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Investigation of Scents on Cheeks and Foreheads of Large Felines in Connection to the Facial Marking Behavior

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Abstract We investigated head- and cheek-rubbing behavior in four species of large felines, lions (*Leo panther*), leopards (*Panthera pardus*), tigers (*Panthera tigris*), and cougars (*Puma concolor*), in captivity. Preliminary behavioral observations found that lions and tigers, but not leopards and cougars, showed behavioral responses to cardboard rubbing samples from head and cheek areas from conspecific felines, compared to the blank cardboard controls. In this context, surface samples on the facial areas of each species were collected to analyze volatile organic compounds that could be involved in the facial marking of felines. Previously developed stir bar surface sampling methodology was used. From all cheek and forehead samples, 100 volatile organic compounds were identified or tentatively identified. Among these, 41 have been previously reported to be present in feline urine and marking secretions. Several new compounds were identified on facial surfaces. Some of the compounds showed substantial quantitative differences among the species. One compound, that has not been reported previously in mammals, 3-acetamidofuran, was found in all investigated species. It was synthesized and tested for behavioral responses. No responses were elicited in a preliminary test. Future research will test other potential signaling compounds and their mixtures for ability to elicit behavioral responses.

Keywords Felidae · Facial marking behavior · Chemical signals · Lion · Tiger · Cougar · Leopard · Volatile compounds · Gas chromatography–mass spectrometry

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Introduction

Members of the family *Felidae* are essentially solitary in their activities and rarely associate with each other, with the exception of lions, cheetahs, and domestic cats (Kleiman and Eisenberg, 1973). This asocial existence does not eliminate the need for communicative signals. Individuals still need to avoid conflict and find mates, and use a complex repertoire of auditory calls and roars (Schaller, 1972), visual signals, including dirt scrapes and tree scratches (Wemmer and Scow, 1977; Smith et al., 1989), and olfactory marks deposited in the form of urine and feces, to convey information (Schaller, 1972; Kleiman and Eisenberg, 1973; Smith et al., 1989).

Marking territory with urine and feces is thought to be a way in which important volatile compounds (signals) identify individuals and advertise reproductive condition (Schaller, 1972). Urine and anal sac-derived volatile compounds have been partially characterized for many large cats, including tigers (Brachmachary and Dutta, 1981; Brachmachary et al., 1992; Poddar-Sarkar et al., 1994, 2004; Poddar-Sarkar, 1996; Burger et al., 2008), lions (Albone and Eglington, 1974; Anderson and Vulpius, 1999), leopards (Brachmachary and Dutta, 1984; Poddar-Sarkar and Brahmachary, 2004),

cheetahs (Burger et al., 2006), and bobcats (Mattina et al., 1991).

A second marking behavior, not as well understood, is head and cheek rubbing. Rubbing behavior has been documented in several species of captive small cats (Mellen, 1993), domestic cats (Van Den Bos and de Cock, 1994; Penny Bernstein personal communication 2009), wild lions (Schaller, 1972), wild leopards (Bothma and le Riche, 1995), and wild tigers (Smith et al., 1989), but the function of the rubbing is in question. Some suggest the behavior functions as a mechanism to pick up scent from objects or as a visual display (Rieger, 1979). Others suggest that rubbing deposits scent, as well as picking up scent and displaying visual signals (Mellen, 1993). Some volatile compounds, such as long-chain carboxylic acids, in particular, have been identified from lion manes (Poddar-Sarkar et al., 2007). In both domestic cats and lions, there is believed to be a form of tactile communication, as well as a marking behavior, which may serve the same purpose as grooming in primates (Schaller, 1972; Van Den Bos and de Cock, 1994).

The purpose of our study was threefold. We collected facial samples on cardboard squares from captives of four species of large felines, lions (*Leo panther*), leopards (*Panthera pardus*), tigers (*Panthera tigris*), and cougars (*Puma concolor*) to observe whether the samples could elicit behavioral responses from conspecifics, indicating that possible chemical signaling compounds would be transferable. Second, we collected separate samples from the facial areas of felines to determine the volatile organic compounds that are present in the head and cheek areas, and could possibly be involved in the marking behavior. Third, we tested behavioral responses to an unusual compound present on all the feline species.

Methods and Materials

Study Site We conducted this study with captive felines at the Exotic Feline Rescue Center, Center Point, Indiana, USA. This is a 108-acre facility that houses 205 felines, representing 10 species. The cats are housed in fenced enclosures ranging from 100 to 1,600 m^2, depending on the species and numbers of individuals housed together. Each enclosure contains at least one climbing tower, water trough or pond, and shelter boxes with straw. Cats are fed a diet that includes raw meat and provided with Boomer Balls™ (Domestic, Zoo and Exotic Animal Products) and other toys for behavioral enrichment. Male lions are vasectomized to retain manes. Other individuals are spayed or neutered, depending upon enclosure members, to prevent pregnancies, and so both altered and unaltered individuals are included in the population. The study was approved by Bloomington Institutional Animal Care and Use Committee Protocol, Study # 07–039.

Preliminary Scent Collection for Behavior Tests To determine if active compounds could be transferred from the head and cheek areas, samples for behavioral tests were taken by rubbing 10×10 cm generic white paper-based cardboard squares on the head and cheek areas of an unneutered male of each species. Samples were collected in May 2008, stored in plastic bags, and refrigerated. Tests were conducted from 5/15/2008 to 9/10/2008 between the hours of 9 am and 1 pm. Three squares, a blank to control for handling contamination, a head or a cheek sample, were mounted with tape on enclosure fences approximately 1.5 m apart at eye level for each species. Ten minutes of observation began when the first individual approached the squares, and subsequent responses were recorded. Positive responses included sniffing, flehmen, licking, rubbing, and urinating toward the swatch. Twenty seven 10-min sessions were conducted with the animals listed in Table 1. Some enclosures had more than one individual. In those cases, it was possible that one animal could be responsible for most of the responses; however, these observations were carried out as preliminary tests to determine if volatile compounds that were able to elicit responses were present. Responses to head or cheek samples were compared to that to the control square by χ^2 tests of frequencies.

Table 1 Animals used for cardboard square scent tests

Species	Enclosure occupants	Males/Females
Cougar	single	male
Cougar	single	female
Cougar	two	1 male and 1 female
Leopard	single	male
Leopard	two	1 male and 1 female
Leopard	two	female
Leopard	two	1 male and 1 female
Leopard	three	1 male and 2 females
Lion	three	males
Lion	single	female
Lion	single	male
Lion	seven	females
Lion	two	1 male and 1 female
Lion	single	male
Lion	single	female
Lion	single	female
Tiger	four	2 males and 2 females
Tiger	four	2 males and 2 females
Tiger	single	male
Tiger	four	2 males and 2 females
Tiger	four	1 male and 3 females
Tiger	five	2 males and 3 females
Tiger	two	1 male and 1 female

Feline Forehead and Cheek Surface Sampling for the Chemical Analyses After positive preliminary responses were obtained using the cardboard squares, separate samples for chemical analysis were gathered using a polymer-coated, 1 cm long rolling pin (Twister™ stir bar, Gerstel GmbH, Mülheim an der Ruhr, Germany). Information about the felines used for the facial stir bar scent collections is shown in Table 2. This technique was developed previously for *in situ* surface-sampling applications (Soini et al., 2006). Surface samples were collected in July 2008 from the head (above eyes and between ears) and cheek areas (near the dorsal edge of the mouth) of 21 individuals. These included 3 males and 3 females each of lions, tigers, and cougars, and 1 female and 2 male leopards. A preconditioned stir bar with an embedded internal standard was placed between the jaws of the collection device. Two separate 5-cm long stretches of the sampling area were rolled over with a stir bar (10 cm^2 skin area). The stir bar was subsequently dropped from the collection device and placed in a capped Twister™ glass vial. All samples were stored in the refrigerator for 2–3 days until analysis. In previous human skin studies, the collected skin surface samples were chemically stable up to 14 days (Soini et al., 2006; Penn et al., 2007).

Reagents and Materials for Chemical Analyses Standards were purchased from Sigma-Aldrich Chemical Company (Milwaukee, WI, USA) and TCI America (N-acetylglucosamine; Portland, OR, USA). Stir bars (Twister™, 10 mm, 0.5 mm film thickness, 24 µl polydimethylsiloxane) used for sample collection were obtained from Gerstel GmbH (Mülheim an der Ruhr, Germany). The stir bars were conditioned prior to embedding the internal standard in a TC-2 tube conditioner (Gerstel GmbH) at 300°C under high helium stream.

Table 2 Housing, sex and age information for felines used for stir bar facial scent collections for chemical analyses

Subject	Age	Species/Sex	Housing condition
Autumn	16	Cougar/Female	single
Ben	9	Cougar/Male	with 1 female
Tinker	unknown	Cougar/Male	single
Aries	unknown	Cougar/Male	single
Paco	unknown	Leopard/Female	single
King	unknown	Leopard/Male	with 1 female
Rodney	3	Leopard/Male	with 1 female and 1 male
Jackie	5	Tiger/Female	with 2 females and 4 males
Anna	5	Tiger/Female	with 2 females and 1 male
Samara	5	Tiger/Female	with 2 females and 1 male
Tony	unknown	Tiger/Male	single
Max[a]	2	Tiger/Male	with 1 male lion
Casey	8	Tiger/Male	with 3 females
Lauren	5	Lion/Female	with 1 female and 1 male
Gaby	10	Lion/Female	with 3 females and 1 male
Nala	10	Lion/Female	with 3 females and 1 male
Clancy	4	Lion/Male	with 1 female
Petey	8	Lion/Male	single
King	8	Lion/Male	with 2 females

[a] Only one housed with a different species

Standards, 3-acetamidofuran (3-AF) and 3-acetamido-5-acetylfuran (3-A-5AcF), were synthesized by adapting the procedures of C-N cross-coupling methodology, as reported by Padwa et al. (2003). Thus, reactions of 3-bromofuran and 5-acetyl-3-bromofuran (Antonioletti et al., 1985) with acetamide were catalyzed by CuI (10 mol%) in 1,4-dioxane at 110°C. The furan products were isolated following flash silica gel chromatography and were fully characterized by spectroscopic and mass spectral data. Proton and carbon NMR spectroscopy confirmed the assigned structures of 3-AF and 3A-5AcF, by comparison with data from other laboratories (Cambell et al., 1982; Franich and Goodin, 1984). Proton (^{1}H NMR, 400 MHz) and carbon (^{13}C NMR, 101 MHz) spectra were recorded in $CDCl_3$ and used to establish sample purity at >95%. This was further confirmed by subsequent HPLC analysis.

Preparation of 3-Acetamidofuran (3-AF) To a sample of CuI (29 mg, 0.15 mmol, 10 mol%) and K_2CO_3 (890 mg, 6.5 mmol) under argon, was added 1,4–dioxane (10 ml) followed by N,N'-dimethylethylenediamine, 3-bromofuran (234 mg, 1.6 mmol) and acetamide (100 mg, 1.6 mmol). The reaction was heated at 100°C for 24 hr, cooled, and then diluted with dichloromethane (10 ml). After filtration through a plug of silica gel, the mixture was concentrated under reduced pressure, and the crude product was purified by flash silica gel chromatography to give 156 mg (1.25 mmol: 80%) of 3-acetamidofuran, which was confirmed spectroscopically by comparisons to samples prepared by other laboratories (Cambell et al., 1982; Padwa et al., 2003): mp 91–92°C; IR ($CHCl_3$) υ=3,450, 1,675 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.5 (s, CH_3), 6.32 (dd, J=1.5 Hz; J= 0.7 Hz), 7.29 (t, J=1.5 Hz; J=1.5 Hz), 7.65 (br, s; N-H), 7.99 (dd, J=1.5 Hz; J=0.7 Hz), ^{13}C NMR ($CDCl_3$) δ 23.5, 104.8, 124.4, 132.7, 141.5, 168.0; High resolution mass spectrum (HRMS), *m/z* calculated for $C_6H_7O_2N$ (M^+) 125.0471, found 125.0475.

Preparation of 3-Acetamido-5-acetylfuran (3A-5AcF) To a sample of purified CuI (20 mg, 0.10 mmol, 10 mol%) and K_2CO_3 (590 mg, 4.3 mmol) under argon, was added 1,4-dioxane (3 ml) followed by N,N'-dimethylethylenediamine, 5-acetyl-3-bromofuran (190 mg, 1.0 mmol), and acetamide (72 mg, 1.2 mmol). The mixture was heated at 110°C for 24 hr, cooled to 22°C, and diluted with dichloromethane

(10 ml). After filtration through a plug of silica gel, the mixture was evaporated under reduced pressure, and the crude product purified by flash silica gel chromatography to give 98 mg (52%) of 3-acetamido-5-acetylfuran, which was identified and confirmed spectroscopically by comparison with data obtained from a sample previously prepared by another method (Franich and Goodin, 1984): IR ($CHCl_3$) 1690–1670 (broad C=0) cm^{-1}: ^1H NMR ($CDCl_3$) δ 2.13 (s, CH_3), 2,45 (s, CH_3), 7.71 (s,H), 8.15 (s, H), 8.32 (br s, N-H); ^{13}C NMR ($CDCl_3$) δ 23.2, 25.8, 110.2, 126.7, 136.2, 150.6, 168.3, 187.5; HRMS *m/z* calculated for $C_8H_9O_3N$ (M^+) 167.0577, found 167.0574.

Preparation of the Embedded Internal Standard An internal standard, 8 ng of 7-tridecanone (Aldrich, Milwaukee, WI, USA) was added in 5 μl of methanol to pre-cleaned 20 ml vials containing 2.0 ml of high-purity water (OmniSolv® EM Science, Gibbstown, NJ, USA), followed by addition of a preconditioned stir bar. Stirring speed was 850+ rpm on a Variomag Multipoint HP 15 stirplate (H + P Labortechnic, Oberschleissheim, Germany). Prior to extraction, all glassware was washed with acetone and dried at 80°C. After extraction, stir bars were stored for up to 3 days in individual vials inside a refrigerator prior to sample collection. The embedded internal standard is stable in the refrigerator for up to 20 days (Soini et al., 2006).

Analytical Instruments The gas chromatograph-mass spectrometer (GC-MS) used for analysis was an Agilent 6890N gas chromatograph connected to a 5973i MSD mass spectrometer (Agilent Technologies, Inc., Wilmington, DE, USA), with a Thermal Desorption Autosampler and Cooled Injection System (TDSA–CIS 4 from Gerstel). Positive electron ionization (EI) mode at 70 eV was used with a scanning rate of 2.41 scans.sec^{-1} over the range of 40–350 amu. The MSD transfer line temperature was set at 280°C, and the ion source and quadrupole temperatures 230°C and 150°C, respectively. A DB-5MS (30 m×0.25 mm, i.d., 0.25 μm film thickness) capillary column from Agilent (J&W Scientific, Folsom, CA, USA) was used for separations. Samples were thermally desorbed in a TDSA automated system, followed by injection onto the column with a cooled injection assembly, CIS-4. The TDSA was operated in splitless mode, and used a temperature program for desorption of 20°C (hold for 0.5 min), then a 60° C.min^{-1} ramp to 270°C (final hold of 5 min). Temperature of the transfer line was set at 280°C. The CIS-4 was cooled with liquid nitrogen to −80°C. After desorption and cryotrapping, the CIS-4 was heated at 12°C.sec^{-1} to 280°C, with a hold time of 10 min. The CIS-4 inlet was operated in the solvent vent mode, with a vent pressure of 9.0 psi, a vent flow of 50 ml. min^{-1}, and a purge flow of 50 ml.min^{-1}. The temperature program for the GC oven was 40°C for 5 min, then increased to 200°C at 2°C.min^{-1} (hold time 10 min). The carrier gas had a constant flow of 1.0 ml.min^{-1}.

Quantitative Comparisons of Compound Levels Peak areas (PAs) were integrated from post-run selected ion chromatograms and divided by the peak area of the embedded internal standard (7-tridecanone) from the ion chromatogram for *m/z* 113 in the corresponding run (normalized peak area, NPA). For 2-ketones, *m/z* 58, phenol and 2-pyrrolecarboxaldehyde *m/z* 94, linear carboxylic acids *m/z* 73, linear alcohols *m/z* 55, and for acetamidofurans *m/z* 125, were used.

N-Acetylglucosamine-Derived Compounds and Tests Two unusual compounds, 3-acetamidofuran (3-AF) and 3-acetamido-5-acetylfuran (3-A-5AcF), which had been reported previously as thermal degradation products of N-acetylglucosamine in the ratio 2.5:1 (Franich and Goodin, 1984; Chen et al., 1998), were identified in our samples. In order to verify whether these compounds were thermal degradation or metabolic products, we conducted an experiment to degrade N-acetylglucosamine under the same conditions that the feline samples were collected and analyzed. Water solutions of N-acetyl glucosamine (8 μg/μl) were acidified with acetic acid, to mimic the presence of organic acids on the feline hair, and allowed to stand for 15 min, 24, 48, or 72 hr. An aliquot of 300 μl of the acidified solution was placed on aluminum foil and rolled over repeatedly with the roller pin device, in similar fashion as to how the facial samples were collected.

Synthetic 3-AF was used as a behavioral test compound. We tested unneutered males and unspayed females, to ensure neutering did not affect the response to the compound, on 7/8/2009, between 9 am and 12 pm. The individuals tested included three lions in two separate enclosures, one vasectomized male with a female, and one female alone, four tiger males, all housed alone, two leopard males, housed alone, and two cougar males, housed alone. A control solution of 10% glycerine in water and a test solution of 10^{-5} M 3-AF in 10% glycerine in water, were swabbed on the upper half of 25.4 cm hard plastic Boomer Balls ™, which were then placed within 2 m of each other in the center of an enclosure. When the animals were released back into the enclosure, positive responses to the solutions, including sniffing, flehmen, licking, rubbing, and urinating, were recorded for 5 min after initial contact. After that, the balls were usually moved as they were contaminated with other substances within the enclosure.

Results

Preliminary Scent Tests We observed a total of 54 positive responses to cardboard scent squares presented to lions. There were more responses ($X_1^2 = 7.4$, P=0.006) to head samples than to the control, but no difference ($X_1^2 = 0.4$, P= 0.54) in numbers of responses to cheek samples and

controls. Tigers showed 110 positive responses to the squares. There were more responses to head ($X_1^2 = 14.5$, P=0.001) and cheek samples ($X_1^2 = 16.0$, P=0.001) than to the control (Fig. 1). Forty positive responses by leopards showed no preference for either head ($X_1^2 = 0.92$, P=0.34) or cheek samples ($X_1^2 = 0.15$, P=0.70) over controls. Thirteen positive responses from cougars showed no differences between either head samples ($X_1^2 = 1.0$, P=0.32) and controls, or cheek samples ($X_1^2 = 0.4$, P=0.53) and controls. No preferences by leopards and cougars could indicate that the compounds deposited on the cardboard squares were more volatile and possibly lost during storage, or that facial compounds are not used as chemical signals in these species. Regardless, identifying facial compounds for comparison was deemed important.

Chemical Analysis of the Facial Surface Samples Reproducibility of the surface sampling analysis has been established previously at 6–10%, RSD (relative standard deviation) for different volatile compound classes (Soini et al., 2006). In this study, the repeatability for the embedded internal standard peak area was 8.6% (RSD, N=17).

In the samples, 100 volatile organic compounds were identified through matches with authentic compounds, or tentatively identified through spectral searches. Table 3 summarizes identified or tentatively identified compounds,

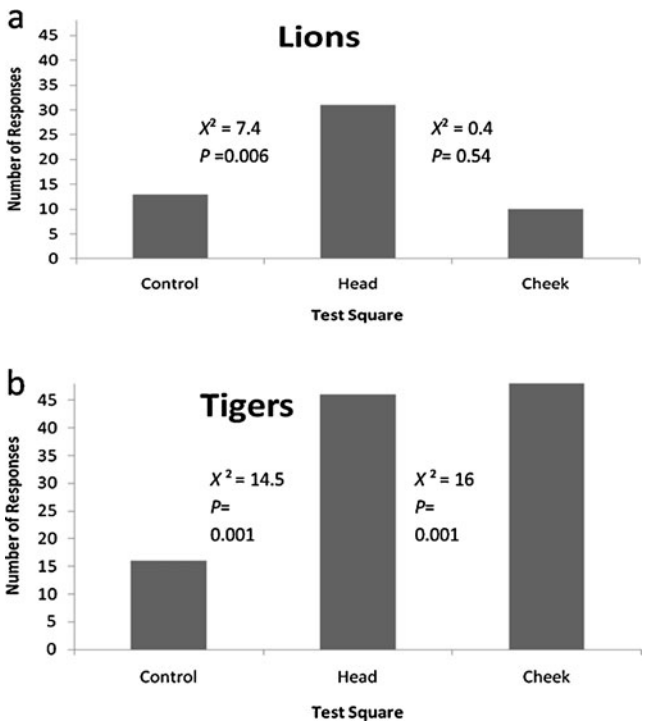

Fig. 1 Numbers of positive responses (including rubbing, spraying, flehmen, licking, and sniffing) to scent (from control, head or cheek of conspecific felines) deposited on cardboard squares for (**a**) lions and (**b**) tigers

organized by compound class. Compound identities were verified by authentic standards (59), except those (41) marked with an asterisk (Table 3), which are called "tentatively identified compounds", because a reference standard was not available for final verification. For tentative identification, NIST reference library match and in-house retention index and mass fragmentation interpretation databases were used. The same compounds were found in both forehead and cheek samples, but the cheek samples generally contained lower amounts (10–80% less, data not shown). The same compounds were found in all four species, although some compound levels varied substantially among species. In general, compound levels in lions and tigers were higher than in leopards and cougars, and may reflect differences seen among behavioral responses. Figure 2 illustrates the total ion chromatogram differences among lions, tigers, leopards, and cougars.

We found 41 compounds, including carboxylic acids, aldehydes, alcohols, ketones, amides, hydrocarbons, and aromatic compounds, that had been identified previously in both feline marking fluid compounds (a mixture of a lipid-based substance from anal sac mixed with urine), urine, and lion manes (Table 3). We also found several compounds not previously reported in feline studies. These included heterocyclic nitrogen and oxygen compounds, such as pyrroles, pyrrolidines, pyrrolidones, and furans. Acetamidofuran and its derivatives were also found. Previously, we found 3-AF on the hair of domestic cats at a much lower concentration (unpublished results). Selected chemical structures for these compounds are shown in Fig. 3.

Compound Level Differences Among Species, Individuals and Sexes Quantitative differences were seen among species for some compounds. 2-Pentadecanone and 2-heptadecanone levels in tiger foreheads were consistently high (peak area ratios 0.2–0.9), while the other species showed only trace levels (peak area ratios<0.002) of these compounds (Fig. 2, peaks 15 and 18). Species level differences for three linear carboxylic acids (tetradecanoic, hexadecanoic, and octadecanoic acids) also were found, with lions and tigers showing substantially higher levels than leopards and cougars (Fig. 2, peaks 16, 20, and 24).

A large amount of individual variation was seen within each species regarding the above compounds. For example, tiger 2-pentadecanone and 2-heptadecanone levels differed four-fold among some individuals (Fig. 4). There were also some interesting differences between the sexes (see Fig. 4, e.g., Anna and Tony). Higher levels of carboxylic acids and acetamidofurans were found in cougar females than in males, but the opposite was found for leopards. There were mixed results with lions and tigers, and there seemed to be no general trend among species. With small sample sizes of

Table 3 Volatile compounds identified from four feline species

	Compound	Retention time (min)	Reported in felines
MW	**Carboxylic acids**		
60	Acetic acid	3.63	[b]tiger, [c]leopard, [i]lion
102	3-Methylbutanoic acid (isovaleric acid)	9.46	[i]lion
116	Hexanoic acid	18.61	[b]tiger, [c]leopard
130	Heptanoic acid	25.10	[b]tiger, [c]leopard
144	Octanoic acid	32.14	[b]tiger, [c]leopard
158	Nonanoic acid	38.48	[b,d]tiger, [c]leopard
172	Decanoic acid	44.76	[b,d]tiger
200	Dodecanoic acid	56.96	[b,d]tiger
214	Tridecanoic acid	62.12	[e]lion
228	Tetradecanoic acid (myristic acid)	67.25	[b,f]tiger, [e]lion, [g]cheetah
242	Pentadecanoic acid (branched)[a]	71.13	
242	Pentadecanoic acid	72.31	[b]tiger, [e]lion
254	Palmitoleic acid ((Z)-9-hexadecenoic acid)	76.12	[b,f]tiger, [g]cheetah
256	Hexadecanoic acid (palmitic acid)	77.28	[b,f]tiger, [e]lion, [j]bobcat
270	Heptadecanoic acid	82.16	[b]tiger, [e]lion
280	Linoleic acid (9,12-octadecadienoic acid)	85.25	
282	Oleic acid ((Z)-octadecenoic acid)	85.33	[b]tiger, [e]lion, [j]bobcat
284	Octadecanoic acid (stearic acid)	86.54	[b,f]tiger, [e]lion
MW	**Aldehydes**		
96	Furfural[a]	8.33	
114	Heptanal	12.17	[b]tiger, [h]lion
106	Benzaldehyde	15.76	[b]tiger, [g]cheetah
110	5-Methylfurfural[a]	15.76	
128	Octanal	18.91	[b]tiger, [g]cheetah, [h]lion
95	2-Pyrrolecarboxaldehyde[a]	19.62	
126	2-Octenal	22.73	[b]tiger, [h]lion
124	4-Ethyl-2-furaldehyde[a]	24.42	
142	Nonanal	26.20	[b]tiger, [g]cheetah, [h]lion
140	2-Nonenal	30.05	
112	Decanal	33.41	[b]tiger, [g]cheetah
154	2-Decenal	37.19	
168	2-Undecenal	46.41	
MW	**Alcohols**		
102	3-Hexanol	7.21	
98	Furfuryl alcohol[a]	9.46	[b]tiger
90	2-(2-Ethoxyethyl)-ethanol[a]	18.81	
130	2-Ethyl-1-hexanol	20.74	
144	1-Octanol	23.80	[b]tiger
158	1-Decanol	38.01	
214	1-Tetradecanol	62.69	[b,f]tiger
242	1-Hexadecanol	73.32	[b,f]tiger
270	1-Octadecanol	83.04	[b,f]tiger
284	1-Nonadecanol	87.79	
298	1-Eicosanol (branched)[a]	90.02	
386	Cholesterol	92.13	
298	1-Eicosanol[a]	94.00	[f]tiger
MW	**Ketones**		
74	Hydroxyacetone[a]	4.22	

Table 3 (continued)

	Compound	Retention time (min)	Reported in felines
116	4-Hydroxy-4-methyl-2-pentanone[a]	9.30	
84	2(5H)-Furanone	12.66	
98	Cyclohexanone	13.69	[g]cheetah, [h]lion
126	6-Methyl-5-hepten-2-one	17.59	
120	Acetophenone	23.00	[b]tiger, [g]cheetah
156	2-Decanone	32.37	[b]tiger
194	Geranylacetone	49.18	[b]tiger
226	2-Pentadecanone	63.75	[b]tiger
254	2-Heptadecanone	74.24	[b]tiger
282	2-Nonadecanone	83.75	
MW	**Amides**		
59	Acetamide	6.62	[b]tiger
101	N,N-Diethylformamide	14.35	
129	Heptanamide[a]	26.91	
283	Stearamide (octadecanamide)[a]	86.91	[j]bobcat
MW	**Heterocyclic nitrogen ring compounds**		
71	Pyrrolidine[a]	13.37	
109	N-Acetylpyrrole[a]	15.37	
113	2-Acetylpyrrolidine[a]	16.19	
125	Z-N-Propenylpyrrolidinone[a]	18.41	
125	E-N-Propenylpyrrolidinone[a]	19.05	
141	An alkylpyrrolidine[a]	19.59	
126	2-(3-Pentenyl)imidazole[a]	23.56	
153	N-Pentenylpyrrolidinone[a]	27.99	
123	An acetylmethylpyrrole[a]	29.88	
113	2-Propylpyrrolidine[a]	29.89	
137	2-Methyl-5-propionylpyrrole[a]	33.27	
MW	**Heterocyclic oxygen ring compounds**		
128	γ-Heptanolactone[a]	28.57	
125	3-Acetamidofuran	34.86	
139	4-Acetamido-2-methylfuran[a]	40.68	
125	4-Amino-2-acetylfuran[a]	41.18	
141	3-Acetamido-4-hydroxyfuran[a]	42.09	
167	3-Acetamido-5-acetylfuran	59.96	
MW	**Hydrocarbons**		
142	Decane	18.75	[b]tiger, [g]cheetah, [h]lion
170	Dodecane	33.02	
182	1-Tridecene	39.32	
184	Tridecane	39.88	
196	1-Tetradecene	45.88	
198	Tetradecane	46.42	[b]tiger
210	1-Pentadecene	52.10	
212	Pentadecane	52.58	
410	Squalene[a]	96.40	[b]tiger
MW	**Esters**		
126	A methyl imidazolecarboxylate[a]	24.09	
250	2-Ethyl-1-hexyl salicylate[a]	70.70	
262	3,3,5-Trimethylcyclohexyl salicylate [a]	72.82	
290	2-Ethyl-1-hexyl x-methoxycinnamate[a]	85.99	

Table 3 (continued)

	Compound	Retention time (min)	Reported in felines
290	2-Ethyl-1-hexyl y-methoxycinnamate[a]	95.19	
MW	**Aromatic compounds**		
94	Phenol	17.33	[b]tiger, [g]cheetah, [h]lion
134	*p*-Cymene	20.29	
117	Phenylacetonitrile[a]	28.24	
138	2-Phenoxyethanol[a]	33.90	
135	Benzothiazole	34.00	
131	Benzenepropanenitrile[a]	35.22	
117	Indole	38.77	[j]bobcat
151	An acetamidophenol[a]	46.88	
167	An acetamidoresorcinol[a]	52.29	
167	An acetamidoresorcinol[a]	53.61	
228	A benzoyl-methoxyphenol[a]	79.42	

[a] Tentative identification based on NIST library match and internal retention index and mass fragmentation databases

[b] Burger et al. 2008. Tiger marking fluid (MF)

[c] Poddar-Sarkar and Brahmachary 2004. Leopard marking fluid (MF)

[d] Poddar-Sarkar et al. 2004. Tiger marking fluid (MF)

[e] Poddar-Sarkar et al. 2007. Lion mane

[f] Poddar-Sarkar 1996. Tiger marking fluid lipids.

[g] Burger et al. 2006. Cheetah urine

[h] Anderson and Vulpius 1999. Lion urine

[i] Albone and Eglington 1974. Lion anal sac

[j] Mattina et al. 1991. Bobcat urine

only three individuals of each sex, non-parametric statistical analysis showed no differences.

N-Acetylglucosamine-Derived Compounds and Tests Acetamidofuran compounds, which have been reported as biodegradation and thermal degradation products from chitin and its monomer N-acetylglucosamine (Stankiewicz et al., 1996), were considered as possible sources of scent cues, because they were present in facial samples from all investigated species. In the N-acetylglucosamine thermal degradation tests, measurable levels of 3-AF were obtained only after 24 and 48 hr of treatment, while 3-A-5AcF appeared as a trace signal after 72 hr treatment with acetic acid. The ratios of 3-AF to 3-A-5AcF in feline forehead samples varied from 2.2 to 8.9:1 for lions, 3.8–7.3:1 for tigers, 5.1–32.5:1 for cougars, and 3.3–5.8:1 for leopards, which deviate from the ratio of 2.5:1 reported for pure thermal degradation (Franich and Goodin, 1984). This suggests that 3-AF and 3-A-5AcF on feline foreheads are derived from metabolic production. Figure 5 illustrates the average normalized peak areas for 3-AF and 3-A-5AcF in the feline species.

In preliminary behavioral tests with 3-AF, we found no differences in positive responses to the control vs. test Boomer Ball™ for lions (23 responses; $X_1^2=1.08$, $P=0.30$), tigers (57 responses; $X_1^2=0.16$, $P=0.69$), and leopards (19 responses; $X_1^2=0.06$, $P=0.81$). Cougars only responded four times; this sample size was too small for statistical analysis. Behavioral tests with 3-A-5AcF were not conducted.

Discussion

Behavioral Responses to Scents Head- and cheek-rubbing behavior of felines is generally difficult to study in the wild, because most feline species are solitary and nocturnal. In the wild, rubbing on objects has been documented at marking sites for tigers (Smith et al., 1989) and leopards (Bothma and le Riche, 1995), and lions are known to rub other individuals and objects (Schaller, 1972). In several species of small felines in captivity, head- and cheek-rubbing on objects has been recorded, but the rubbing was not directed at individuals (Mellen, 1993). In our preliminary study, head- and cheek-rubbing could be observed systematically in captive large felines. Preliminary tests with cardboard scent squares indicated that volatile compounds deposited by rubbing elicited responses from lions and tigers. Leopard

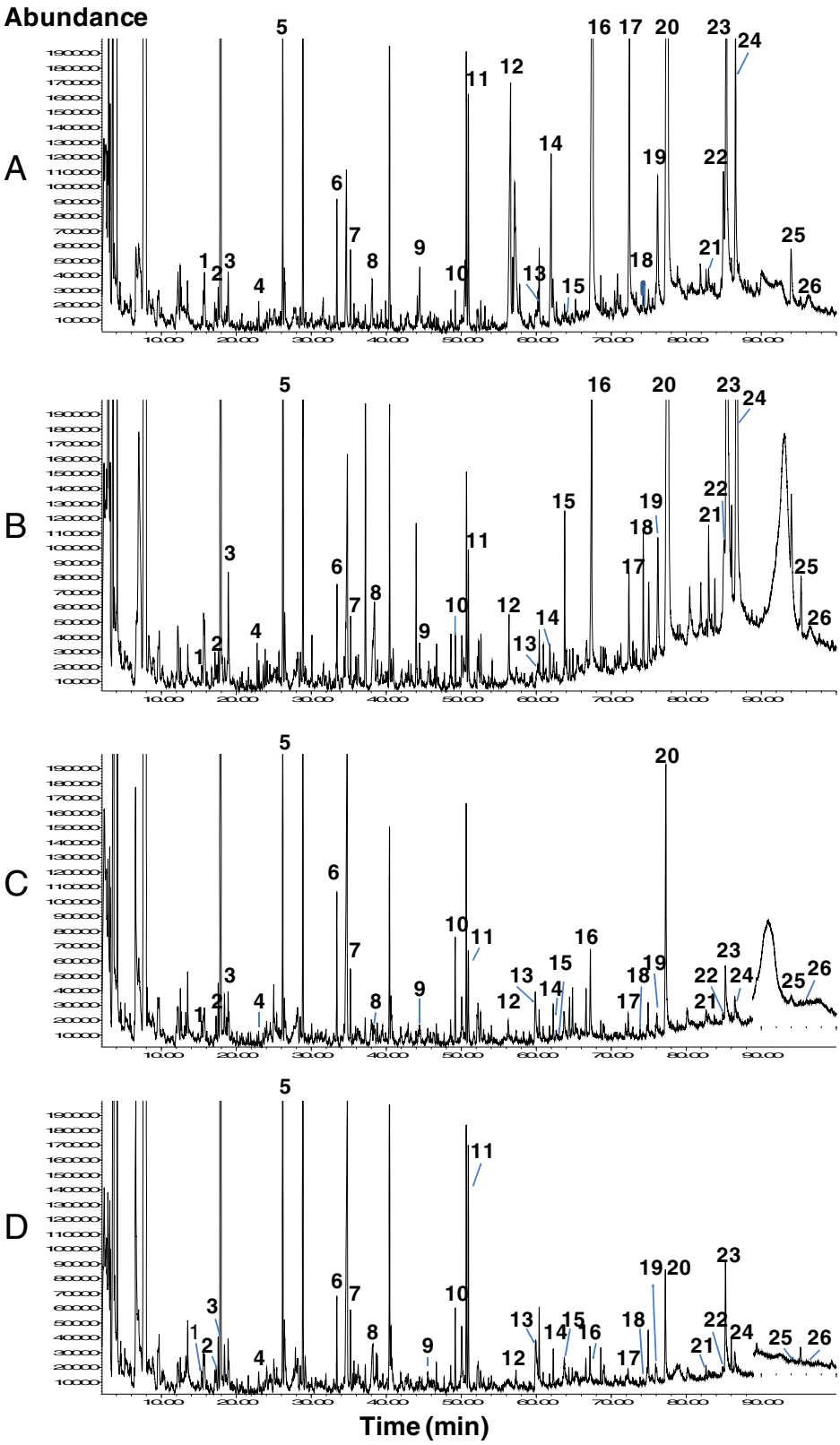

Fig. 2 Total ion chromatograms of compounds from male **a**: lion, **b**: tiger, **c**: leopard, **d**: cougar. Selected compounds indicated in the graphs are 1: benzaldehyde, 2: phenol, 3: octanal, 4: acetophenone, 5: nonanal, 6: decanal, 7: 3-acetamidofuran (3-AF), 8: nonanoic acid, 9: decanoic acid, 10: geranylacetone, 11: undecanoic acid, 12: dodecanoic acid, 13: 3-acetamido-5-acetylfuran (3-A-5AcF), 14: tridecanoic acid, 15: 2-pentadecanone, 16: tetradecanoic acid, 17: pentadecanoic acid, 18: 2-heptadecanone, 19: palmitoleic acid, 20: hexadecanoic acid, 21: 1-octadecanol, 22: linoleic acid, 23: oleic acid, 24: octadecanoic acid, 25: 1-eicosanol, 26: squalene

and cougars rubbed objects, but did not respond to rubbings, which may indicate that urine and other secretions may be a more important volatile compound source for these species, or that important volatiles may have evaporated from samples before they were presented to the cats. The compound 3-AF did not elicit any behavioral response from any of the

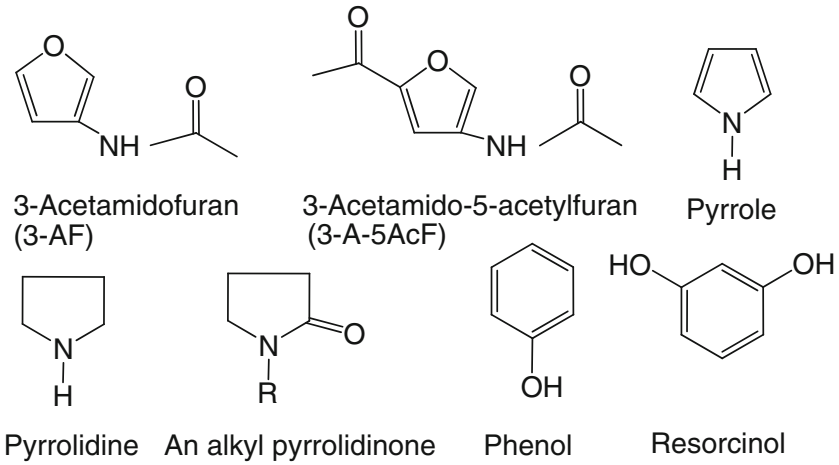

Fig. 3 Selected chemicals found on the foreheads of large felines

species. Future tests with 3-AF and other compounds, in different concentrations and blends, need to be conducted to determine what substances are important to each of the species. Also, other scent presentation methods need to be tested to rule out possible background scent disturbance from the Boomer Balls™.

Facial Volatile Compounds Previous authors suggested that volatile compounds may act as important scent cues of health and physical condition. Carboxylic acids identified in our facial samples are of particular interest. These acids are commonly found in mammalian samples, including human axillary secretions (Natsch et al., 2006; Penn et al., 2007). They have been identified in feline urine, marking fluids, and lion manes (Poddarr-Sarkar et al., 1994, 2007; Burger et al., 2008). These short-chain acids are products of fatty acid metabolism and bacterial activity (Natsch et al., 2006), and may be indicators of health and metabolic condition. Lipid metabolism differences have been documented for tiger, lion, cougar, and cheetah urine in a zoo environment (Asa, 1993). In our study, the higher levels of carboxylic acids found in tigers and lions, compared to the other species, may reflect total lipid metabolism differences among the species, as well as differences among individuals within a species. Our findings of higher levels of linear carboxylic acids in tigers and lions in facial scent samples are in accord with the previous findings in urine.

Methylketones (2-ketones) also are related to fatty acid metabolism and are enzymatic conversion products from linear carboxylic acids (Pannell and Olson, 1991). Other smaller and more volatile 2-ketones, such as 2-octanone,

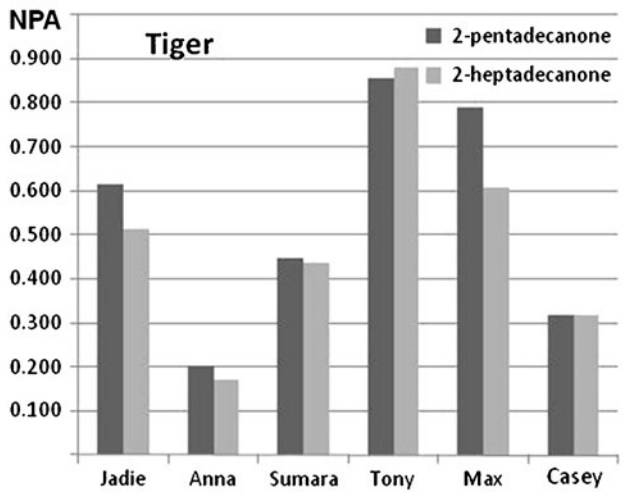

Fig. 4 Normalized peak areas (NPA) from post-run selected peak areas of *m/z* 58 for 2-pentadecanone (Rt 63.75 min) and 2-heptadecanone (Rt 74.24 min) in tiger forehead samples

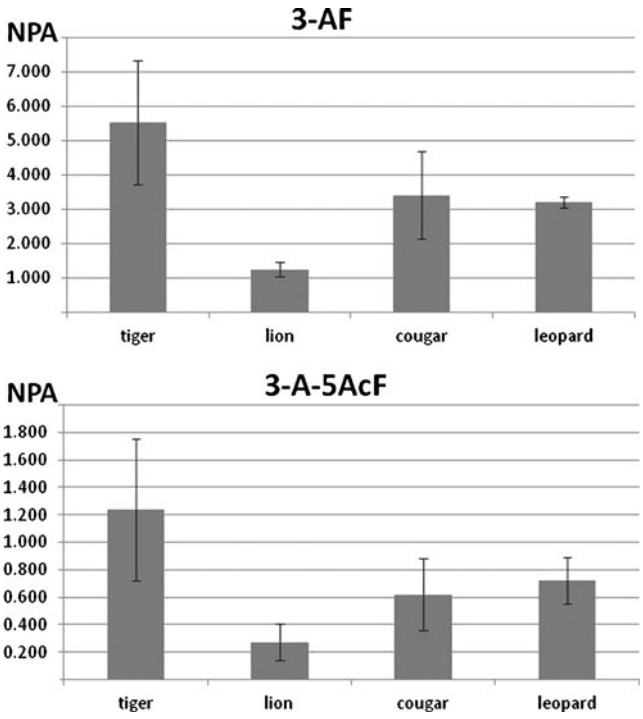

Fig. 5 Averages (±SEM) for normalized peak areas (NPA) for 3-acetamidofuran (3-AF) and 3-acetamido-5-acetylfuran (3-A-5AcF) in males and females in four feline species, measured from post-run *m/z* 125 peak areas (Rt 34.86 min)

2-nonanone, and 2-undecanone, also have been reported in cheetah urine (Burger et al., 2006), while 2-pentanone and 2-heptanone were found in lion urine (Anderson and Vulpius, 1999). In our study, high levels of 2-pentadecanone and 2-heptadecanone were found in captive tigers. These are the same compounds found in territorial marking fluid of Bengal tigers (Burger et al., 2008), and may function as indicators of tiger-specific fatty acid metabolism and in chemical signaling.

Heterocyclic oxygen and nitrogen compounds have not been identified previously in felines. Furans have been reported in a wide range of mammalian samples. Rat urine (Holland et al., 1983) and rat preputial gland secretions (Pohorecky et al., 2008), as well as wolf urine (Raymer et al., 1986) and anal-sac secretions (Raymer et al., 1985), contained furan alkyl and acetyl derivatives, but not the amino derivatives found in this study. N-Acetylpyrrole, identified here, has been reported in human skin (Penn et al., 2007) and rat urine (Holland et al., 1983). Pyrrole levels in urine and skin may reveal individual health, as increased pyrrole levels have been reported in human urine after stress (Jackson et al., 1997).

Two aromatic compounds, phenol and indole, have been identified in the urine of several felines (Mattina et al., 1991; Anderson and Vulpius, 1999; Burger et al., 2006, 2008). Indole is often identified in carnivore samples, but also has been found in Siberian Hamster urine (Soini et al., 2005).

N-Acetylglucosamine was originally identified as a monomer of chitin, which is part of the support structures of fungi, parasites, and bacteria (Stankiewicz et al., 1996). Recently, mammalian acidic chitin-degrading chitinase has been found in humans and mice (Renkema et al., 1995; Boot et al., 2001). The authors suggested that chitin-degrading metabolic pathways might be connected to mammalian pathogen resistance mechanisms.

In summary, this comparative study of four large felines has identified a list of possible volatile compounds associated with their behavior. In prior chemical signaling research, apart from the few compounds identified in lion manes and some indication of sexual differences in lion urine compounds, no reports exist linking such volatile compounds to behavior. More work is needed in identifying individual compounds and determining which of those compounds may induce important behavioral cues.

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