered compounds (Table 1) were four ketones (peak 7, 4-heptanone; peak 9, 2-heptanone; peak 40, *o*-aminoacetophenone; peak 48, dimethoxyacetophenone), 2,5-dimethylpyrazine (peak 12), quinoline (peak 38), and 4-methylquinazoline (peak 42), while 27, 28, and 45 represent unidentified compounds.

In the anal gland secretions from three males and three females, 3-ethyl-1,2-dimethyl-1,2-dithiolane (compound 22) was found only in females. Certain compounds were more abundant in females, while others were more enriched in males. For example, compounds *E*-2,3-dimethylthietane and *E*- and *Z*-3,4-dimethyl-1,2-dithiolane (compounds 5, 26, and 30) were more abundant in females. The abundant male compounds were 2-propylthietane, 3,3-dimethyl-1,2-dithiolane, 3-propyl-1,2-dithiolane, and a nitrogen-containing compound, *o*-aminoacetophenone (peaks 14, 23, 37, and 40, respectively). The numbers are referenced in Table 2 and Figure 3. Figure 5 shows differences in the abundance of sulfur-containing compounds in male and female ferret anal glands, as measured by GC-MS.

Sex-related individual variations in volatile urinary profiles

Hierarchical linkage cluster (average linkage) analysis was performed on the normalized, integrated GC PAs of 30 relevant volatile compounds found in male and female ferret urine. Urine samples "a" were collected from four male (M_x a) and four female (F_x a) ferrets (subscript x indicates the number of a particular animal). After 1–2 weeks, the urine samples "b" were collected from males and females (M_x b and F_x b). The average linkage dendrogram based on normalized data as variables is shown in Figure 6. The closer the vertical linkage between individual ferrets (or ferret groups) is positioned to zero in the cluster distance axis (x-axis), the more similar data properties for individuals or groups are measured (in this case a volatile compound profile of 30 relevant compounds). In males, cluster analysis has shown that urine composition in samples a and b remained relatively constant across all individuals (cluster distance <3). Also, males 1, 3, and 4 were more similar than male 2 (clustered separately), with respect to their urine composition. Female urine composition showed dissimilarities between a and b samples (variable cluster distances). This may indicate that female urine composition fluctuates

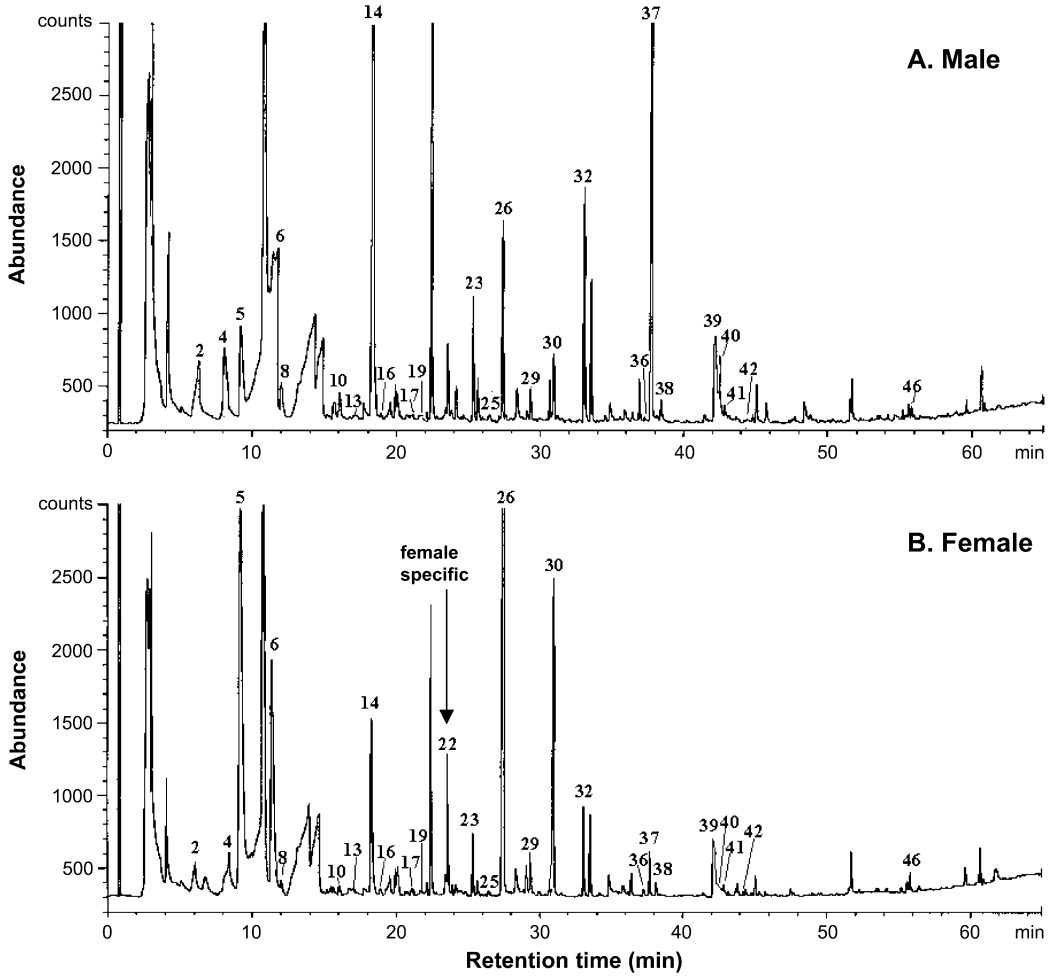

Figure 3 Representative GC profiles of volatile compounds from anal gland secretions of **(A)** a male and **(B)** a female ferret (*Mustela furo*) measured by the element-specific GC-AED carbon line at 193 nm. The number for each peak corresponds to peak numbers given in Table 2.

substantially within a relatively short time. Females 4 and 5 appeared to be more similar to each other than to other females (cluster distance <5). The urinary profiles in females 4 and 5 were relatively similar, except for 2,5-dimethylpyrazine which was twice as high in females 5a and 5b than in females 4a and 4b (data not shown).

Discussion

Comparison of anal gland and urinary volatiles

Although many compounds were common to urine and anal gland secretions, none of the major anal gland secretion constituents (thietanes, dithiolanes, and indole) were found in ferret urine. This suggests that anal gland secretions do not contribute to urinary odors, implying that urine may have its own significance in chemical signaling. Previous chemical and behavioral tests revealed that the above-mentioned major compounds originating from ferret anal glands could act as chemosignals for sex discrimination (Crump, 1980b; Crump and Moors, 1985; Clapperton *et al.*, 1988, 1989; Cloe *et al.*, 2004; Woodley *et al.*, 2004). At present, it is not known whether any of the common volatile compounds found to be present in anal glands and urine affect social communication.

Three of the compounds that were common to both urine and anal glands (38, quinoline; 41, 2-methylquinoline; and 42, 4-methylquinazoline) showed statistically significant differences between males and females in their urinary concentrations (see Table 1). Consequently, these compounds may be important in communicating information about sex. In other carnivore species, 2-methylquinoline was found in the anal glands of skunks (Wood *et al.*, 2000) and in the urine of red fox (*Vulpes vulpes*) and coyote (*Canis latrans*) (Jorgenson *et al.*, 1978; Raymer, 1984).

An abundance of sulfur-containing compounds has been observed in the anal gland secretions of certain carnivores, for example, genus *Mustela* of *Mustelinea* subfamily and in three other genera: *Conepatus*, *Mephitis*, and *Spilogale* in *Mephitinae* subfamily (Brinck *et al.*, 1983; Wood *et al.*, 2002). Sulfur compounds cannot be viewed as "signature

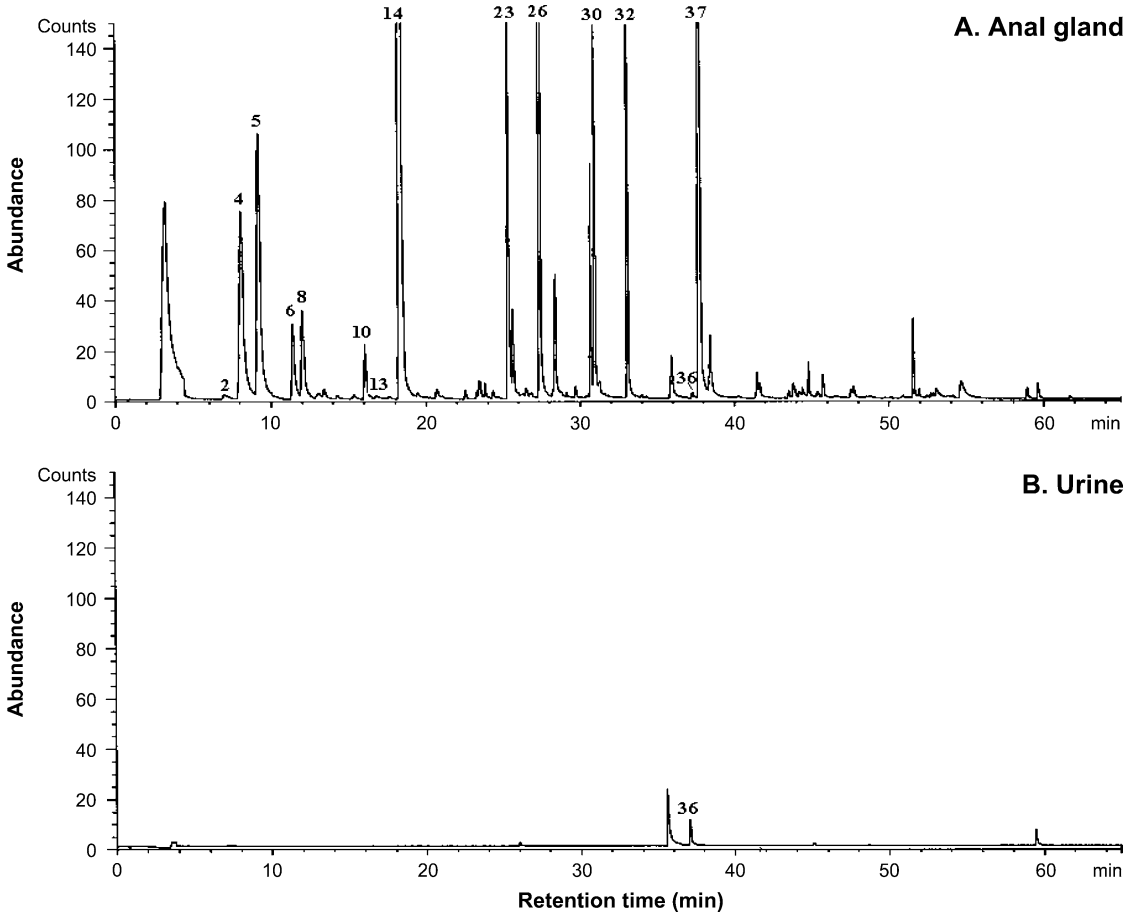

Figure 4 Sulfur compound profiles in male ferret (*Mustela furo*) from **(A)** an anal gland and **(B)** urine, measured by element-specific GC-AED at 181 nm. The number for each peak corresponds to the peak numbers given in Tables 1 and 2, respectively.

compounds'' for carnivores since numerous carnivores lack such compounds in their urine and anal gland secretions. Thus, urinary and subcaudal gland volatiles in badgers (*M. meles*) did not contain sulfur compounds (Service *et al.*, 2001; Buesching *et al.*, 2002a,b), and major malodorous compounds in the anal gland secretions of *Martes* genus are nonsulfur-containing organic acids (Schildknecht and Birkner, 1983). Anal gland secretions of *Canis* and *Vulpes* genus, for example, dogs (*Canis familiaris*), coyotes (*C. latrans*) (Preti *et al.*, 1976), wolves (*Canis lupus*), and red foxes (*V. vulpes*), also lack sulfur-containing compounds, although high concentrations of sulfur-containing compounds were found in urine of these species (Jorgenson *et al.*, 1978; Raymer, 1984; Raymer *et al.*, 1985, 1986; Schultz *et al.*, 1988). While ferret anal gland secretions were rich in sulfur-containing compounds, the absence of such compounds in urine (other than the ubiquitous benzothiazole seen in this study) was striking (Figure 4). Some sulfur-containing compounds have also been found in noncarnivore urine. Male house mouse (*Mus musculus*) urine contains heterocyclic thiazol derivatives and a disulfide (Harvey *et al.*, 1989), while male black-tailed deer (*Odocoileus virginianus*), a typical herbivore, exhibits urinary ethyl thiolacetate and dimethylsulfide (Miller *et al.*, 1998). In the current study, a nitrogen heterocyclic quinoline (compound 38) was a predominant compound in the urine and anal glands of both male and female ferrets. Schildknecht *et al.* (1986) was first to confirm the presence of quinoline in ferret urine through a fractionation approach, although that study did not address quantitative issues.

Several other compounds (benzaldehyde, nonanal, 6-methyl-5-hepten-2-one, 2-octanone, acetophenone, geranylacetone, and benzothiazole), not reported previously in the ferret, were found in urine and anal gland secretions, but these are fairly common metabolic products. Interestingly, *o*-aminoacetophenone, known as a strong ''weasel smell'' compound, was found in this study for the first time in ferret anal glands, although it has been reported in anal gland secretions of male stoat (Crump, 1980a; Brinck *et al.*, 1983), both sexes of the steppe polecat (*M. eversmanni*), and the Siberian weasel (*M. sibirica*) (Zhang *et al.*, 2003). Schildknecht *et al.* (1986) reported the presence of *o*-aminoacetophenone in male and female ferret urine during the breeding season. In the current study, *o*-aminoacetophenone was found at higher

concentrations in male than in female ferret urine. Higher *o*-aminoacetophenone concentrations have also been observed in female steppe polecats and in male Siberian weasels when compared to their opposite sex conspecifics (Zhang *et al.*, 2003). A previous study (Clapperton *et al.*, 1988) indicated that indoles are more prevalent in the anal glands of male than of female ferrets. In the present study, no such difference in indole concentration was observed between the sexes.

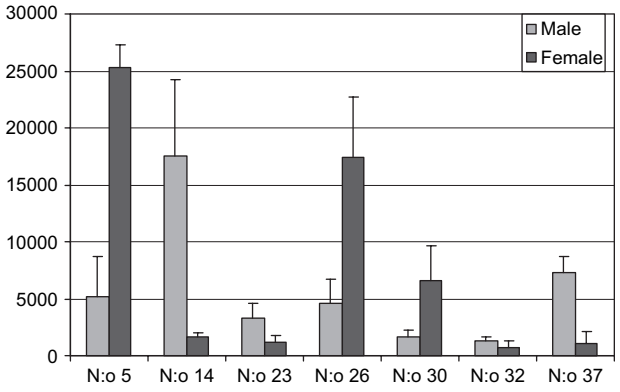

Figure 5 Comparisons of the levels of selected constituents (mean ± SEM, $n = 3$) of anal glands from male and female ferrets. Numbers (see Table 2) refer to the following compounds: 5 = *E*-2,3-dimethylthietane; 14 = 2-propylthietane; 23 = 3,3-dimethyl-1,2-dithiolane; 26 = *E*-3,4-dimethyl-1,2-dithiolane; 30 = *Z*-3,4-dimethyl-1,2-dithiolane; 32 = 2-pentylthietane; and 37 = 3-propyl-1,2-dithiolane. PAs were measured by GC-MS (analytical conditions described in text).

Concentration of volatiles and sex recognition

The ability of ferrets to discriminate between the two sexes probably depends on the detection of relevant sexually dimorphic anal scent and/or urinary odorants. In *M. musculus*, more abundant 2-sec-butyl-4,5-dihydrothiazole and dehydro-exo-brevicomin, α- and β-farnesene, present in male urine, have been shown to attract female mice (Harvey *et al.*, 1989; Jemiolo *et al.*, 1985, 1991). The male lion (*Panthera leo*) has more 2-butanone and less acetone in their urine than females (Andersen and Vulpius, 1999). No sex differences in urinary constituents were found in *Castoreum* of *Castor canadensis* and *Castor fiber* (Sun and Müller-Schwarze, 1999; Rosell and Sundsdal, 2001), but there are sex differences in anal gland volatile patterns, except in the mink (Crump, 1980a,b; Schildknecht and Birkner, 1983; Zhang *et al.*, 2002, 2003). Behavioral tests have indicated that ferrets were attracted by the odor of opposite-sex anal gland secretion (Clapperton *et al.*, 1988; Cloe *et al.*, 2004).

Ferrets are thought to be a domesticated form of the European polecat (*M. putorius*) (Corbet and Hill, 1980). Male polecats seem to prefer sniffing female urine to male urine (Sokolov and Rozhnov, 1983), and female ferrets prefer sniffing male as opposed to female urine (Cloe *et al.*, 2004). These observations suggest that ferret urine may also be used for sex recognition. Moreover, other behavioral results (Clapperton, 1987) suggest that information about sex is conveyed both by anal gland secretions and by urine or preputial gland odors.

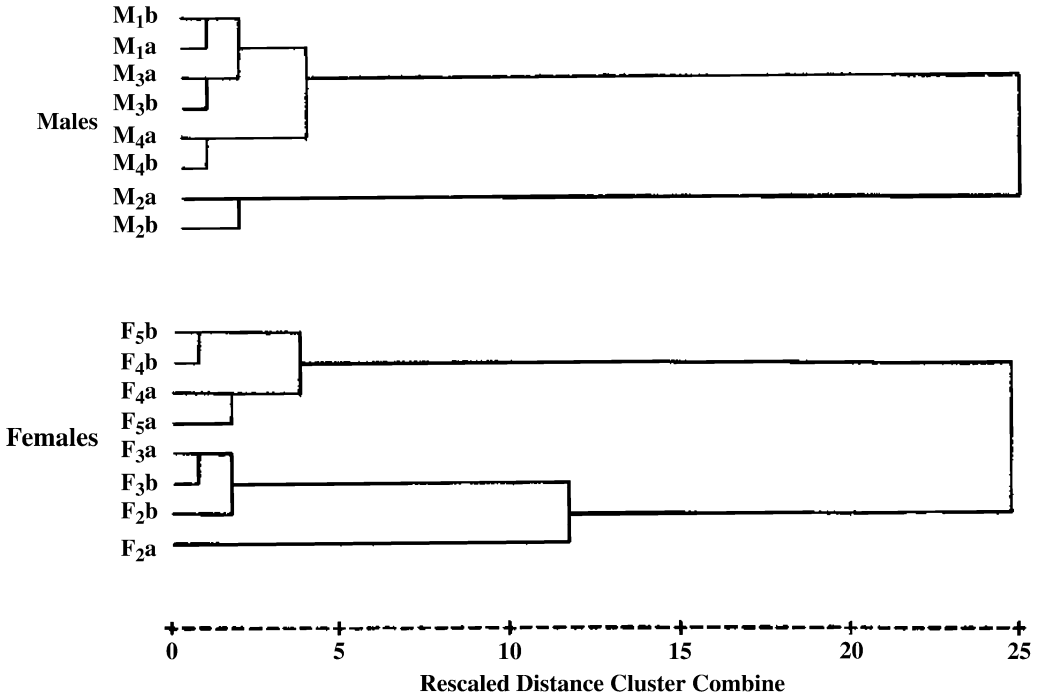

Figure 6 Hierarchical linkage cluster (average linkage) dendrogram of urinary volatile profiles from male (M), and female (F) ferrets (*Mustela furo*). Subscript numbers indicate a different, individual ferret; letters a and b indicate urine samples collected 1–2 weeks apart from the same individuals.

Sex differences in the volatile constituents of anal gland secretion revealed the existence of a female-specific compound, 3-ethyl-1,2-dimethyl-1,2-dithiolane (peak 22). All other sulfur-containing compounds were abundant in both males or females, which is consistent with the previous reports (Schildknecht and Birkner, 1983; Clapperton *et al.*, 1988) on the composition of ferret anal gland secretions.

Individual scent versus chemical composition

Canid species use urine marks to communicate important information about sex, social rank, and individuality (Macdonald, 1985). Also, in lion (*P. leo*), GC volatile profiles of individual urine samples overlapped more within an individual sample than they did between samples from different individuals (Andersen and Vulpius, 1999). In European badgers (*M. meles*), GC profiles of two subcaudal gland secretion samples proved to be highly specific for individuals (Buesching, 2002a,b). In the beaver (*C. canadensis*), the variation in GC profiles of anal gland secretions was smaller within an individual than between individuals. Consequently, anal gland secretions seem suitable for characterizing individuality (Sun and Müller-Schwarze, 1998). Our results suggest that individual recognition on the basis of urine odor likely occurs in the ferret. Schildknecht *et al.* (1986) suggested that individual differences in ferret urine volatiles appeared only in males. However, the hierarchical cluster analysis of female ferret urine profiles in the present study reveals wide variations in the levels of 2,5-dimethylpyrazine, suggesting that differences in female ferret urine composition may contribute to individual recognition in females as well.

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References

Andersen, K.F. and **Vulpius, T.** (1999) *Urinary volatile constituents of the lion,* Panthera leo. Chem. Senses, 24, 179–189.

Baltussen, E., Cramers, C.A. and **Sandra, P.J.F.** (2002) *Sorptive sample preparation—a review.* Anal. Bioanal. Chem., 373, 3–22.

Baltussen, E., Sandra, P., David, F. and **Cramers, C.** (1999) *Stir bar sorptive extraction (SBSE), a novel extraction technique for aqueous samples: theory and principles.* J. Microcolumn Sep., 11, 737–747.

Bicchi, C., Iori, C., Rubiolo, P. and **Sandra, P.** (2002) *Headspace sorptive extraction (HSSE), stir bar sorptive extraction (SBSE), and solid phase microextraction (SPME) applied to the analysis of roasted arabica coffee and coffee brew.* J. Agric. Food Chem., 50, 449–459.

Birks, J.D.S. (1981) Home Range and Territorial Behavior of the Feral Mink (*Mustela vison* Devon) (PhD thesis). University of Exeter, Devon, UK.

Brahmachary, R.L. and **Dutta, J.** (1987) *Chemical communication in the tiger and leopard.* In Tilson, R.L. and Seal, U.S. (eds), Tigers of the World. Noyes Publications, Park Ridge, NJ, pp. 296–302.

Brinck, C., Erlinge, S. and **Sandell, M.** (1983) *Anal sac secretion in Mustelids: a comparison.* J. Chem. Ecol., 9, 727–745.

Brinck, C., Grell, R. and **Odham, G.** (1978) *Anal pouch secretion in mink,* Mustela vison. Oikos, 38, 68–75.

Buesching, C.D., Waterhouse, J.S. and **Macdonald, D.W.** (2002a) *Gas-chromatographic analyses of the subcaudal gland secretion of the European badger* (Meles meles). *Part I: chemical differences related to individual parameters.* J. Chem. Ecol., 28, 41–56.

Buesching, C.D., Waterhouse, J.S. and **Macdonald, D.W.** (2002b) *Gas-chromatographic analyses of the subcaudal gland secretion of the European badger* (Meles meles). *Part II: time-related variation in the individual-specific composition.* J. Chem. Ecol., 28, 57–69.

Clapperton, B.K. (1987) *Preputial glands of the ferret,* Mustela furo. J. Zool. (Lond.), 212, 356–361.

Clapperton, B.K., Minot, E.O. and **Crump, D.R.** (1988) *An olfactory recognition system in the ferret* Mustela furo *L. (Carnivora: Mustelidae).* Anim. Behav., 36, 541–553.

Clapperton, B.K., Minot, E.O. and **Crump, D.R.** (1989) *Scent lures from anal sac secretions of the ferret,* Mustela furo *L.* J. Chem. Ecol., 15, 291–308.

Cloe, A.L., Woodley, S.K., Waters, P., Zhou, H. and **Baum, M.J.** (2004) *Contribution of anal scent gland and urinary odors to mate recognition in the ferret.* Physiol. Behav., 82, 871–875.

Corbet, G.B. and **Hill, J.E.** (1980) A World List of Mammal Species. British Museum (Natural History), London.

Crump, D.R. (1980a) *Thietanes and dithiolanes from the anal gland of the stoat (*Mustela erminea*).* J. Chem. Ecol., 6, 341–347.

Crump, D.R. (1980b) *Anal gland secretion of the ferret* (Mustela putorius furo). J. Chem. Ecol., 6, 837–844.

Crump, D.R. and **Moors, P.J.** (1985) *Anal gland secretions of the stoat* (Mustela erminea) *and the ferret* (Mustela putorius forma furo*): some additional thietane components.* J. Chem. Ecol., 8, 1037–1043.

Erlinge, S., Sandwell, M. and **Brinck, C.** (1982) *Scent marking and its territorial significance in stoat,* Mustela erminea. Anim. Behav., 30, 811–818.

Gerell, R. (1970) *Home range and movements of the mink* (Mustela vison). Oikos, 21, 160–173.

Harvey, S., Jemiolo, B. and **Novotny, M.** (1989) *Pattern of volatile compounds in dominant and subordinate male mouse urine.* J. Chem. Ecol., 15, 2061–2071.

Jemiolo, B., Alberts, J., Sochinski-Wiggins, S. and **Harvey, S.** (1985) *Behavioral and endocrine responses of female mice to synthetic analogues of volatile compounds in male urine.* Anim. Behav., 33, 1114–1118.

Jemiolo, B., Xie, T.-X. and **Novotny, M.** (1991) *Socio-sexual olfactory preference in female mice: attractiveness of synthetic chemosignals.* Physiol. Behav., 50, 1119–1122.

Johnston, R.E. (2003) *Chemical communication in rodents: from pheromones to individual recognition.* J. Mammal., 84, 1141–1162.

Jorgenson, J.W., Novotny, M., Carmack, M., Copeland, G.B., Wilson, S.R., Katona, S. and **Whitten, W.K.** (1978) *Chemical scent constitutes in the urine of the red fox (*Vulpes vulpes*) during the winter season.* Science, 199, 796–798.

Kelliher, K.R. and **Baum, M.J.** (2001) *Nares occlusion eliminates heterosexual partner selection without disrupting coitus in ferrets of both sexes.* J. Neurosci., 21, 5832–5840.

Kelliher, K.R. and **Baum, M.J.** (2002) *Effect of sex steroids and coital experience on ferrets, preference for the smell, sight and sound of conspecifics.* Physiol. Behav., 76, 1–7.

Kelliher, K.R., Baum, M.J. and **Meredith, M.** (2001) *The ferret's vomeronasal organ and accessory olfactory bulb: effect of hormone manipulation in adult males and females.* Anat. Rec., 263, 280–288.

Ma, W., Wiesler, D. and **Novotny, M.V.** (1999) *Urinary volatile profiles of the deermouse (*Peromyscus maniculatus*) pertaining to gender and age.* J. Chem. Ecol., 25, 417–431.

Macdonald, D.W. (1985) *The carnivores: order Carnivora.* In Brown, R.E. and Macdonald, D.W. (eds), Social Odours in Mammals. Oxford University Press, Oxford, pp. 619–722.

Mason, J.R., Epple, G. and **Nolte, D.L.** (1994) *Semiochemicals and improvements in rodent control.* In Galef, B.E., Mainardi, M. and Valsecchi, P. (eds), Behavioral Aspects of Feeding: Basic and Applied Research in Mammals. Harwood Academic, Chur, Switzerland, pp. 327–346.

Miller, K.V., Jemiolo, B., Gassett, J.W., Jelinek, I., Wiesler, D. and **Novotny, M.** (1998) *Putative chemical signals from white-tailed deer (*Odocoileus virginianus*): social and seasonal effects on urinary volatile excretion in males.* J. Chem. Ecol., 24, 673–683.

Novotny, M., Harvey, S. and **Jemiolo, B.** (1990) *Chemistry of male dominance in the house mouse,* Mus domesticus. Experientia, 46, 109–113.

Preti, G., Muetterties, E.L., Furman, J.M., Kennelly, J.J. and **Johns, B.E.** (1976) *Volatile constitutes of dog (*Canis familiaris*) and coyote (*C. latrans*) anal sacs.* J. Chem. Ecol., 2, 177–186.

Raymer, J. (1984) Investigations into the Chemical Nature of Chemo-Olfactory Communication in the Wolf (*Canis lupus*) (PhD thesis). Indiana University, Bloomington.

Raymer, J., Wiesler, D., Novotny, M., Asa, C., Seal, U.S. and **Mech, L.D.** (1985) *Chemical investigations of wolf (*Canis lupus*) anal sac secretion in relation to breeding season.* J. Chem. Ecol., 11, 593–608.

Raymer, J., Wiesler, D., Novotny, M., Asa, C., Seal, U.S. and **Mech, L.D.** (1986) *Chemical scent constituents in urine of wolf (*Canis lupus*) and their dependence on reproductive hormones.* J. Chem. Ecol., 12, 297–314.

Rosell, F. and **Sundsdal, L.J.** (2001) *Odorant sources used in Eurasian beaver territory marking.* J. Chem. Ecol., 27, 2471–2491.

Schildknecht, H. and **Birkner, C.** (1983) *Struktur und Wirkung der Musteliden-Ökomone, III: Analyse der Analbeutelsekrete Mitteleuropäischer Musteliden.* Chemiker-Zeitung, 107, 267–270 (in German).

Schildknecht, H., Stenuf, G. and **Krauss, D.** (1986) *Structure and action of mammalian ecomones. VI. Behavior-active chemical signals from the urine of the ferret (*Mustela putorius furo*).* Chemiker-Zeitung, 110, 185–195 (in German).

Schultz, T.H., Flath, R.A., Stern, D.J., Mon, T.R., Teranishi, R., Kruse, S.M., Butler, B. and **Howard, W.E.** (1988) *Coyote estrous urine volatiles.* J. Chem. Ecol., 14, 701–712.

Service, K.M., Brereton, R.G. and **Harris, S.** (2001) *Analysis of badger urine volatiles using gas chromatography-mass spectrometry and pattern recognition techniques.* Analyst, 126, 615–623.

Soini, H.A., Bruce, K.E., Wiesler, D., David, F., Sandra, P. and **Novotny, M.V.** (2005) *Stir bar sorptive extraction: a new quantitative and comprehensive sampling technique for determination of chemical signal profiles from biological media.* J. Chem. Ecol., 31, 377–392.

Sokolov, V.E., Albone, E.S., Flood, P.F., Heap, P.E., Kagan, M.Z., Vasilieva, V.S., Roznov, V.V. and **Zinkevich, E.P.** (1980) *Secretion and secretory tissues of the anal sac of the mink,* Mustela vison*: chemical and histological studies.* J. Chem. Ecol., 6, 805–825.

Sokolov, V.E. and **Rozhnov, V.V.** (1983) *Information value of scent markers of mustelidae: urine and excrement of* Mustela putorius *and* Mustela vison. Dokl. Akad. Nauk SSSR Ser. Biol., 269, 220–222.

Sun, L. and **Müller-Schwarze, D.** (1998) *Anal gland secretion codes for relatedness in the beaver,* Castor canadensis. Ethology, 104, 917–927.

Sun, L. and **Müller-Schwarze, D.** (1999) *Chemical signals in the beaver: one species, two secretions, many fuction?* In Johnston, R.E., Müller-Schwarze, D. and Sorensen, P.W. (eds), Advances in Chemical Signals in Vertebrates. Kluwer Academic, New York, pp. 281–288.

Tienpont, B., David, F., Bicchi, C. and **Sandra, P.** (2001) *High capacity headspace sorptive extraction.* J. Microcolumn Sep., 12, 577–584.

Tienpont, B., David, F., Desmet, K. and **Sandra, P.** (2002) *Stir bar sorptive extraction-thermal desorption-capillary GC-MS applied to biological fluids.* Anal. Bioanal. Chem., 373, 46–55.

Weiler, E., Apfelbach, R. and **Farbman, A.I.** (1999) *The vomeronasal organ of the male ferret.* Chem. Senses, 24, 127–136.

Wood, W.F., Sollers, B.G., Dragoo, G.A. and **Dragoo, J.W.** (2002) *Volatile components in defensive spray of the hooded skunk,* Mephitis macroura. J. Chem. Ecol., 28, 1865–1870.

Woodley, S.K. and **Baum, M.J.** (2003) *Effects of sex hormones and gender on attraction thresholds for volatile anal gland odors in ferrets.* Horm. Behav., 44, 110–118.

Woodley, S.K. and **Baum, M.J.** (2004) *Differential activation of glomeruli in the ferret's main olfactory bulb by anal scent gland odours from males and females: an early step in mate identification.* Eur. J. Neurosci., 20, 1025–1032.

Woodley, S.K., Cloe, A.L., Waters, P. and **Baum, M.J.** (2004) *Effects of vomeronasal organ removal on olfactory sex discrimination and odor preferences in female ferrets.* Chem. Senses, 29, 659–669.

Zhang, J., Ni, J., Ren, X., Sun, L., Zhang, Z. and **Wang, Z.** (2003) *Possible coding for recognitions of sexes, individuals and species in anal gland volatiles of* Mustela eversmanni *and* M. sibirica. Chem. Senses, 28, 371–378.

Zhang, J., Sun, L., Zhang, Z., Wang, Z., Chen, Y. and **Wang, R.** (2002) *Volatile compounds in the anal gland of Siberian weasels (*Mustela sibirica*) and steppe polecats (*M. eversmanni*).* J. Chem. Ecol., 28, 1287–1297.

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