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Volatile constituents of wolf *(Canis lupus)* urine as related to gender and season[1]

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Summary. The volatile constituents of wolf urine were examined via capillary gas chromatography and compared among male, female, and castrate male. Several compounds including methyl isopentyl sulfide, 3,5-dimethyl-2-octanone, and acetophenone were clearly associated with the gender of the animal and many displayed a seasonal dependence. In addition, 2 long-chain aldehydes isolated from urine samples by an HPLC procedure also correlated with the endrocrine status of the animal.

Communication in the canidae is quite complex[3]. This complexity varies directly with the degree of sociability of the species such that a gregarious animal like the wolf *(Canis lupus)* appears to have a more complex communication system than the more solitary red fox *(Vulpes vulpes)*. The wolf utilizes visual, vocal, and tactile as well as chemo-olfactory modes of communication[3]. A major mode of chemo-olfactory communication (scent-marking) in the wolf is urination, and the way urine is deposited relates to behavior displayed during marking[4,5]; urination, and the chemical scent constituents of urine, are intimately involved in communication in general. Following our previous characterization and behavioral testing of the chemical scent constituents in red fox urine[6-8], the present study deals with the urinary scent chemistry of the wolf as related to sex, endocrine status and seasonality. Capillary gas chromatography (GC), gas chromatography/mass spectrometry (GC/MS), and gas chromatography/Fourier-transform infrared spectroscopy (GC/FTIR) were used to identify and quantitate typical wolf urinary components. For comparison, urinary scent profiles of arctic foxes *(Alopex lagopus)* and common dogs *(Canis familiaris)* were also investigated.

Urine samples were collected from several captive wolves maintained near Minneapolis, Minnesota. The collection procedure involved anesthetizing the animals with ketamine hydrochloride and promazine hydrochloride to provide adequate relaxation for urine withdrawal by catheter. Aliquots of about 10 ml were discharged into acid-washed vials, which were immediately closed with a Teflon-lined cap. The samples were shipped on dry ice to our laboratory at Indiana University where they were stored frozen until analysis. Similar procedures were employed for collections from dogs and foxes.

Wolf urine samples from every 2 or 3 weeks, from 31 October 1979 to 30 October 1980, were chosen. To minimize individual variations, aliquots of 2–7 (mean = 4.4) samples were combined when both date and class (i.e., male, female, or castrate male) were alike.

The profiles of urinary volatiles resulting from the GC separation of the samples were obtained through a headspace sampling procedure estabished in our laboratory[9,10]. Following thermal desorption of volatiles from a porous polymer (Tenax GC, Applied Science Laboratories, State College, Pennsylvania) onto a 60 m × 0.25 mm, inner diameter, glass capillary column coated with a polypropylene glycol fluid, UCON 50-HB-2000, the separations were followed with the flame ionization detector (FID). Compound identifications were initially aided by the nitrogen-sensitive (thermionic) and the sulfur-sensitive (flame-photometric) GC detectors. Data obtained from combined capillary GC/MS provided the major information leading to a positive identification of the urinary constituents. GC/MS data were obtained with a Hewlett-Packard 5982A dodecapole instrument. For several component identifications, a combination of capillary GC with FTIR was essential[11]. The compound identifications discussed below were verified through both retention and mass-spectral data of synthesized compounds.

Representative FID chromatograms are shown in figure 1 for normal male, normal female and castrate male urine volatiles. The numbered peaks and their corresponding identities in the table were selected for discussion in this paper; the components showing no clear relationship to sex or season are excluded. The sulfur-containing compounds were examined with great interest, because similar ones (or the same in the case of Δ^3-isopentenyl methyl sulfide) were seen in red fox urine[6] and later shown to induce scent-marking by foxes in the wild[7,8].

The ketones are discussed here for 2 reasons: a) these compounds appear to be unique to the wolf, or closely related canids[12]; and, b) this series of compounds is closely associated with the male. Some of these ketones were seen (in lesser amounts, however) in the dog, while there was no evidence of this series in the arctic fox. Acetophenone was of particular interest because of its obvious association with the sex of the animals. Ketones **7**, **9**, **11**, and **12**, which have never been reported to occur naturally, were synthesized by conventional methods as mixtures of diastereoisomers. One can do no more than speculate regarding stereochemistry, but it is of special interest that in each case the earliest of the synthetic diastereo-

isomers to be eluted was the one identical in retention time with the natural product.

The areas of the peaks corresponding to the listed compounds from all of the chromatographic data representing 1 year's worth of samples were plotted as a function of time. To obtain an indication of the amount of a compound excreted in one year and generalize concerning the relative abundances in male, female, and castrate male, the area-vs-time curves were integrated for each of the compounds. The integrated values for the male were divided by those of both female and castrate male for each compound. A ratio greater than one indicates a compound associated more with male, while a lower ratio indicates a compound more closely associated with female or castrate male (table).

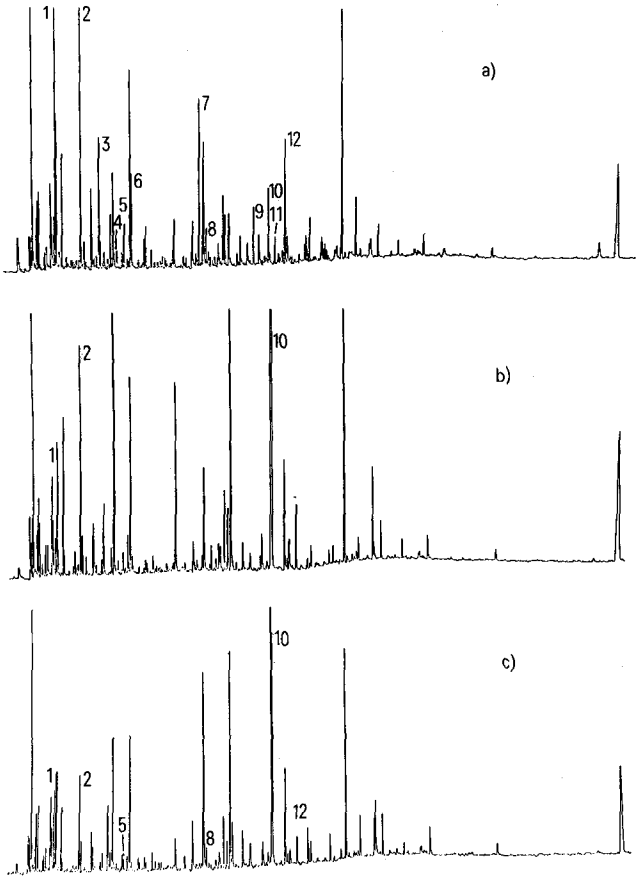

Figure 1. Volatile urinary profiles recorded by capillary gas chromatography with the flame ionization detector. *a* Normal male; *b* normal female; *c* castrate male. For identification, see table.

Isopentyl methyl sulfide and the aliphatic ketones are associated with normal (intact) male wolf. An abundance of acetophenone is, on the other hand, consistent with female or castrate male. Bailey et al.[13] also reported the presence of Δ^3-isopentenyl methyl sulfide in red foxes from the British Isles. More importantly, they reported isopentyl methyl sulfide and demonstrated its seasonal variation. The wolf isopentyl methyl sulfide also displays a seasonal dependence, peaking in March, one month after the breeding season (fig. 2). Seasonal changes indicated in this paper are derived from samples representing one year, as noted above. The general gender differences have, however, been supported by many other experiments with samples from other years.

Carbonyl compounds numbers **7** and **8** appear to have peaks in mid-December. Compound **6** has no obvious cycle but peaks mid-December and early March. Compounds **9** and **11** display maxima in November. Acetophenone in the female displays 2 maxima; one occurs in early June, while the large increase occurs in early August. The sulfur compounds other than isopentyl methyl sulfide display no clear maxima.

The results of experiments recently completed indicate that the levels of methyl propyl sulfide, 4-methyl-3-heptanone, 3,5-dimethyl-2-octanone, 3,5,7-trimethyl-2-nonanone, 3,5-dimethyl-2-decanone, and 3,5,7-trimethyl-2-decanone (peaks **1**, **6**, **7**, **9**, **11**, **12**) were initially small or non-existent in two castrate males, but treatment with testosterone brought these levels up to those observed in normal males. The mechanism of this hormone dependence is not clear. This dependence, however, increases the probability that these compounds may be important in chemical communication. Jorgenson et al.[6] hypothesized that the Δ^3-isopentenyl methyl sulfide from the red fox is derived from isopentenyl pyrophosphate and methionine via a

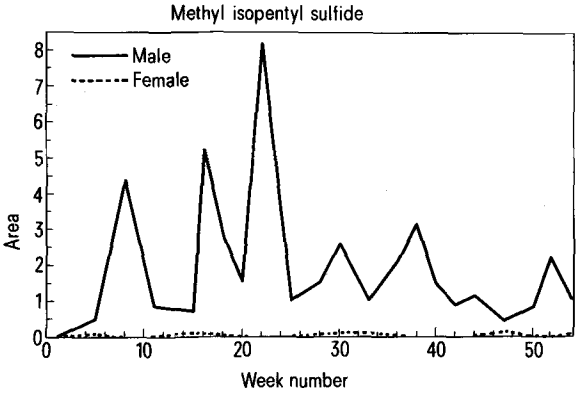

Figure 2. Area of peak corresponding to isopentyl methyl sulfide versus time for normal male and normal female. Week number 1 corresponds to 31 October 1979.

Identification of peaks labeled in figure 1.

Peak number	Compound	Molecular weight	Integrated male/female	Area ratios male/castrate male
1	Methyl propyl sulfide	90	1.80	2.11
2	Methyl butyl sulfide	104	1.50	0.92
3	Methyl isopentyl sulfide	118	22.5	5.62
4	Methyl pentyl sulfide	118	1.20	1.18
5	Δ^3-Isopentenyl methyl sulfide	116	1.70	0.46
6	4-Methyl-3-heptanone	128	3.70	11.2
7	3,5-Dimethyl-2-octanone	156	36.3	54.5
8	3-Nonanone	142	10.0	2.3
9	3,5,7-Trimethyl-2-nonanone	184	37.0	52.8
10	Acetophenone	120	0.01	0.52
11	3,5-Dimethyl-2-decanone	184	83.3	12.5
12	3,5,7-Trimethyl-2-decanone	198	2.20	18.0

S-methylsulfonium ion intermediate. Isopentenyl pyrophosphate[14] is a key compound in the biosynthesis of long-chain terpenes and, ultimately, steroid hormones. A simple hydrogenation of it would produce isopentyl methyl sulfide.

Finally, we noticed another sex-related difference involving carbonyl compounds in wolf urine. Utilizing isolation and selective derivatization of carbonyl compounds[15] from wolf urine, followed by separation and quantitation by reversed-phase HPLC, samples from male, female, castrate male, and ovariectomized female urines (pooled from November 1981 to February 1982) were compared. The most notable differences between chromatograms were in the peaks identified (through retention times and MS) as hexadecanal and octadecanal. Peak areas reported in arbitrary units were 1.80 and 1.49, respectively, for the normal male, 0.11 and 0.15, respectively, for the normal female, 0.17 and 0.11 for the castrate male, and 0.13 and 0.10 for the ovariectomized female. Thus, these 2 long-chain aldehydes could also play a role in chemical communication in the wolf. Behavioral testing with these compounds is currently being conducted.

While it seems clear[3,5] that the wolf utilizes olfactory means of communication effectively, a possible role of the urinary constituents reported here must now be elucidated. Behavioral tests are currently being designed and carried out for compounds identified and synthesized in this work in either synthetic mixtures or as a part of natural samples.

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D-3-Dodecanoyltetradecanoic acid as a constituent of lipid A from the lipopolysaccharide of *Yersinia pseudotuberculosis*

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Summary. D-3-Dodecanoyltetradecanoic acid has been separated from the lipid A of *Yersinia pseudotuberculosis* and its structure has been established by chromato-mass-spectrometry and ^{13}C NMR spectroscopy, by comparison with authentic samples.

It was recently shown that an acyloxy fatty acids both ester-bound[1] and amide-linked[2] are constituents of lipid A from the lipopolysaccharides (LPS) of gram-negative bacteria. Together with other fatty acids they are considered to be responsible for the endotoxic properties of lipid A such as pyrogenicity, toxicity, mitogenicity, and others. A mild alkaline treatment of lipid A or LPS decreases or abolishes the aforementioned activities[3] and leads to formation of free fatty acids. The aim of the present work was the isolation of acyloxy fatty acid from the lipid A of *Y. pseudotuberculosis*.

The lipid A was prepared by mild acid hydrolysis of *Y. pseudotuberculosis* LPS (IB serovar 598 strain) with 1% acetic acid[4]. The acyloxy acid together with other fatty acids was obtained by mild alkaline hydrolysis of lipid A (0.25 N NaOH, 56 °C, 15 min)[1]. The fatty acid mixture obtained was separated on the column with silica gel L (40/100 μ, CSSR, 1 × 12 cm) in the system: hexane (20 ml), hexane: diethyl ether (99:1, 20 ml; 95:5, 20 ml; 50:50, 20 ml). As a result dodecanoic ($M^+ = 214$, m/z 199, 183 for methyl ester[5]), D-3-hydroxytetradecanoic ($[\alpha]_D^{20}$ −11.8° (c 0.7, $CHCl_3$)[1]; $M^+ = 258$, m/z 240, 227, 208, 103 for methyl ester[5]), and D-3-dodecanoyltetradecanoic ($M^+ =$ 440, m/z 409, 240, 241, 208, 209 for methyl ester[2]) acids were obtained. Treatment of the latter with sodium methylate yielded dodecanoic and D-3-hydroxytetradecanoic acids in the ratio 1:1. It should be noted that the same mixture of fatty acids is formed during the preparation of the lipid A from LPS by acid hydrolysis and it may also be used for isolation of acyloxy acids. A lightness of the formation of these fatty acids points to their ester linkage with a residue of glucosamine.

^{13}C NMR spectroscopy data on some D,L-3-hydroxytetradecanoic acid derivatives

Atom	Shifts in compound (ppm)							
	I	Ia	II	IIa	III	IV	IVa	lip A
C-2	41.2	41.3	38.9	39.0	39.0	39.0	39.2	38.8
C-3	68.1	68.1	70.6	70.6	70.2	70.0	70.3	70.2
C-4	36.6	36.6	34.0	34.1	34.3	34.5	34.6	34.5
C'-2					34.0	34.1	33.9	34.0
C'-3					24.9	25.0	25.0	24.9
C'-4					29.7	29.7	29.7	29.7
C=O	177.7		174.8	170.1	176.2	177.6	173.1	174.0
C'=O		173.5	170.7	170.7	173.4	172.7	170.8	170.6