ODORANT SOURCE USED IN EURASIAN BEAVER TERRITORY MARKING

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Abstract—Mammals use urine, feces, or the secretion of specialized skin glands to mark their territories. These sources can carry different information and, thus, have different functions. Presently it is not known if beavers (*Castor* spp.) deposit castoreum (primarily a mixture of secondary metabolites from urine) from the castor sacs and secretion from the anal glands (AGS) together or alone when scent marking their territories. We hypothesized that castoreum would be the main scent signal used in the defense of beaver territories during winter and predicted that castoreum would be deposited more often than AGS. A total of 96 scent marks on snow were collected from January 1 to March 31, 1997–1999 in the Bø River, Telemark County, Norway. In order to obtain control material, we chemically analyzed AGS and castoreum from 60 dead beavers collected during January–May 1997–1999. We compared the compounds found in the dead beavers with compounds found in the scent marks on snow. Samples were analyzed by using gas chromatography–mass spectrometry (GC-MS). All 96 scent marks contained compounds from castoreum, whereas compounds from AGS were found in only four scent marks. This suggests that beavers do not specifically deposit AGS on scent mounds as they do with castoreum and that the AGS compounds we found probably were remnants of AGS from the feet or fur following pelt lubrication or coprophagy behavior. We conclude that castoreum is the main scent signal used in the defense of beaver territories during winter.

Key Words—Beaver, *Castor fiber*, anal gland secretion, castor sacs, castoreum, skin glands, snow-secretion.

INTRODUCTION

Communication and social recognition in many mammals are based on olfactory signals (Wynne-Edwards, 1962; Ralls, 1971; Schulte et al., 1994), and they use urine, feces, or the secretion of specialized skin glands to mark their territories (e.g., Müller-Schwarze, 1983; Gorman, 1984). Recent studies have demonstrated that scent types can carry different information and, thus, have different functions (Johnston et al., 1993). For instance, the study by Gorman et al. (1978) on otters (*Lutra lutra*) showed that deposits of spraints and urine may be used in the maintenance of otter territories, while the deposits of anal gland secretion (AGS) sometimes found at the latrines appear to have another function. The primary roles of skin glands of carnivores are the maintenance of the pelage and thermoregulation (Gorman and Trowbridge, 1989). The same scent may also code for different information and, thus, serve multiple functions (e.g., Quay and Müller-Schwarze, 1971; Epple et al., 1979; Johnston, 1985), while different scents may carry the same information (Baldwin and Meese, 1977; Roeder, 1980; Martin and Beauchamp, 1982).

Eurasian beaver (*Castor fiber*) and North American beaver (*C. canadensis*) possess two pairs of organs: castor sacs and anal glands (Svendsen, 1978; Walro and Svendsen, 1982). Both are suspected to be used for scent marking during territory defense (e.g., Rosell and Bergan, 1998). These are located in two cavities between the pelvis and base of the tail (Walro and Svendsen, 1982; Valeur, 1988). The anal gland is a holocrine secretory gland, but the castor sac is only a pocket lined with a layer of nonsecretory epithelium. They both open into the urogenital pouch (cloaca) (Svendsen, 1978). The castor sac is used to store what is believed to be a mixture of secondary metabolites from urine, collectively called castoreum (Walro and Svendsen, 1982). Copious amounts of castoreum deposited on scent mounds result from a process not dissimilar to urination except that the urine flushes through the contents of the castor sacs. This material can be deposited on the scent mound without the animal contacting the substrate with the cloacal region. The anal gland papillae, however, must be rubbed on the substratum in order to deposit the exudates (Wilsson, 1971; Svendsen, 1978). All age classes (except kits younger than 5 months) and both sexes defend their territories by scent marking (Wilsson, 1971; Svendsen, 1980; Welsh and Müller-Schwarze, 1989; Nolet and Rosell, 1994; Buech, 1995). Scent is usually secreted by the Eurasian beaver onto small piles of mud and debris scraped together and placed close to the water's edge and at the borders of the territory throughout the entire year (Rosell and Nolet, 1997; Rosell et al., 1998).

Both beaver species are suspected to use castoreum more frequently than AGS when scent-marking the territories (Schulte et al., 1994, 1995; Rosell and Bergan, 1998). Schulte (1993) discovered by chemical analysis that many compounds in the North American beaver scent mounds ($N = 4$) were common to castor sacs and castor fluid collected from live beavers. The presence of AGS compounds

was not examined. Field observations indicate that beavers scent mark using only castoreum, only AGS, or sometimes both (Rosell and Bergan, 1998). The odor of beaver scent marks, as detected by humans, varies greatly within site over time. Whether this is due to different beavers or different scents (castoreum and AGS smell different), or to a change in the compounds, is uncertain (Rosell and Bergan, 1998).

Castoreum consists of phenolic, neutral, basic, and acidic components (Tang et al., 1995). No clear sex difference has been detected so far (Pedersen, 1999; Sun and Müller-Schwarze, 1999). Chemical analyses of the AGS of Eurasian beavers have revealed that AGS contains free fatty acids, fatty alcohols, coprostanone, cholestenols, cyclic triterpenes, sterol esters, and wax esters. Wax esters are found only in males, whereas females possess fatty acids (Grønneberg, 1978–1979; Grønneberg and Lie, 1984). Most of the compounds in castoreum have a low molecular weight (Tang et al., 1993, 1995; Pedersen, 1999), while most of the lipids in AGS have a molecular weight above 300 (Grønneberg, 1978–1979; Grønneberg and Lie, 1984; Sun, 1996). The upper size limit for airborne pheromones is a molecular weight of about 300 (e.g., Wilson, 1963; Bradbury and Vehrencamp, 1998). Thus, AGS will normally not be volatile enough to act as an effective chemical messenger through air. One function suggested for AGS is that it serves to waterproof the fur (Walro and Svendsen, 1982). Excision of the anal glands seems to reduce the ability of the pelage to repel water (Walro and Svendsen, 1982). Prohibition of autogrooming in Eurasian beaver produces similar results (Wilsson, 1971). Both the glands that produce a secretion that waterproofs and maintains the fur and the motor patterns associated with application of the secretion would be expected to develop early in semiaquatic animals. Such is the case in beavers (Walro and Svendsen, 1982). However, several researchers have found that AGS can elicit territorial responses, similar to those shown to castoreum (Hodgdon, 1978; Müller-Schwarze et al., 1986). The secretion could be well suited to long-term signaling and may be deposited around the territory borders for the purpose of territory defense.

Castoreum may be an ideal substance for scent marking the territory because it has a minimal energetic cost to the signaler. Selection for effective signal-sending behavior harnesses odors that are already available at no extra cost (Müller-Schwarze, 1999). The large number of phenolics and terpenes in castoreum (Tang et al. 1993, 1995), undoubtedly diet-derived, may, therefore, constitute an honest signal, advertising the physical condition of the individual and, indirectly, the food supply in the territory (Müller-Schwarze, 1999). As such, beavers may have evolved a unique organ to store and excrete the secondary defense compounds produced by plants, which may in turn be used in territorial scent marking.

Presently it is not known if beavers deposit castoreum and AGS together or alone when scent marking their territories. Neither is it known how often beavers deposit castoreum compared with AGS. The aim of this study was to investigate and

to search for characteristic chemical compounds from the castor sacs and the anal glands in scent deposited on snow in an effort to resolve this issue. We hypothesized that castoreum is the main scent signal used in the defense of beaver territories during winter, and predicted that castoreum is deposited more often than AGS.

METHODS AND MATERIALS

Study Area and Animals. The study was conducted on two distinct sections of the Bø River in the municipalities of Bø and Sauherad (59°25′N, 09°03′–04′E) in Telemark County, Norway. Section 1 was 13 km long, section 2 was 11 km, and the sections were 5 km apart. The sections of the river studied averaged 35 m in width, and most of the river was ice-free during winter due to hydroelectric regulation further upstream. This provided us with the opportunity to study scent-marking behavior of beaver uninhibited by the usual constraints of winter ice (Rosell et al., 1998). Rosell et al. (1998) and Rosell and Bergan (2000) observed that scent was deposited on snow during winter in our study area. Snow, in contrast to the mud and debris normally used to build scent mounds, provides a suitable substrate for determining the deposition frequency of castoreum and AGS on scent mounds. Beavers have occupied the river since the 1920s (Olstad, 1937), and colony density was believed to be near maximum during our field studies (0.54–0.73 colonies/stream km in 1998). During October–December 1996–1998, the study area was ground-censused for active colonies by recording food caches, new mud on the lodges, and fresh feeding sites. Eight active colonies were found on section 1 in 1996 and 1997, and 7 in 1998. Eight active colonies were present on section 2 in 1998 (section 2 was not studied in 1996 and 1997). During autumn 1995, the average colony size on the Bø River (section 1) was 4.0 ± 0.6 (SD) (Rosell et al., 1998). Territorial boundaries were drawn based on the location of scent mound concentrations (Rosell and Nolet, 1997; Rosell et al., 1998) and from sight observations of animals moving up- and downstream of the lodge (Rosell et al., 1998).

Sample Collection. We collected a total of 96 scent samples on snow (hereafter called snow-scent sample, SSS) between January 1 and March 31, 1997–1999 during 14 trips in the study area. Eighteen samples were collected during 1997, 35 during 1998, and 43 during 1999. A SSS was defined as a scent mark directly on snow or in the ice (frozen), situated on a snow-covered tussock or snow mound scraped together where fluid or secretion from the castor sacs and/or anal glands had been deposited (Rosell et al., 1998). All scent marks had a detectable odorant to the human nose at 2 cm or more. Minimum distance between two different scent marks was 10 cm (Rosell and Bergan, 2000). All scent marks were collected between 08:00 and 13:00 hr and within 24 hr after the last snowfall. Beavers usually had one night without snowfall during which they scent-marked before

the marks were collected. A binocular was used to spot the scent marks from a canoe. All tracks from the water in the snow and a range of colors in the snow were investigated.

Samples from 1997 and 1998 were collected using a plastic bag as a glove to prevent contamination of the samples from skin contact. As much of the affected snow as possible was collected, judging from the light yellow to red colored stains seen at depths of 1–3 cm. Frozen samples were loosened with a clean axe. None of the samples contained debris or organic compounds (e.g., soil, mud, twigs, grass, leaves, or conifer needles) normally used to build scent mounds. The plastic bags were sealed and samples thawed at room temperature in the laboratory, emptied into sterile 100 ml plastic or glass vials with caps to minimize bacterial contamination, and then swirled thoroughly to assure a homogeneous mixture. In 1999, samples were collected directly into a plastic or glass cup with airtight caps and swirled thoroughly. The samples were then immediately frozen and stored at -20°C until analyzed. On each sampling day, we also collected control samples of scent-free snow ($N = 14$) into one vial in all territories where we found SSS with the same methods in order to check for contamination from the plastic and glass equipment used to collect the samples.

We collected castor sacs and anal glands from 60 animals shot locally during the normal hunting season from January 28 to May 6, 1997–1999 (Table 1) (Parker and Rosell, 2001), and used the castoreum and AGS from these animals as the basis for comparison with castoreum and AGS found in the SSS. We opened the castor sacs with a surgical blade and scraped the castoreum from the inside surface with a metal scapula. AGS was collected from the glands by cutting off the last 2–3 mm of the papillae and squeezing out the secretion (Rosell and Sun, 1999; Rosell et al., 2000). Sun and Müller-Schwarze (1998a) found no significant variation in the chemical compounds from the right and left gland of the North American beaver, and we, therefore, collected all secretion in the same glass vial. All samples were stored at -20°C until analyses. We sexed the animals by checking for the presence

TABLE 1. CONTROL SAMPLES OF CASTOREUM AND ANAL GLAND SECRETION (AGS) FROM DEAD MALE (M, $N = 30$) AND FEMALE (F, $N = 30$) EURASIAN BEAVERS OF DIFFERENT AGE[a]

Age[b]	Male		Female	
	Castoreum	AGS	Castoreum	AGS
<1 year (6 M, 7 F)	2	5	5	5
≥1 to 2 year (10 M, 6 F)	9	8	5	5
2.5–14 year (14 M, 17 F)	8	7	10	10
$\sum$	19	20	20	20

[a] Samples were collected from January 28 to May 6, 1997–1999.
[b] See methods and materials.

or absence of the os penis (Osborn, 1955). Age was determined by examining tooth root closure and annual cementum and dentine layers of the first molar (van Nostrand and Stephenson, 1964).

Control Experiment. We conducted a control experiment during April 2000 to check whether our methods could detect the compounds from the castor sacs and the anal glands in the SSS. We simulated beaver scent-marking activity by constructing artificial scent mounds of snow, on snow, and applied castoreum and AGS to them. We used scent material from the same animals (all adults 2.5–14 years old plus the number of male subadults necessary to reach a sample size of $N = 10$, see Table 1) as described above. A sample was taken from a castor sac or an anal gland, divided in two, and distributed randomly to the snow control experiment (SCE) and the lab control experiment (LCE). Ten mounds were treated with castoreum (0.5 g) from males, 10 with castoreum (0.5 g) from females, 10 with AGS (0.25 ml) from males, and 10 with AGS (0.25 g) from females. AGS from females has a high viscosity, so we used a spatula to take out the secretion from the vials and weighed it on an electronic balance. We collected the artificial scent marks 20 hr after being made using the same methods as described above for collection.

*Sample Preparation.*The SSS, including the scent-free controls, and the SCE were thawed by putting the bottles into a water bath at 35–40°C for 10 min. Afterwards, the samples were transferred to a 250-ml Erlenmeyer flask with ground joint. We added 10 ml of toluene, and the solution was swirled using a magnetic stirrer at high velocity (about 1400 rpm) for 10 min. The solution was then transferred to a separatory funnel, where it remained for 15–20 min. to allow the phases to separate. The lower water phase was tapped out and the upper organic phase filtered through a filter paper (Schleicher and Schuell no. 595, Dassel, Germany) into an evaporating flask. A PTFE sleeve for ground joints was set in the flask neck to avoid locking. The sample was evaporated in a Heidolph WB 2001 rotary evaporator to a volume of variable size (therefore, we only carried out qualitative analyses), and 1 ml of toluene was added. The solution was transferred to a vial with a snap cap that was kept in a refrigerator (<24 hr) at 4°C until the analysis was done. We also analyzed the water phase. Some of the compounds from the organic phase were found in the water phase as well, but in lower concentrations. However, we found no new compounds in the water phase.

AGS and Castoreum. From the control material (the dead animals), we transferred 0.25 ml AGS from the males and 0.25 g from the females into smaller glass vials before adding 7.5 ml of a mixture of toluene–methanol 3:1. The solutions were filtered through a filter paper (Schleicher and Schuell no. 595) and kept in a refrigerator (< 24 hr) at 4°C until analyses.

We weighed 0.5 g castoreum and added 10 ml of toluene–methanol 3:1. The sample was extracted in a Milestone MLS 1200 Mega microwave oven. The extraction program had the following cycle: 5 min at 250 W and 100°C, 5 min at

400 W and 130°C, and 5 min at 400 W and 160°C. All compounds were dissolved. The sample was then treated the same way as the AGS solution.

Chemical Analysis. We injected 1 μl of the resulting solution into a Hewlett-Packard 6890 Series II gas chromatograph equipped with a nonpolar HP-5 MS 5% phenyl-methyl-siloxane column (30.0 m long $\times$ 0.25 mm ID $\times$ 0.25-μm film thickness) connected to a Hewlett-Packard 5973 Series mass spectrometer detector with a split/splitless inlet used in the splitless mode. Helium was used as the carrier gas at a constant flow of 0.7 ml/min. The following temperature program was employed for all analyses: 130°C to 310°C at 4°C/min and kept at 310°C for 15 min. To avoid that the solvent damaged the detector, a delay of 2 min was set for every run. We used the first 60 min as the collection time because few compounds were detected after 60 min and most of these peaks stemmed from the column material. The column was regularly baked out at 315°C to remove any remaining low-boiling-point compounds. The instrument was regularly calibrated to detect possible changes in the sensitivity of the instrument. This prevented unstable conditions during the work. We characterized each compound in a sample by its GC retention time and mass spectrum, and we determined the structures of some of the compounds with a computer-aided compound search of the Wiley 275 Library, which contains about 70,000 known compounds. In addition, all mass spectra from the samples and from the library were visually compared to see if the suggestions from the computer were reasonable. We also made our own library of the compounds not found in the Wiley library, i.e., we saved 12 compounds from the castor sacs and 128 compounds from the anal glands from the dead individuals. We could, therefore, recognize many compounds from individual to individual, and also from individuals to the SSS. Since the main focus of our study was to determine if the beaver used castoreum and/or AGS in their scent-marking behavior, positive identification of these compounds was not attempted, i.e., we did not compare the compounds with a known standard. All major peaks were analyzed, but excessively small peaks were not, i.e., peaks less than twice the noise level were not analyzed. After numerous injections, we could see a small shortening in the retention times of the peaks because the column aged a little. We, therefore, presented the retention time found when the column was new. Peak retention times were rounded off to the first decimal.

Statistics. Since the data did not fit assumptions of distribution and homogeneity of variance for parametric analysis (Sokal and Rohlf, 1995), we used nonparametric statistics (Siegel and Castellan, 1988). A Mann-Whitney U test was used to compare the median number of peaks between two independent groups. We corrected for compounds in the plastic and glass equipment used to collect the samples when counting the number of castoreum and AGS compounds and omitted the smallest peaks (see above). Probability values are two-tailed and 5% was used as the level of significance. All data were treated in Minitab version 12.1 for Windows.

RESULTS

Castoreum. Chemical analysis of the 96 SSS revealed that castoreum compounds were present in all samples ($\bar{X} \pm \mathrm{SD} = 6.75 \pm 4.18$, range = 1–17). Figure 1a is a typical example. We found 24 different compounds from castoreum in the SSS (Table 2). None of the compounds was found in all SSS, and 17 of the compounds were found in 10 or more of the SSS (Table 2). Forty-seven of the 50 (94.0%) compounds detected in the castoreum from the dead animals had a molecular weight below 300 (Table 3).

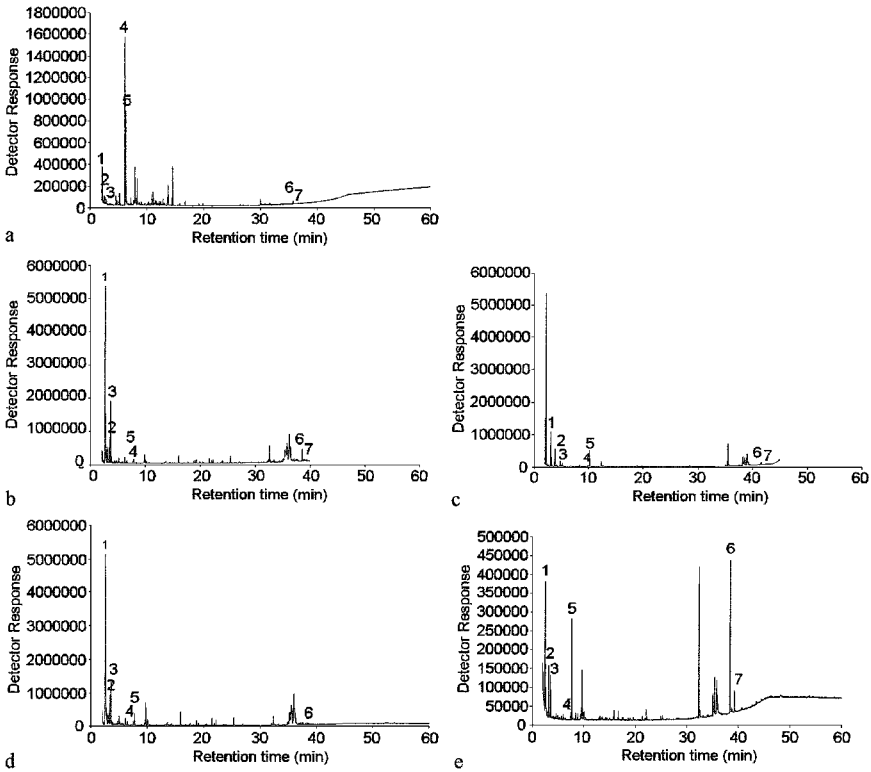

FIG. 1. Typical chromatograms of castoreum from (a) a beaver snow-scent sample, (b) a dead adult male, (c) a dead adult female, (d) from the snow control experiment with the same male as in b and (e) the same female as in c. Compound marked 1–7 in the chromatograms are tentatively identified as: 1, ethylphenol; 2, prophylphenyl; 3,4-ethyl-2-methoxyphenol; 4,4-(4-hydroxyphenyl)-2-butanone; 5, 4-(4-hydroxyphenyl)-2-butanol; 6, cholest-5-ene-3-ol; and 7 cholest-7-ene-3-ol (3β,5-α). Note that identities of the compounds have not been verified with known samples (see Methods and Materials). The *x* axis is the time in minutes, and the *y* axis is in arbitrary units.

TABLE 2. TENTATIVELY IDENTIFIED COMPOUNDS FOUND IN 96 SNOW-SCENT SAMPLES FROM EURASIAN BEAVERS

No.	Tentatively identified compound[a]	Retention time (min)	Samples (N)	Source[b] (Sex)[c]
1	Ethylphenol	2.2	75	c
2	Borneol	2.3	3	c
3	α-Terpineol	2.4	5	c
4	Myrthenol	2.4	12	c
5	Methoxymethylphenol	2.4	6	c
6	Verbenone	2.5	35	c
7	3-phenylpropanol	2.6	34	c
8	Propylphenol	2.7	66	c
9	2-Hydroxybenzylalcohol	2.8	29	c
10	4-Ethyl-2-methoxyphenol	2.9	33	c
11	Unknown	3.6	7	c
12	4-Hydroxyacetophenone	4.6	33	c
13	Unknown	5.0	12	c
14	4-Hydroxy-3-metoxyacetophenone	5.3	54	c
15	4-Hydroxy-3-methoxybenzoic acid, methylester	5.7	38	c
16	4-(4-Hydroxyphenyl)-2-butanone	6.3	49	c
17	4-(4-Hydroxyphenyl)-2-butanol	6.5	44	c
18	4-(4-Hydroxy-3-methoxyphenyl)-2-butanone	8.0	13	c
19	Unknown	8.1	10	c
20	4-(4-Hydroxy-3-methoxyphenyl)-2-butanol	8.5	15	c
21	Unknown	14.0	4	c
22	Unknown	14.7	10	c
23	Hexadecanoic acid	16.5	1	a (F)
24	Unsaturated C_{14} hydrocarbon	18.3	3	a (M)
25	Double unsaturated C_7–C_7 ester	25.1	2	a (M)
26	Double unsaturated C_7–C_8 ester	26.3	2	a (M)
27	Unknown	30.3	7	c
28	Hydrocarbon	31.7	2	a (F)
29	Unsaturated C_{30} hydrocarbon	32.3	3	a (F)
30	C_{29} wax ester	37.2	1	a (M)
31	A steroid	37.8	1	a (M, F)
32	Cholestan-3-ol (3-β,5-α)	37.8	1	a (M, F)
33	Unknown	38.0	1	a (M, F)
34	Saturated C_{15}–C_{15} wax ester	38.1	1	a (M)
35	Unsaturated C_{31} wax ester	38.4	3	a (M)
36	A steroid	38.5	1	a (M, F)
37	Cholest-7-ene-3-ol (3-β,5-α)[d]	39.2	4	a (F) or c
38	4-α-Methylcholest-8(14)-ene-3-β	39.8	1	a (M, F)
39	Saturated C_{31} wax ester	40.3	1	a (M)
40	A steroid	41.4	1	a (F)
41	A steroid	42.0	1	a (F)
42	Unknown	44.8	1	a (M)
43	A steroid	45.8	1	a (M, F)

[a] The identities have not been verified with known samples.
[b] Also found in the castoreum (c) or anal gland secretion (a) samples from dead beavers (control material) (see Tables 3 and 4).
[c] M = male and F = female.
[d] Note that this compound was found in both castoreum and anal gland secretion of females (no. 50 in Table 3 and no. 107 in Table 4).

TABLE 3. TENTATIVELY IDENTIFIED COMPOUNDS FOUND IN CASTOREUM FROM DEAD MALE ($N = 19$) AND FEMALE ($N = 20$) EURASIAN BEAVER[a]

No.	Tentatively identified compound[b]	Retention time (min)	Samples (N)	
			Male	Female
1	Cyclohexandiol	1.8	6	7
2[c]	2-Methylphenol	1.8	13	14
3	Undecane	1.9	1	0
4[c]	Benzenemethanol	2.0	17	19
5[c]	Ethylphenol (1)	2.2	19	20
6[c]	Benzoic acid	2.2	2	5
7[c]	Borneol	2.3	12	12
8[c]	1,2-Benzenediol	2.3	4	7
9[c]	α-Terpineol	2.4	11	11
10[c]	Myrthenol	2.4	14	14
11	2-Hydroxybenzoic acid	2.4	1	0
12	Methoxymethylphenol	2.4	2	3
13[c]	Verbenone	2.5	16	16
14[c]	3-Phenylpropanol	2.6	9	13
15[c]	Propylphenol (2)	2.7	18	18
16[c]	2-Hydroxybenzylalcohol	2.8	13	17
17[c]	Myrthanol	2.8	3	1
18[c]	1,4-Benzenediol	2.8	1	0
19[c]	4-Ethyl-2-methoxyphenol (3)	2.9	17	16
20[c]	Unknown	3.2	1	2
21[c]	Benzenepropanoic acid	3.4	3	4
22[c]	Aromatic carboxylic acid	3.4	3	5
23	Phenyl propanoic acid	3.4	3	1
24	Unknown	3.6	3	2
25[c]	2-Methoxy-4-propylfenol	3.6	6	9
26[c]	5-Methyl-1,3-benzenediol	3.6	2	6
27[c]	4-Ethyl-1,3-benzenediol	3.8	3	8
28[c]	3,4-Dihydro-2H-1-benzophyrane-2-one	4.1	9	9
29[c]	4-Methoxy benzoic acid	4.4	5	5
30[c]	2-(4-Hydroxybenzene)-ethanol	4.4	9	12
31[c]	4-Hydroxyacetophenone	4.6	9	12
32[c]	Unknown	5.0	14	15
33[c]	3-Hydroxy benzoic acid	5.2	8	7
34[c]	4-Hydroxy-3-methoxy acetophenone	5.3	13	11
35	4-Hydroxy-3-methoxybenzoic acid, methylester	5.6	3	1
36[c]	4-Hydroxy-3-methoxymethanoic acid	5.7	6	10
37[c]	4-(4-Hydroxyphenyl)-2-butanone (4)	6.3	19	20
38[c]	4-(4-Hydroxyphenyl)-2-butanol (5)	6.5	19	20
39	Unknown N-compound	7.2	2	3
40[c]	4-(4-Hydroxy-3-methoxyphenyl)-2-butanone	8.0	2	4
41	4-Hydroxy-3-methoxy-benzoacetic acid	8.0	0	1

TABLE 3. CONTINUED

No.	Tentatively identified compound[b]	Retention time min	Samples (*N*) Male	Female
42[c]	Unknown	8.1	14	18
43[c]	4-(4-Hydroxy-3-methoxyphenyl)-2-butanol	8.5	7	7
44[c]	unknown	14.0	11	14
45[c]	Unknown	14.7	10	11
46[c]	Unknown	30.3	13	18
47[c]	Unknown	33.5	17	18
48[c]	Unknown	33.8	19	20
49[c]	cholest-5-ene-3-ol (6)	36.1	11	14
50[c]	cholest-7-ene-3-ol (3-beta, 5-alpha) (7)	39.2	5	9

[a] Numbers in parentheses match those in Figure 1.
[b] The identities have not been verified with known samples.
[c] Compounds were found both in the snow control experiment and in the dead animals used in the control experiment.

We found no difference in median number of castoreum compounds between dead males and females (males: $\bar{X} \pm \text{SD} = 22.53 \pm 2.86$, range = 18–29, $N = 19$; females: $\bar{X} \pm \text{SD} = 23.25 \pm 3.95$, range = 12–29, $N = 20$) ($W = 338.0$, $P = 0.241$). Figure 1b and 1c show typical chromatograms from a dead adult male and female. Forty-six (92%) of the detected compounds were found in both sexes. Three compounds were detected in only males and one in only females (Table 3). However, they were found in only one animal (Table 3). Forty-one (82%) compounds were found both in the SCE and in the LCE (Table 3) (compare Figure 1b and d, and 1c and e). This shows that our method could detect the compounds from the castor sacs in the SSS.

AGS. Chemical analysis of the 96 SSS revealed that AGS compounds were present in only four samples ($\bar{X} \pm \text{SD} = 7.50 \pm 4.93$, range = 2–14). Figure 2a is a typical example. These four scent marks were found on January 19, 1997; February 5 and March 13, 1998; and on March 15, 1999; they were inside four different territories. Three of the SSS were deposited directly on snow, while one was deposited on a snow-covered tussock. We found that two of the scent marks (January 19, 1997, and March 15, 1999) contained compounds from only females, and two contained compounds that were typical for both sexes. We found 20 different compounds from AGS in the SSS (Table 2).

More compounds were found in the AGS from dead males ($\bar{X} \pm \text{SD} = 55.40 \pm 10.81$, range = 29–69, $N = 20$) compared with females ($\bar{X} \pm \text{SD} = 22.70 \pm 4.93$, range = 14–30, $N = 20$) ($W = 607.5$, $P = 0.0001$). Figure 2b and c show typical chromatograms from a dead adult male and female. Overall, 56 different compounds were found in the female AGS and 126 compounds in the

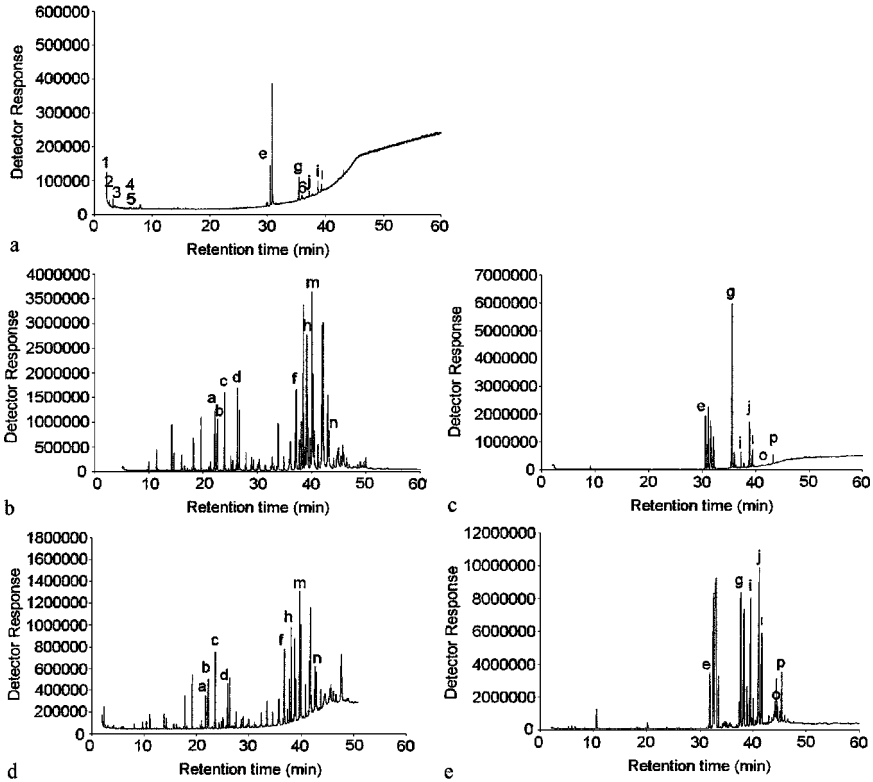

FIG. 2. Typical chromatograms of anal gland secretion from (a) a beaver snow-scent sample deposited by a female (contains both compounds form castoreum and AGS), (b) a dead adult male, (c) a dead adult female, (d) from the snow control experiment with the same male as in b and (e) the same female as in c. Compunds marked form a–p in the chromatograms are tentatively identified as: a, hexadecadiene; b, unknown; c, unknown; d, long hydrocarbon chain; e, unsaturated C_{30} hydrocarbon; f, C_{29} wax ester; g, unknown; h, usaturated wax ester; i, a steroid; j, 4-α-methylcholest-8(14)-ene-3-β; k, unknown; p, a steroid; m, unsaturated C_{32} wax ester; n, saturated C_{33} wax ester; o, a steroid; and p, a steroid. Note that the identities of the compounds have not been verified with known samples (see Methods and Materials). The *x* axis is the time in minutes, and the *y* axis is in arbitray units.

male AGS (Table 4). Nineteen compounds were found in both males and females (Table 4). Only 7 (12.5%) of the compounds detected in AGS of females and 41 (32.5%) of the compounds in the males had a molecular weight below 300 (Table 4). One-hundred thirty-three (89.3%) compounds were found both in the SCE and in the LCE (both sexes combined) (Table 4) (compare Figure 2b and d, and 2c and e). Therefore, our method detected the compounds from the anal gland in the SSS.

TABLE 4. TENTATIVELY IDENTIFIED COMPOUNDS FOUND IN ANAL GLAND SECRETION OF DEAD MALE ($N = 20$) AND FEMALE ($N = 20$) EURASIAN BEAVER[a]

No.	Tentatively identified compound[b]	Retention time (min)	Samples (N)	
			Male	Female
1[c]	Dodecanoic acid	7.6	4	0
2[c]	1,13-Tetradecadiene	9.8	8	0
3[c]	Tetradecanoic acid, methyl ester	9.8	5	0
4[c]	Unsaturated or cyclic hydrocarbon	10.0	12	0
5[c]	1-Tetradecene	10.0	2	0
6	3-Tetradecene	10.0	1	0
7[c]	Unknown	10.3	1	17
8[c]	Pentadecanoic acid, methyl ester	11.2	5	0
9[c]	Pentadecene	11.5	7	0
10[c]	Unknown	11.7	7	0
11	Unknown	11.7	0	2
12[c]	Tetradecanoic acid	11.9	8	3
13[c]	Pentadecanoic acid	13.4	5	3
14[c]	Hexadecanoic acid, methyl ester	13.8	6	0
15[c]	Hexadecadiene (a)	14.2	11	0
16	Methyl hexadecanate	14.2	0	3
17	Hexadecanoic acid	14.3	1	0
18[c]	1-Hexadecene or cyclohexadecane	14.7	9	0
19[c]	Unknown	16.1	5	0
20	Heptadecadiene	16.1	1	0
21	1-Heptadecene	16.1	1	0
22[c]	Unsaturated C_{16} fatty acid ($C_{15}H_{29}COOH$)	16.2	6	0
23	Hexadecanoic acid	16.5	0	3
24[c]	Unknown	16.7	11	0
25	Unknown	17.2	3	0
26[c]	Unknown hydrocarbon	18.0	8	0
27[c]	Unsaturated C_{14} hydrocarbon	18.3	20	0
28[c]	Octadecanoic acid, methyl ester	18.7	5	3
29[c]	Unknown (b)	19.6	13	0
30[c]	Unknown	19.7	4	0
31	Nonadecanoic acid, methyl ester	20.0	2	0
32[c]	Unknown	20.5	0	14
33[c]	Unknown	21.2	9	0
34[c]	N-compound	21.4	18	0
35	Unknown	22.0	1	0
36[c]	Hydrocarbon	22.5	12	0
37[c]	Unknown	22.7	15	0
38[c]	Unknown (c)	24.0	18	0
39[c]	Unknown	24.2	2	0
40	Aromatic compound	24.3	1	0
41[c]	Double unsaturated C_7–C_7 ester	25.1	12	0
42[c]	Unknown	25.2	20	0
43[c]	N-compound	25.6	19	0

TABLE 4. CONTINUED

No.	Tentatively identified compound[b]	Retention time (min)	Samples (*N*)	
			Male	Female
44[c]	Unknown	26.1	16	0
45[c]	Double unsaturated C_7–C_8 ester	26.3	5	0
46[c]	Long hydrocarbon chain (d)	26.4	19	0
47[c]	Unknown	26.7	8	0
48	Docosanoic acid, methyl ester	27.4	1	0
49[c]	Double unsaturated C_8–C_6 ester	27.7	11	0
50[c]	Unknown	27.8	14	0
51[c]	Wax ester	27.9	16	0
52[c]	Unknown	29.0	20	0
53[c]	Unknown	29.1	9	0
54[c]	Unknown	29.2	10	0
55[c]	Hexadecyl octanat (wax ester)	29.3	8	0
56[c]	Unknown	30.1	11	0
57[c]	Unknown	30.2	1	0
58[c]	Unknown	30.4	15	0
59[c]	Unknown	31.5	16	0
60[c]	Unknown	31.6	0	8
61	Hydrocarbon	31.7	0	1
62[c]	A steroid	31.7	5	0
63[c]	Unsaturated C_{30} hydrocarbon (e)	32.3	0	20
64[c]	Unknown	32.3	0	8
65[c]	A steroid	32.4	5	1
66	Unknown	32.4	3	0
67	Unknown	32.5	1	0
68[c]	Hexamethyl-tetracosatetraene	32.5	5	7
69[c]	A steroid	32.6	2	0
70[c]	C_{26} wax ester	32.8	11	0
71[c]	Wax ester	32.8	6	0
72[c]	Unsaturated C_{30} hydrocarbon	32.9	0	18
73	Unknown	33.0	0	1
74[c]	A steroid	33.0	4	0
75[c]	Unsaturated hydrocarbon	33.2	8	0
76	A steroid	33.2	0	1
77[c]	Unknown	33.3	0	11
78[c]	Saturated C_{14}–C_{14} wax ester with side chain	33.3	5	0
79[c]	Unknown	33.4	0	1
80	Unknown	33.5	0	2
81[c]	Wax ester	33.6	11	0
82[c]	Unknown	33.9	2	0
83[c]	Wax ester	33.9	18	0
84[c]	Unknown	33.9	0	3
85[c]	Unknown	33.9	0	1
86[c]	Unknown	34.0	0	10
87[c]	Unknown	34.3	4	0
88	Unknown	34.4	0	3

TABLE 4. CONTINUED

No.	Tentatively identified compound[b]	Retention time (min)	Samples (*N*)	
			Male	Female
89[c]	Unknown	34.9	15	0
90[c]	A steroid	34.9	3	0
91[c]	Unknown	35.9	11	0
92[c]	Tetradecyl-tetradecanoate (wax ester)	36.1	12	0
93[c]	Vitamine E	36.6	3	0
94[c]	Unknown	36.9	12	0
95[c]	C_{29} wax ester (f)	37.2	20	0
96[c]	Unsaturated C_{30} wax ester	37.5	1	0
97[c]	Cholestan-3-ol-(3-β,5-α)	37.8	5	11
98[c]	A steroid	37.8	9	13
99[c]	Unknown (g)	38.0	11	19
100[c]	Saturated C_{15}–C_{15} wax ester	38.1	19	0
101[c]	Unsaturated C_{31} wax ester	38.4	4	0
102[c]	A steroid	38.5	17	17
103	Dihydrocholesterol	38.6	3	0
104[c]	A steroid	38.8	13	12
105	A steroid (coprostan-3-ol)	39.0	0	1
106[c]	Unsaturated wax ester (h)	39.2	17	0
107[c]	Cholest-7-ene-3-ol (3-β,5-α)	39.2	0	13
108[c]	Unknown	39.3	3	0
109[c]	A steroid	39.3	5	0
110[c]	A steroid (i)	39.5	0	6
111[c]	A steroid	39.8	2	0
112[c]	4-α-Methylcholest-8 (14)-ene-3-β (j)	39.8	13	17
113[c]	Wax ester	39.9	4	0
114[c]	Unknown (k)	40.1	17	0
115	A steroid	40.1	0	3
116[c]	4-Methylcholest-7-ene-3-one	40.1	0	9
117[c]	Unknown	40.3	0	16
118[c]	Saturated C_{31} wax ester	40.3	19	0
119[c]	A steroid	40.4	0	14
120[c]	Cholest-4-ene-3-one	40.5	1	0
121[c]	A steroid	40.9	0	13
122[c]	Unknown	41.0	0	5
123[c]	Dihydrolanosterol	41.2	0	12
124[c]	Heptadecanoic acid, pentadecyl ester	41.2	20	0
125[c]	A steroid	41.4	0	9
126[c]	A steroid	41.4	2	1
127[c]	4-β-Methyl-24(*R*)-methylcholest-8 (14)-ene-3-beta	41.5	0	3
128[c]	Lanosta-8,24-diene-3-ol (3-β)	41.9	0	19
129[c]	Double unsaturated C_{32} wax ester	42.0	20	0
130[c]	A steroid (l)	42.0	0	18
131[c]	Unsaturated C_{32} wax ester (m)	42.2	20	0
132[c]	Hexadecanoic acid, hexadecyl ester	42.3	19	0

TABLE 4. CONTINUED

No.	Tentatively identified compound[b]	Retention time (min)	Samples (N)	
			Male	Female
133	9-Octadecenoic acid, hexadecyl ester	42.8	2	0
134[c]	Unsaturated C_{33} wax ester	43.0	19	0
135	Cholest-3-ene (5-α)	43.0	0	2
136[c]	Saturated C_{33} wax ester (n)	43.2	18	0
137[c]	Unknown	43.4	3	0
138[c]	A steroid	43.4	3	0
139	Octadecanoic acid, hexadecylester	43.5	2	0
140[c]	Unknown	44.1	18	0
141[c]	Unknown	44.4	0	5
142[c]	A steroid	44.5	0	8
143[c]	Unknown	44.6	8	0
144[c]	A steroid	44.7	3	9
145	Unknown	44.8	4	0
146[c]	Double unsaturated C_{34} wax ester	44.9	8	0
147[c]	Unsaturated C_{34} wax ester	45.0	6	0
148[c]	A steroid (o)	45.0	12	16
149[c]	A steroid	45.5	5	4
150[c]	Unknown	45.5	9	6
151[c]	A steroid (p)	45.8	17	19
152[c]	A steroid	45.8	1	0
153[c]	Unknown	46.1	11	0
154[c]	A steroid	46.6	12	2
155[c]	Unknown	47.1	9	0
156	A steroid	48.6	2	0
157	Unknown	48.9	5	0
158[c]	Unknown	49.1	14	0
159[c]	Wax ester	49.4	3	0
160[c]	A steroid	49.8	11	0
161[c]	A steroid	50.1	18	0
162	A steroid	55.2	0	1
163	A steroid	57.3	0	1

[a] Letters in parentheses match those in Figure 2.
[b] The identities have not been verified with known samples.
[c] Compounds were found both in the snow control experiment and in the dead animals used in the control experiment.

DISCUSSION

Our results support the prediction that castoreum is most frequently deposited on scent marks (96 of 96) and appears, therefore, to be the main scent signal used in the defense of Eurasian beaver territories during January–March. AGS, however, was deposited only on 4 of 96 scent marks. This suggests that beavers do not specifically deposit AGS on scent mounds, but that the compounds we found

possibly were remnants of AGS from the feet and/or fur after pelage lubrication (Walro and Svendsen, 1982). Beavers may also get AGS on their feet and fur following coprophagy (Wilsson, 1971). AGS may, therefore, have other functions.

Beaver scent marks with castoreum might be a volatile alerting signal for attracting attention (Müller-Schwarze, 1999). Alerting signals contain no information about an individual or even a species (Müller-Schwarze, 1999). Responses to single compounds support the hypothesis that castoreum is used for signaling territorial occupancy, which requires only one bit of information in the signal for making a decision by receivers, i.e., whether the territory is occupied or not (Müller-Schwarze and Houlihan, 1991; Schulte et al., 1994; Sun and Müller-Schwarze, 1999). It may be that the lighter, volatile compounds in the castoreum direct receivers toward the less volatile but potentially more informative chemical components still present at the scent mark. This is supported by the fact that 94% of the compounds had a molecular weight below 300.

Schulte (1998) found that North American beavers discriminated among castor-fluid scents from family, neighbor, and nonneighbor adult males. The Eurasian beaver can also discriminate among scents (castoreum and AGS) from neighbor and non-neighbor individuals (Rosell and Bjørkøyli, unpublished data). However, no significant difference was found in the number of castoreum compounds between dead males and females whose castoreum chromatograms were similar (see also Pedersen, 1999). Likewise, Sun and Müller-Schwarze (1999) failed to find any consistent difference between male and female castoreum profiles in North American beaver and concluded that castoreum is unlikely to be used for sex recognition. This conclusion is in accordance with the evidence that castoreum compounds are mainly dietary derivatives, which do not differ between the two sexes (Müller-Schwarze, 1992). By contrast, the composition of AGS in both the North American and Eurasian beaver exhibits chemical sexual dimorphism (Grønneberg, 1978–1979; Grønneberg and Lie, 1984; Sun and Müller-Schwarze, 1999; this study). Whether the Eurasian beaver uses the sex difference in AGS to distinguish between individuals of different sex, needs further study.

AGS also contains information about individuality, kinship, and family membership (Sun and Müller-Schwarze, 1999). Sun and Müller-Schwarze (1997) have shown that North American beavers use AGS to discriminate between unfamiliar sibling and unfamiliar nonrelatives and that this discrimination was not shown when castoreum samples were tested. Sun and Müller-Schwarze (1998b) showed that beavers' response to AGS from unfamiliar adult males remained at about the same level, but their response to castoreum showed a descending trend. The descending trend in response to the same signal without matching the signaler demonstrates a declining importance of the signal over time, i.e., the scent-matching hypothesis (Gosling, 1982) was supported. The scent-matching hypothesis predicts, among other things, that the territory owner should make itself available for scent matching by the intruder (Gosling, 1982). Sun and Müller-Schwarze (1998a)

recently documented that related individuals shared more features in the chemical AGS profile than did unrelated individuals, and Sun and Müller-Schwarze (1998c) also demonstrated that it is possible to use some AGS compounds to classify different families.

AGS may act as a chemical messenger in the water territory (Grønneberg and Lie, 1984) sensed through close range or contact with the animal. The latter is supported by the fact that only 12.5% and 32.5% of the compounds detected in AGS of females and males, respectively, had a molecular weight below 300. It could be advantageous for a swimming mammal such as the beaver to present chemical signals in the form of lipid substances that would concentrate at the air–water interface (Albone, 1984). By lubricating the fur with AGS, which would be released into the water, beaver could also act as a "living scent mark." As AGS is insoluble in water (Svendsen, 1978), beavers downstream would receive a concentrated flow of chemical scent information in the surface film from upstream territories (Rosell et al., 1998). The recently discovered vomeronasal organ in Eurasian beavers may play a significant role here (Rosell and Pedersen, 1999). Furthermore, anal glands, which are located in the anus (Svendsen, 1978), may add AGS to the feces when beavers defecate in the water. For instance, the large complex of sebaceous and apocrine glands located in and around the anus of many species of antelope may add individual-specific secretion to feces (Barrette, 1977; Mainoya, 1980; Gosling, 1982).

However, Rosell and Bergan (1998) observed on July 21 two adult Eurasian beavers depositing AGS at the border of their territory by everting the "cloaca," protruding the anal gland openings and rubbing them against the surface as the animal walked over the scent mound. Therefore, further analyses need to clarify if beavers use the AGS on scent marks of other times of the year.

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