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Potential semiochemicals in urine from free ranging wolverines (*Gulo gulo* Pallas, 1780)

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A B S T R A C T

Urine deposition has been observed as an important scent-marking behaviour among wolverines (*Gulo gulo*, Mustelinae, Mustelidae). Solid phase microextraction (SPME) of headspace volatiles of the urine from free ranging wolverines were examined by gas chromatography–mass spectrometry (GC–MS). Urine samples were collected directly from the bladder of live-trapped animals or from frozen samples deposited in snow. Nineteen potential semiochemicals were identified in the headspace from 22 urine samples. The composition of these volatile compounds varied by type and amount with each sample, but a number of chemicals were regularly found in many samples. The most commonly found constituents were the ketones; 2-heptanone, 4-heptanone and 4-nonanone; and the terpenes: α-pinene, β-pinene, limonene, linalool and geraniol. Mammalian urinary discharge of ingested α-pinene, β-pinene, limonene and other hydrocarbon terpenes is unusual, as these compounds are usually oxidized before excretion. The source of the hydrocarbon monoterpenes likely includes conifer needles, as they have been found in wolverine scat.

1. Introduction

The volatile compounds in urine are considered important as mammalian semiochemicals. Reviews by Albone (1984), Gorman and Trowbridge (1989), Müller-Schwarze (2006), and Burger (2005) cite numerous investigations on urine volatiles from mammalian species, but note that there have been few investigations on chemical constituents of carnivore urine.

Studies on carnivore urine constituents include, reports about several species of felids: the lion (*Panthera leo*, Andersen and Vulpius, 1999) the bobcat (*Lynx rufus*, Mattina et al., 1991) and the cheetah (*Acinonyx jubatus*, Burger et al., 2006). Three species of canids have had urine volatiles investigated: the wolf (*Canis lupus*, Raymer et al., 1986), the coyote (*Canis latrans*, Schultz et al., 1988) and the red fox (*Vulpes vulpes*, Jorgensen et al., 1978). Mustelid urine has been investigated from two species, the European badger (*Meles meles*, Service et al., 2001) and the ferret (*Musetela furo*, Zhang et al., 2005). In these studies, the only urine examined from free ranging animals was that from the red fox and the European badger.

Urination as a scent-marking agent in wolverine has been discussed (Hash, 1987; Koehler et al., 1980; Pulliainen and Ovaskainen, 1975). Hash (1987) describes wolverine urine being deposited on trees, rocks or other prominent objects as part of scent marking. We report potential semiochemicals in the headspace volatiles from the urine of free ranging wolverines

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using solid phase microextraction (SPME). SPME has recently been shown to be an efficient method to identify urine headspace volatiles (Kayali-Sayadi et al., 2005).

2. Materials and methods

Urine was collected from free ranging wolverines in 2004 and 2005 during the months of February and March in Glacier National Park, Montana, U.S.A. Five of the urine samples were taken by insertion of a needle into the bladder of anaesthetized animals that had been caught in live traps (wolverine immobilization was conducted under the purview of the University of Montana Institutional Animal Use and Care Committee). Three of these samples were from different females and two were from the same male captured on two different occasions. Seventeen of the samples were collected frozen from the snow during wolverine tracking sessions (following released animals or recognition of wolverine snow tracks). Care was taken with these snow samples to exclude collection of any extraneous materials (plant parts or extra snow). A snow sample was taken as a control. Of these samples, nine were deposited by two different males, and eight were from animals of undetermined sex. Samples were placed in 50 ml plastic screw cap centrifuge tubes (Fisherbrand®) and kept frozen at $-20\,^{\circ}C$ until they could be analyzed. A plastic screw cap centrifuge tube that had not been used to store urine was used as a control for chemical analyses.

Headspace volatiles were collected using a Supelco solid phase microextraction (SPME) apparatus, equipped with a 100 μm polydimethylsiloxane fibre. Before each urine sample analysis, the fibre was cleaned by heating in a GC inlet at 205° for 20 min. Cleaning was confirmed by a preliminary gas chromatograph-mass spectrometer (GC–MS) run. For analysis, the absorbent tip of the SPME apparatus was placed about 2 cm above a 2 ml sample of wolverine urine at 20 °C for 15 min. The SPME sample was immediately analyzed. The volatiles were desorbed from the SPME apparatus for 0.5 min in the 205 °C inlet of a Hewlett-Packard G-1800C GC–MS. Analyses were done in the splitless mode (0.5 min), using a 30 m $\times$ 0.25 mm cross-linked phenyl methyl silicone capillary column (HP-5MS). The gas chromatograph was programmed so the oven temperature was kept at 40 °C for 4 min, then increased to a final temperature of 300 °C at a rate of 30 °C/min and held at this temperature for 2 min. Mass spectral fragments below $m/z = 39$ were not recorded and impurities identified from plastic storage tubes not used to collect urine or from control snow samples were not reported. Compounds were initially identified by comparison of mass spectra in the NIST 1998 computerized mass spectral library. All identifications were confirmed by comparisons of spectra and retention times of commercially available samples purchased from Fischer Scientific. Where the relative amounts of individual chemicals were reported, the compound with the lowest value of the total ion current (TIC) was arbitrarily set to the value 1.

3. Results

Headspace analysis of 22 wolverine urine samples showed 25 different compounds by GC–MS, of which 19 were identified (Table 1). The number of components seen in the urine headspace ranged from 1 to 10 compounds per sample but no two

Table 1
Occurrence of volatile compounds identified in the headspace of wolverine urine.

Compound	Retention time	Occurrence (in 22 samples)
4-Heptanone	5.8	14
Unknown	5.9	1
2-Heptanone	6.0	14
Unknown	6.4	1
4-Methyl-3-heptanone	6.4	1
α-Pinene	6.5	10
Benzaldehyde	6.7	1
Camphene	6.7	1
β-Pinene	6.8	6
3-Carene	7.1	4
Limonene	7.3	14
o-Cresol	7.4	2
4-Nonanone	7.5	6
Unknown	7.6	1
Linalool	7.7	11
Isopentyl isovalerate	7.7	3
Unknown	8.0	1
Camphor	8.0	1
4-Ethylphenol	8.1	1
Unknown	8.1	3
Borneol	8.1	2
Menthol	8.2	1
Unknown	8.3	2
Geraniol	8.6	14
Bornyl acetate	8.8	1

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Table 2
Volatile compound occurrence in the headspace of wolverine urine by sample source.

Compound	Trapped wolverine[a] (5 samples)	Snow deposit[b] (17 samples)
4-Heptanone	5	9
Unknown		1
2-Heptanone	5	9
Unknown		1
4-Methyl-3-heptanone	1	
α-Pinene	2	8
Benzaldehyde		1
Camphene		1
β-Pinene	1	5
3-Carene	1	3
Limonene	2	12
o-Cresol		2
4-Nonanone	2	4
Unknown		1
Linalool	4	7
Isopentyl isovalerate		3
Unknown	1	
Camphor		1
4-Ethylphenol		1
Unknown	2	1
Borneol		2
Menthol	1	
Unknown		2
Geraniol	4	10
Bornyl acetate		1

[a] Urine taken from bladder of trapped animal.
[b] Urine frozen in snow.

samples had the same composition. A similar pattern of compounds was observed for the five samples taken directly from the bladder of trapped animals and the 17 collections from frozen snow deposits (Table 2). The difference in the number of compounds identified between the two types of sample collection may be due to unequal sample size. Also, no distinct pattern for male or female urine volatiles was noted (Table 3), however, 4-nonanone, present in many of the male urine samples was not seen in the female urine samples. Since the samples size is small, it is not possible to tell if this is significant. Eleven of the identified compounds are terpenes, five of which are the hydrocarbons: α-pinene, β-pinene, camphene, 3-carene and limonene. Four of the terpenes are the alcohols: linalool, geraniol, menthol, and borneol; and the two remaining compounds are camphor and bornyl acetate. The most common compounds in the urine headspace and their percent occurrence were 4-heptanone (64%), 2-heptanone (64%), limonene (64%), geraniol (64%), linalool (50%), α-pinene (45%), β-pinene (27%), and 4-nonanone (27%).

The 10 urine samples were collected from the same male (Table 4), one in 2004 from a snow deposit and nine in 2005. Two of the 2005 samples were taken from the wolverine's bladder on capture, a week apart, and the other 7 were collected randomly from the snow over the period of a month. Table 4 reports the relative peak areas of each compound detected in the headspace by SPME and was normalized so the component with the lowest peak area (TIC) was given the value of 1. The amounts of each component in these analyses varied greatly, and no clear pattern was seen. The ketones, 4-heptanone, 2-heptanone, and 4-nonanone were seen in many of these samples, as were the terpenes, α-pinene, limonene, linalool, and geraniol. The hydrocarbon terpenes; α-pinene, β-pinene, camphene and limonene, as well as, borneol and bornyl acetate varied greatly in amounts when present.

4. Discussion and conclusion

The finding of free hydrocarbon terpenes in the urine of a carnivore was unexpected. The source of the hydrocarbon monoterpenes likely includes conifer needles, as they have been found in wolverine scat (Copeland, 1996), but other plants could be sources as well. Borneol, bornyl acetate, menthol and camphor, compounds only found in one or two of the samples, are also typical found in plants. Geraniol and linalool may be from plant sources, but more likely are biosynthesized by wolverines since these compounds are formed in the first steps of mammalian terpene biosynthesis.

The wolverine's excretion of ingested terpenes as free hydrocarbons is unusual. The relative amounts of these hydrocarbon terpenes varied widely from each urine sample, but for some analyses, these compounds were the major volatiles present (Table 4). Hydrocarbon terpenes have been reported in only one other carnivore's urine. α-Pinene, β-pinene, myrcene, *p*-cymene and limonene were minor components in coyote oestrous urine (Schultz et al., 1988). Hydrocarbon monoterpenes are widely found in plants where they protect plants from insect attack. These compounds are not highly toxic to mammalian herbivores (Brattsten, 1983) but are usually excreted in the urine after oxidation, typically as glucuronic acid or other conjugates and not as hydrocarbons. Humans that have been exposed to α-pinene fumes, oxidize it and excrete it as *cis*- and

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Table 3
Occurrence of volatile compounds identified in the headspace of wolverine urine by sex.

Compound	Sex		
	Male (11 samples)	Female (3 samples)	Unknown (8 samples)
4-Heptanone	9	3	2
Unknown			1
2-Heptanone	9	2	2
Unknown			1
4-Methyl-3-heptanone		1	
α-Pinene	5	1	4
Benzaldehyde			1
Camphene			1
β-Pinene	3		3
3-Carene	1		3
Limonene	5	1	8
o-Cresol	1		1
4-Nonanone	6		
Unknown			1
Linalool	7	3	1
Isopentyl isovalerate	1		2
Unknown		1	
Camphor			1
4-Ethylphenol	1		
Unknown	1	2	
Borneol			2
Menthol	1		
Unknown	1		1
Geraniol	9	2	3
Bornyl acetate			1

trans-verbenol conjugated with glucuronic acid and other oxygenated terpene derivatives (Eriksson and Levin, 1990, 1996). Rabbits convert α-pinene, β-pinene, 3-carene, and camphene to many different oxygenated derivatives (Ishida et al., 1979, 1981). Rat urine has been shown to contain 10 oxidized metabolites of the monoterpene limonene (Regan and Bjeldanes, 1976). Woodrats (genus *Neotoma*) rapidly detoxify ingested α-pinene using cytochrome P450 (Haley et al., 2007). Also, woodrats have been shown to have low blood concentrations of α-pinene after ingestion indicating detoxification in the first circulatory pass through the liver (Sorensen and Dearing, 2003).

4-Heptanone and 2-heptanone were always found together in the 14 samples that contained these compounds. Another ketone, 4-nonanone, found in 6 of these 14 samples, was only identified in male wolverine urine. Since only three samples

Table 4
Relative peak areas[a] of headspace compounds of multiple collections from a single male.

Compound	Collection number and source[b,c]									
	1	2	3	4	5	6	7	8	9	10
	Snow	Snow	Snow	Snow	Snow	Snow	Snow	Snow	Trap	Trap
4-Heptanone		43		17	176	80	88	39	78	49
Unknown	308									
2-Heptanone		5		2	20	9	18	4	9	6
Unknown	46									
α-Pinene	197	2			4	3				6
Camphene	578									
β-Pinene	322	5								3
3-Carene		3								3
Limonene	356	3	1			3				5
o-Cresol				1						
4-Nonanone						4	1	1	5	2
Linalool				5	23	30	17	9		8
Borneol	187									
Menthol							6			
Unknown						3				
Geraniol		125		1	38	6	5	16	382	21
Bornyl acetate	316									

[a] Relative amounts are based on the smallest area count of the GC–MS TIC being reported as 1.
[b] Trap = urine taken from bladder of trapped animal.
[c] Snow = urine frozen in snow.

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could be identified as being from females, it is unclear if only males excrete this compound. 4-Heptanone, 2-heptanone and/or other straight-chain ketones have been identified from carnivore urine: lion (Andersen and Vulius, 1999), cheetah (Burger et al., 2006), wolf (Raymer et al., 1986), coyote (Schultz et al., 1988), European badger (Service et al., 2001) and ferret (Zhang et al., 2005).

The physiology and behaviour of chemical communication in carnivores are well documented (see reviews in Albone, 1984 and Müller-Schwarze, 2006), while a paucity of information exists regarding the composition and intent of the chemical message. Interspecific responses of scent-gland compounds and urine (Bramley and Waas, 2001; Bramley et al., 2000; Burwash et al., 1998; Sullivan et al., 1985) provide important insight into chemical communication but likely reflect passive, rather than active responses to the deposited material. Understanding the composition of potential semiochemicals from wolverine urine provides a foundation for future investigations designed to elucidate the intended message.

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