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# Chemical composition of Eurasian lynx urine conveys information on reproductive state, individual identity, and urine age

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**Abstract** In mammals, the chemical profiles of individuals are complex and variable mixtures, and animals perceive information based on variation in the overall quality of these mixtures. A variety of compounds potentially involved in chemical communication have been characterized in the urine of different felid species, but little is known about the information content of felid scent marks. In this study, we investigated whether chemical composition of Eurasian lynx *Lynx lynx* urine was related to sex, reproductive state, and individual identity. We further analysed if elemental sulphur in lynx urine could serve as a dietary cue or as an indicator for the freshness of a scent mark. We collected urine from captive and wild Eurasian lynx, and analysed volatile constituents of urine by means of solid phase microextraction and gas chromatography-mass spectrometry. Our results show that lynx scent profiles contain sex-specific information on reproductive state, as well as individual identity cues. Urine marks are, therefore, well-suited to fulfil a role in reproductive behaviour and social organisation of wild lynx populations. Relative sulphur content was unrelated to time since last feeding, but decreased with age of the urine sample. The influence of diet and body condition on scent profiles should be further investigated by means of experimental studies, and may shed more light on the messages encoded in carnivore scent-marks.

**Keywords** *Lynx lynx* · Chemical communication · Urine · Scent profile · Individual identity · Dietary cues

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## Introduction

In mammals, the chemical profiles of individuals consist of molecules produced by different scent-sources on the animal's body, together with molecules acquired from other group members or from the environment (Wyatt 2014). The resulting odours are complex and variable mixtures, and animals perceive information based on the overall quality of these scent mixtures (Johnston 2003; Wyatt 2014). Variation in the relative proportions of chemical compounds of a scent profile may provide information about the donor's species, sex, individual identity, reproductive status, genetic quality, or kinship (Buesching et al. 2002; Penn 2002; Johnston 2003; Charpentier et al. 2010; Roberts et al. 2014). For example, many male mammals are attracted to scent marks of females, and preferences for odours of oestrus females have been detected in several species, e.g. in rats *Rattus norvegicus*, mice *Mus musculus*, dogs *Canis familiaris*, Asian elephants *Elephas maximus* (Petrulis 2013), giant panda *Ailuropoda melanoleuca* (Swaisgood et al. 2000), and ringtailed lemur *Lemur catta* (Scordato et al. 2007). On the other hand, females may choose males based on information on health, dominance status, or genetic compatibility encoded in their scent

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profile (Penn 2002; Petrulis 2013; Roberts et al. 2014). Many studies on rodents have provided evidence that animals do not only respond to specific chemical cues, but learn to recognize individual scent profiles from related or familiar individuals at certain stages of their lives (reviewed in Johnston 2003). Odour recognition has been shown to be important for kin recognition, for mate choice, and other behaviour in group-living and even in less social species (Wyatt 2014).

Most wild felid species are solitary and territorial, and communication by means of scent marking with urine, faeces, or gland secretions is an important component of their social behaviour (Smith et al. 1989; Mellen 1993; Allen et al. 2015b). Scent-marks are often enhanced by a visual component, which is thought to facilitate detection of the mark (Allen et al. 2014; Vogt et al. 2014). Males mark generally more often than females, and marking-frequency increases during the mating season (Mellen 1993; Vogt et al. 2014; Allen et al. 2015a). Scent-marking is assumed to play a role in territoriality, in mate attraction, and in competition among same sex individuals (Vogt et al. 2014; Allen et al. 2015a, b). In recent years, a variety of compounds in urine and facial scent samples of several felid species, that are potentially involved in chemical communication, have been characterized by means of gas chromatography-mass spectrometry (GCMS); for example hydrocarbons, ketones, aldehydes, fatty acids, alcohols, lactones, and S- or N-containing substances such as thioethers, sulphones, amines, and amides (Mattina et al. 1991; Andersen and Vulpius 1999; Poddar-Sarkar and Brahmachary 2004; Burger et al. 2006, 2008; Soini et al. 2012). However, only a small number of experimental studies have so far investigated the possible information content of scent-marks: Sokolov et al. (1996) reported that captive Eurasian lynx *Lynx lynx* sniffed the urine of conspecifics longer than control urine samples from other species. They further found that female lynx head rubbed longer on urine of males than of females, and that lynx of both sexes smelled urine samples of unknown individuals longer than those of familiar individuals. Natoli (1985) found that both male and female domestic cats *Felis catus* spent more time sniffing urine sprayed by a strange tomcat, than urine sprayed by a male from the same group. These studies show that lynx and domestic cats perceive information about sex in urine of conspecifics, and are able to recognize odours of familiar individuals.

Apart from information about sex, reproductive status, or genotype, the chemical composition of a scent-mark can also convey information on health, body condition, and diet of an individual (Ferkin et al. 1997; Buesching et al. 2002; Johnston 2003; Munoz-Romo et al. 2011). Dietary cues are known to act as signals of quality to conspecifics, e.g. female meadow voles prefer chemosignals of males fed on a protein rich diet (Ferkin et al. 1997). But information on diet may also be exploited by other species. For example, many fish and amphibian species are known to adapt their anti-predatory behaviour in response to dietary cues emitted by their predators (Laurila et al. 1997; Murray and Jenkins 1999; Chivers and Mirza 2001). In carnivores, dietary cues most likely stem from their protein or fat metabolism. One well described metabolic pathway is the conversion of the amino acids methionine and, more importantly, cysteine to felinine (Hendriks et al. 2001), a process catalysed by the felid-specific protein cauxin. Felinine is then degraded into several organosulphur compounds in felid urine, namely into 3-mercapto-3-methylbutanol (Miyazaki et al. 2006), and further into di- and trisulphide derivatives (Mattina et al. 1991). Cauxin has been found in the urine of domestic cats, bobcats *Lynx rufus*, and Eurasian lynx (Miyazaki et al. 2008), as well as in several large felid species (McLean et al. 2007; Burger et al. 2008). 3-mercapto-3-methylbutanol and other malodorous organosulphur compounds are responsible for the typical catty odour of domestic cat urine, and are known to elicit aversive responses in prey animals (Lewison et al. 1993; Mattina et al. 1991). Burger et al. (2006) found that these organosulphur compounds were totally or almost absent from the urine of cheetah *Acinonyx jubatus.* Instead, for the first time in a mammalian species, the authors described excretion of large amounts of elemental sulphur in cheetah urine (ca. 1 µg/ml urine). Sulphur was later also detected in the urine of tigers and Iberian wolves *Canis lupus signatus*, although at much lower concentrations (tiger: ca. 80 ng/ml urine, Burger et al. 2008; Iberian wolf: 1 % of TIC, Martín et al. 2010). Burger et al. (2006) investigated a function of sulphur as pheromone, but no reactions to sulphur could be elicited either in cheetahs or in other tested carnivores. In fact, they found that cheetah urine elicited practically no responses of either cheetah or several other felid species. Elemental sulphur is described as an odourless substance which led Burger et al. (2006) to the alternative hypothesis that a conversion of sulphur-containing compounds to elemental sulphur could serve to "chemically camouflage" cheetah urine from detection by larger sympatric predator species. However, given that organosulphur compounds are products of amino acid metabolism, this mechanism might also serve to hide information on diet from conspecifics or potential prey.

In this study, we investigated the chemical composition and the information content of the volatile fraction of Eurasian lynx urine. Eurasian lynx are solitary and occupy large home ranges. The home ranges of resident males encompass those of one or two resident females, but there is little overlap between the home ranges of same sex individuals (Breitenmoser-Würsten et al. 2001). Subadult lynx may not yet occupy stable home ranges, and move as

"floaters" among residents (Breitenmoser and Breitenmoser-Würsten 2008). During mating season, male lynx will mate-guard oestrous females. Scent-marking with urine occurs in both male and female lynx, is especially frequent during mating season, and is thought to play an important role in reproduction and the maintenance of spatial and social organisation of wild lynx populations (Breitenmoser and Breitenmoser-Würsten 2008; Vogt et al. 2014). We predicted that, to fulfil these proposed functions, lynx urine should contain information on sex, social status (resident adult, subadult, juvenile), and reproductive state. The formerly demonstrated ability of lynx to discriminate between urine of familiar and unfamiliar individuals (Sokolov et al. 1996; see above) further suggests that scent profiles also convey information on individual identity.

Eurasian lynx are stalking predators of medium-sized ungulates, and avoidance of eavesdropping by prey has been shown to partially shape spatial patterns of scent-marking (Vogt et al. 2016). In an earlier study, G. Zachariae (pers. comm.) found that organosulphur compounds were present only in very low amounts in fresh lynx urine samples, similar to cheetah urine (Burger et al. 2006). He observed that these substances reached peak amounts 2–4 days after urine deposition, and were not detectable after about 14 days. He hypothesised that they were continually released from a low volatile source in the urine, possibly via the metabolism of microbes living on the substrate of the scent-mark. It is conceivable that dietary cues may be masked in fresh lynx urine, for example by conversion of organosulphur compounds to elemental sulphur. To test this hypothesis, we specifically investigated whether lynx urine contained elemental sulphur, and whether the amount of sulphur was related to food condition and age of the urine-mark.

## Methods

### Collection of urine samples from wild lynx

From November to April 2012/13 and 2013/14, we collected 29 urine samples of ten adult Eurasian lynx individuals (5 males, 5 females) and two subadult male lynx in the Northwestern Swiss Alps. All lynx had previously been fitted with GPS/GSM-collars (GPS Plus Mini-1 C collars, Vectronic Aerospace GmbH, Berlin, Germany; Wild Cell SL/SD GPS-GSM collars, LoTek wireless, Ontario, Canada). Urine samples were collected by following lynx tracks in the snow, starting from a known GPS-fix, to assure proper assignment of the urine sample to the individual. Lynx scent-mark at visually conspicuous objects, such as small spruce trees or cut tree trunks, by means of urine spraying (Vogt et al. 2014). Collection of frozen urine sprays from snow covered objects was possible up to 3 days after deposition, depending on snow and temperature conditions. The snow-urine mixtures were collected directly into 20 ml headspace vials with PTFE-lined screw caps (Gerstel GmbH & Co KG, Switzerland). After each urine sample, we also collected a blank of untainted snow from the same object (ca. 30 cm away from the urine spray). All samples were frozen after snow tracking at −20 °C and transferred to a −80 °C freezer within 2–6 months, where they were kept until chemical analysis. Headspace vials were rinsed before use once with dichloromethane (Rotisolv, GC Ultra Grade, CARL ROTH GmbH + Co. KG, Switzerland), acetone (Rotisolv, UV/IR-Grade, CARL ROTH GmbH + Co. KG, Switzerland), and n-heptane (Rotisolv, UV/IR-Grade, CARL ROTH GmbH + Co. KG, Switzerland), respectively.

### Collection of urine samples from captive lynx

Between August 2013 and November 2014, we collected 35 urine samples from seven adult, captive Eurasian lynx (3 males, 4 females) held at five different zoos in Switzerland (Tierpark Dählhölzli, Natur- und Tierpark Goldau, Tierpark Lange Erlen, Wildnispark Zürich Langenberg, Tierpark Biel). Four of these lynx came from the Carpathian population which is also the source population of the reintroduced wild lynx population in Switzerland. For one male and two female lynx, the population of origin was unknown. Urine was collected by means of a collection device modified from a system used for Iberian lynx *Lynx pardinus* (Jewgenow et al. 2009). The collector consists of a stainless steel panel with a funnel at the bottom, mounted on a post, and placed in front of lynx scent-marking sites in the enclosure. When lynx spray urine against the panel, the urine is collected in a 1 dl glass container (Emmi, Luzern) at the bottom of the funnel. In enclosures with more than one lynx, we mounted a collector with an automatic closing system (prototype made at Theodor-Kocher Institute, University of Bern): when liquid drops into the sample glass with two diodes, an electric circuit was activated and the sample glass was closed with a steel lid. This prevented the urine sample from being mixed with urine from another over-marking individual. We observed the collector with a Reconyx RC55 infrared camera trap (Reconyx, Inc., Wisconsin), to determine age of the sample and assign samples to the proper individuals. Zoo keepers checked the collector once per day and changed panels and glass ware if urine had been collected. Glasses with urine samples were closed with a plastic lid lined with aluminium foil and immediately frozen at −20 °C. They were transferred to a −80 °C freezer within 2–6 months, where they were kept until chemical analysis. All glassware was cleaned before use as described in the

section above. Panels were washed with soft soap (oecoplan, Coop, Basel) and water, and then rinsed once with distilled water (hypotonic, pyrogen free, CARL ROTH GmbH + Co. KG, Switzerland) and once with ethanol (Merck KGaA, Darmstadt) to remove urine residues. After each sampling round, we rinsed the cleaned panels with distilled water and collected this as blank.

### Collection of bladder urine samples

We also analysed six samples of urine collected by gently pressing the bladder of one adult female, one adult male, and three juvenile lynx coming from the wild lynx population in Switzerland. Urine of adult lynx was collected while the animals were captured and anaesthetized for radio-tagging in the frame of our long-term monitoring programme for the lynx population in the Northwestern Swiss Alps. Urine of juvenile lynx was collected during routine veterinary check-ups of three orphaned lynx kept at the wildlife rehabilitation centre Schloss Landshut, Switzerland. Urine was collected directly into 20 ml headspace vials with PTFE-lined screw caps (Gerstel GmbH & Co KG, Switzerland) and treated like all other samples.

### Chemical analysis

Urine volatiles were collected using solid phase microextraction (SPME) in the headspace of 4.5 ml urine buffered with 0.5 ml acetate. If there were less than 4.5 ml of urine in a sample, distilled water (hypotonic, pyrogen free, CARL ROTH GmbH + Co. KG, Switzerland) was added until all samples had the same volume. Before extraction, 20 µl 2-Heptadecanone (200 ng/20 µl Acetone; Sigma Aldrich) was added as an internal standard. Volatile adsorption was carried out with a Gerstel MPS2 XL Twister Multi-Purpose Sampler at 70 °C for 240 min using a SPME fibre with 85 µm CAR/PDMS coating (Gerstel GmbH & Co KG, Switzerland). During adsorption, samples were agitated for 5 s every minute. Chemical analysis was conducted with a GC/MS (Agilent 7890A coupled to a Agilent 5975C inert XL MSD) fitted with a 30 m HP-5 ms capillary column (0.25 mm internal diameter and 0.25 µm film thickness; Agilent, CA, USA) with a helium flow rate of 1.4 ml/min. Injector temperature was 250 °C, and was operated in splitless mode. Initial oven temperature was 45 °C held for 2 min. Oven temperature was increased at a rate of 10 °C/min to 70 °C and subsequently at 4 °C/min to 200 °C and at 30 °C/min to 300 °C, where temperature was kept for 10 min. Chromatograms were analysed using ChemStation software (Agilent, CA, USA). To tentatively identify volatile compounds, we visually compared the mass spectra of peaks with similar retention time among different samples, and with the mass spectra of putative compounds supported by NIST08 library match results (Gaithersburg, MD, USA). For the interested reader we provide the mass spectra of the peaks as electronic supplementary material.

We identified 13 lynx-specific peaks by visually comparing urine samples to the corresponding blanks (snow/urine collector blanks, respectively). Manual integration was chosen for quantification because some of the urine samples contained large amounts of $S_2$–$S_8$ sulphur species, which eluted in one to three broad smears. When present in large amounts, several long-chain fatty acids could also form broad smears, which sometimes overlapped other peaks. If sulphur smears were overlapping other compounds, we integrated what was visible of the target peak above the raised baseline caused by the sulphur smear. If fatty acids overlapped other substances, we integrated the overlapping peaks together, as well as the area of the non-target peak(s) separately, and then subtracted the latter from the total peak area, assuming the typical broad, fronting shape of the fatty acid peaks.

Relative quantities of the 13 peaks in each sample were calculated in relation to the peak area of the internal standard (IS) as (peak area compound)/(peak area IS) × 200 ng. Consequently, all compounds were expressed as relative quantities standardized for the peak area of the IS, and we make no claims about absolute quantities. If a compound was not detectable in a sample, we assumed a very small peak area of 100, assuming that the compound was in reality present, but at an amount below detection threshold.

For each of the 13 compounds, we calculated the urine sample/blank ratios of their absolute quantities. We excluded compound 3 (nonanoic acid) and 5 (dodecanoic acid) from further analysis because the medians of their urine sample/blank ratios were below 1, i.e. there was often more of this compound in snow/collector blanks than in urine samples. Compound 1 (nonanal) was excluded because it also occurred in water blanks, has been described as a common human skin volatile (Dormont et al. 2013), and we therefore suspected nonanal concentrations in urine to be partly falsified by contamination. During the GC-analysis conducted in 2013, we ran one blank with distilled water (hypotonic, pyrogen free, CARL ROTH GmbH + Co. KG, Switzerland) between two sample runs ($N = 32$), to minimize carry-over from one sample to the next. Nonetheless, there was still carry-over for cyclic octatomic sulphur. Sulphur peaks in GC-runs of $H_2O$ blanks averaged 12.2 % of the peak area of sulphur in the corresponding urine sample (8.1–16.3 % CI). In 2014, we ran two water blanks between each of two sample runs ($N = 38$), thereby reducing $S_8$ carry-over to a mean of 5.8 % of the peak area of the corresponding urine sample (4.2–7.4 % CI). We included all lynx-specific compounds

into our analysis, irrespective of their volatility at room temperature, since also low volatile compounds can be transported to and detected by the vomeronasal organ (VNO) by means of flehmen (Doving and Trotier 1998). This behaviour has been described for many felid species (e.g. Mellen 1993; Allen et al. 2014), and was also observed by us in Eurasian lynx. All compound identifications were confirmed by careful visual inspection of the mass spectra, but since we were primarily interested in overall differences in scent profiles related to social and dietary factors, we refrained from further validation with pure compounds.

### Statistical analysis

For each urine sample, we recorded sex, social status (adult: >2 years, subadult: 1–2 years, juvenile: <1 year), and identity of the lynx, and determined whether the sample had been collected during or outside the lynx mating season (15th of February to 15th of April, according to Breitenmoser and Breitenmoser-Würsten 2008). We further calculated age of the urine sample (time difference in days between urine deposition and sample collection), as well as time since the last feeding (in days). For captive lynx, feeding schedules were known, and we assumed that lynx fed immediately after food was provided. For wild lynx, we searched the last kill the radio-tagged lynx had made. To find kills, we searched GPS location clusters (GLC's), similar as in Krofel et al. (2012) and in Svoboda et al. (2013). A GLC was defined as a set of at least 2 GPS locations within 100 m and a maximum time span of 72 h between consecutive fixes in the same GLC. Within each GLC, we searched until we found prey remains or until all GPS locations and a radius of 30 m around each location was searched. Time since last feeding was then calculated as the time difference between the last GPS location in the GLC containing the last kill and the time of urine deposition estimated from GPS-telemetry and snow tracking data. In total, 70 urine samples were analysed, but different subsets of the whole dataset were used depending on the type of analysis conducted (Table 1).

To reduce the dimensionality of the compositional data, we first conducted a principal component analysis (PCA) on the transformed relative quantities of 10 lynx-specific chemical compounds using the function prcomp() in R. Since the distributions of compound quantities were heavily skewed, they were raised to the exponent 0.2 for transformation. The transformed variables were standardised to a mean = 0 and standard deviation = 1 using the scaling option of the function prcomp(). We chose the first three PC's with eigenvalues >1 for further analysis in linear mixed models (LMM) fitted using restricted maximum likelihood (REML). Bladder samples and samples which could not unambiguously be attributed to one lynx individual were excluded from the analysis resulting in $N = 57$ samples. Models were fitted to each principal component separately, including sex, mating season, and their interaction as fixed factors and individual identity nested in collection method (snow, collector) as random factors.

We evaluated the scope for individual discrimination based on the amount of variation in scent-profile composition explained by individual identity as a random factor in the LMM. For a heuristic estimate of individual discrimination, we subsequently applied a heteroscedastic discriminant analysis (HDA) using the function hda() of the package *hda* in R. HDA was performed on 25 samples of two male and two female lynx for which we could collect at least five urine samples per individual. Lynx identity was the group identifier, and the second and third PC's were the discriminant variables. The hda() function uses the naive Bayes classifier to make predictions. The percentage of correct classification was interpreted against the chance percentage of correct assignment expected for four individuals (chance of correct classification of four groups by random drawing = 25 %).

We were specifically interested in the relationship between the proportion of sulphur in urine (logit-transformed) and the factors time since last feeding and age of the urine sample. We conducted a separate LMM fitted by REML, where we included all samples for which time since last feeding and sample age were known ($N = 45$). Time since last feeding (linear and quadratic term) and age of the urine sample were included as fixed factors, and individual as random factor. The quadratic term was entered in the model to allow for a non-linear relationship

**Table 1** Subsets of the whole dataset used for the different analyses (subsets overlap partly)

| Type of analysis | PCA | LMM (PC1, PC2, PC3—reprod. state, sex) | LMM ($S_8$ content—feeding time, sample age) | HDA (lynx identity—PC2, PC3) |
|---|---|---|---|---|
| Sample size | 70 | 57 | 45 | 25 |
| Used samples | all samples | unambiguous individual identification, no bladder samples | unambiguous individual identification, time since last feeding known | only individuals with ≥5 samples |

with sulphur content in urine (i.e. sulphur may take a certain time to enter urine via amino acid metabolism). Sample type was not included as random factor, since it was already represented by 0 values for sample age in bladder urine. All linear mixed models were calculated using the function lme() of the package *nlme* in R (version 3.1.0, R Development Core Team 2013).

## Results

### Scent profiles and PCA

We analysed the compounds from the headspace of 70 urine samples coming from 23 lynx individuals. 13 compounds were present in more than trace quantities in most urine samples, and their mass spectra corresponded to the same compound identifications (Table 2).

The first three principal components of the PCA jointly explained 82.2 % of the total variance in relative compound quantities (PC1: 57.3 %, PC2: 14.4 %, PC3: 10.5 %). The contributions of each compound to the three PC's are shown in the loading table (Table 3). The loadings of all substances showed the same sign for PC1. Hence, this component can be thought of as mainly reflecting differences in urine quantities or overall compound concentrations among samples, resulting in higher or lower abundance of all urinary constituents in a given sample. Conversely, the second and third PC's showed loadings of opposite sign which implies that they reflect different aspects of compound composition. PC2 was mainly characterized by negative loadings for 4,8-dimethyl nonanol and 6,10-dimethyl 2-undecanone, as well as positive loadings for n-Hexadecanoic acid and cyclic octaatomic sulphur. PC3 was characterized by negative loadings for the three unknown aldehydes (Table 2), and positive loadings for 4,8-dimethyl nonanol and dodecanoic acid, isooctyl ester.

### Reproductive state, sex and social status

We found a significant correlation between PC2 and mating season (Table 4) suggesting that chemical composition of lynx urine varied with reproductive state. PC2 most strongly reflected changes in concentrations of cyclic octaatomic sulphur and nonanol, 4,8-dimethyl (Table 3). Thus, the observed association is partially due to lower relative abundances of cyclic octaatomic sulphur during the mating season ($\phi$ 12.3 %, 9.5–15.1 % CI) compared to outside the mating season ($\phi$ 16.5 %, 14.5–18.5 % CI). Although males showed lower values for PC1 than females (Fig. 1), we did not find significant correlations of PC1, PC2, or PC3 with sex. However, there was a significant interaction between sex and season on PC3, and a nearly significant interaction on PC2 (Table 4). The scent profiles of both males and females changed independently: during the mating season, males showed lower values for PC3 than outside of the mating season, when values for males and females were similar (Fig. 1). Conversely, females showed lower values for PC2 than males during the mating season. Before or after the mating season, their values for PC2 were larger than for males.

Sample size of subadult lynx was too small for statistical testing (three samples from two individuals). Four urine samples of three juvenile lynx were collected by pressing the bladder of anaesthetized individuals, but we collected only two bladder urine samples from adult lynx. Therefore, variation in scent profiles due to social status could not be disentangled from variation due to different sampling procedures. Nonetheless, a PC biplot of the second and third principal components showed that bladder urine samples of juvenile lynx were more closely clustered than bladder urine samples of adults (Fig. 2).

### Lynx identity

Lynx identity explained <0.1, 49.5, and 26.1 % of the variance in the data for PC1, PC2, and PC3, respectively (Table 4). Furthermore, the heteroscedastic discriminant analysis (hda) on PC2 and PC3 enabled the correct classification of 69 % of the urine samples (as opposed to the chance percentage = 25 % for four individuals).

### Dietary cues

Relative sulphur content in urine samples was not correlated to time since last feeding, neither to the linear nor to the quadratic term (Table 5). There was, however, a significant correlation with age of the urine sample (Table 5). The proportion of sulphur in lynx urine decreased with time after urine deposition (Fig. 3).

## Discussion

### Reproductive state, sex and social status

By analysing the headspace of Eurasian lynx urine, we were able to detect constituents belonging to the following compound classes: carboxylic acids, aldehydes, ketones, esters, and elemental sulphur. Many lynx-specific constituents had low volatility at room temperature. This corresponds to field observations suggesting that visual components of urine marks (e.g. scrapes, Allen et al. 2014; conspicuous objects, Vogt et al. 2014) seem to be essential to draw conspecifics close enough to perceive

**Table 2** Compounds present in the urine of male ($N = 11$), female ($N = 9$), and juvenile ($N = 3$) Eurasian lynx

| Compound No. | Retention time (min) | Compound identification | Statistically significant ions (*m/z*) |
|---|---|---|---|
| C1 | 11.2 | Nonanal[a] | 57, 70, 79, 82, 93, 95, 98, 109, 114, 124, 142, 281 |
| C2 | 14.8 | Nonanol, 4,8-dimethyl | 53, 57, 65, 67, 70, 79, 81, 85, 95, 98, 111, 121, 126, 137, 155, 164, 182, 281 |
| C3 | 16.1–16.5 | Nonanoic acid[a] | 51, 53, 55, 56, 57, 58, 60, 61, 63, 65, 67, 69, 70, 71, 72, 73, 74, 75, 77, 78, 79, 80, 81, 82, 83, 84, 85, 87, 88, 91, 93, 95, 96, 98, 99, 101, 105, 111, 115, 122, 129, 137, 141, 150, 158 |
| C4 | 20.4 | 2-Undecanone, 6,10-dimethyl | 51, 53, 55, 58, 65, 67, 69, 71, 79, 81, 85, 91, 95, 107, 109, 115, 119, 123, 129, 133, 140, 147, 153, 161, 165, 180, 183, 189, 198 |
| C5 | 25.1–25.4 | Dodecanoic acid[a] | 51, 53, 55, 57, 60, 65, 67, 69, 71, 73, 83, 85, 87, 91, 97, 101, 105, 111, 115, 121, 125, 129, 138, 143, 154, 157, 164, 171, 183, 200 |
| C6 | 26.5 | Unknown aldehyde | 51, 55, 57, 69, 71, 77, 79, 82, 85, 93, 96, 105, 109, 123, 138, 152, 168, 175, 184, 175, 184, 194, 218 |
| C7 | 26.8–27.9 | Tetradecanoic acid, 12-methyl, methyl ester | 53, 55, 57, 59, 64, 69, 74, 83, 87, 91, 97, 99, 109, 115, 128, 137, 143, 149, 152, 155, 160, 173, 177, 185, 192, 202, 213, 224, 256 |
| C8 | 29.3 | unknown aldehyde | 51, 55, 57, 68, 71, 77, 79, 82, 85, 96, 102, 109, 124, 138, 152, 166, 182, 193, 198, 208, 227 |
| C9 | 30.5–31.1 | Tetradecanoic acid | 51, 53, 55, 60, 67, 69, 73, 79, 81, 83, 87, 93, 97, 111, 115, 125, 129, 140, 143, 152, 157, 160, 166, 171, 181, 185, 192, 199, 211, 224, 228 |
| C10 | 31.9 | unknown aldehyde | 51, 55, 57, 68, 71, 77, 79, 82, 85, 96, 109, 123, 138, 152, 166, 180, 196, 207, 212, 222, 240 |
| *C11* | *34.1* | *2-Heptadecanone (IS)* | 55, 58, 65, 67, 69, 71, 79, 82, 85, 93, 96, 99, 111, 127, 138, 152, 166, 180, 196, 211, 225, 239, 254 |
| C12 | 35.6–36.1 | *n*-Hexadecanoic acid | 51, 57, 60, 65, 67, 71, 73, 79, 83, 85, 87, 97, 101, 111, 115, 125, 129, 143, 149, 157, 171, 177, 185, 194, 199, 205, 213, 223, 227, 239, 256 |
| C13 | 36.9–37.9 | Cyclic octaatomic sulphur ($S_8$) | 54, 64, 66, 68, 74, 80, 96, 98, 110, 128, 153, 160, 192, 224, 256 |
| C14 | 38.2 | Dodecanoic acid, isooctyl ester | 51, 55, 57, 60, 65, 67, 70, 73, 79, 83, 87, 95, 97, 101, 112, 123, 129, 143, 149, 157, 163, 171, 183, 193, 201, 207, 215, 226, 239, 255 |

Compounds presented were found in urine obtained by three different sampling methods (snow, collector, and bladder). Retention time is the time taken for each compound to elute from the GC-column (in minutes). Compounds were tentatively identified by matching their mass spectra with the NIST08 library (Gaithersburg, MD, USA) and by visual inspection of the mass spectra. *IS* internal standard

[a] Compound excluded from further analysis

**Table 3** Loadings of the PCA on 10 lynx-specific compounds ($N = 70$)

| PC1 | PC2 | PC3 | Compound |
|---|---|---|---|
| −0.3140 | **−0.4342** | **0.2742** | Nonanol, 4,8-dimethyl |
| **−0.3302** | **−0.3564** | 0.1807 | 2-Undecanone, 6,10-dimethyl |
| **−0.3351** | −0.2366 | **−0.3287** | Unknown aldehyde (C6) |
| **−0.3678** | 0.1261 | 0.2354 | Tetradecanoic acid, 12-methyl, methyl ester |
| **−0.3519** | −0.1767 | **−0.2708** | Unknown aldehyde (C8) |
| **−0.3302** | 0.2340 | 0.1083 | Tetradecanoic acid |
| −0.2321 | 0.0400 | **−0.7394** | Unknown aldehyde (C10) |
| **−0.3443** | **0.3200** | −0.0340 | *n*-Hexadecanoic acid |
| −0.1616 | **0.6327** | 0.0387 | Cyclic octaatomic sulphur (S8) |
| **−0.3369** | 0.1494 | **0.3072** | Dodecanoic acid, isooctyl ester |

Compounds with loadings >|0.25| are highlighted in bold and considered to be of biological relevance for this PC. Compound numbers refer to Table 2

chemosignals. Compounds with low volatility would also have the advantage of persisting longer on the substrate than highly volatile substances. High levels of carboxylic acids and ketones were identified in the urine of tigers and lions (Andersen and Vulpius 1999; Burger et al. 2008), and were suggested to have a function in chemical

**Table 4** Parameter estimates of the linear mixed models (LMMs) for the first three principal components

| | *DF* | *F* value | *P* |
|---|---|---|---|
| PC1 | | | |
| Intercept | 36 | 0.019 | 0.892 |
| Sex = male | 16 | 4.044 | 0.062 |
| Mating season = yes | 36 | 3.005 | 0.092 |
| Sex × season | 36 | 1.260 | 0.269 |
| PC2 | | | |
| Intercept | 36 | 2.800 | 0.103 |
| Sex = male | 16 | 3.051 | 0.100 |
| Mating season = yes | 36 | 7.608 | **0.009** |
| Sex × season | 36 | 3.402 | 0.073 |
| PC3 | | | |
| Intercept | 36 | 0.035 | 0.852 |
| Sex = male | 16 | 0.048 | 0.829 |
| Mating season = yes | 36 | 1.083 | 0.305 |
| Sex × season | 36 | 8.971 | **0.005** |

The LMMs were fitted to the data assuming a normal error distribution and using restricted maximum likelihood (REML). The first three principal components (PC1, PC2, PC3) derived from a PCA on the absolute quantities of 10 compounds found in lynx urine were set as response variables. *DF* denominator degrees of freedom. $P = p$ values of Wald tests for model terms (values <0.05 are indicated in bold script). Levels of the fixed factors are compared as follows: sex = male is compared to sex = female, mating season = yes is compared to mating season = no. The analysis was conducted on data from 57 urine samples. Lynx identity nested in sample type (snow, collector) was included as random effect. Sample type explained 16.5 % (PC1, $\sigma = 0.819$), <0.1 % (PC2, $\sigma < 0.001$), and 16.4 % (PC3, $\sigma = 0.154$) of the variance in the data. Lynx identity explained <0.1 % (PC1, $\sigma < 0.001$), 49.5 % (PC2, $\sigma = 0.363$), and 26.1 % (PC3, $\sigma = 0.245$) of the variance in the data

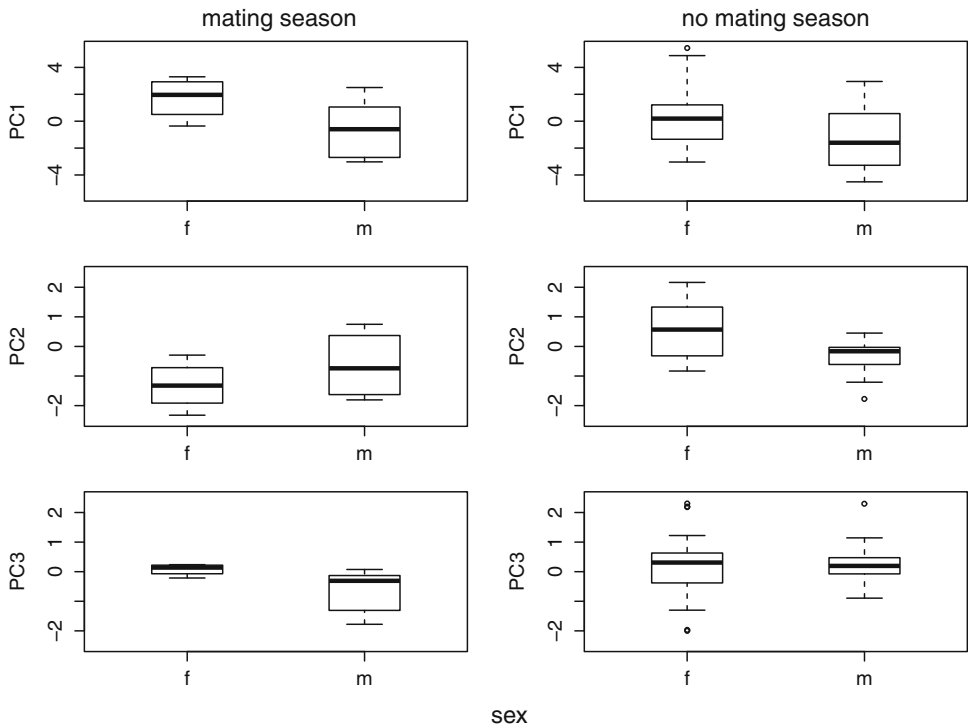


**Fig. 1** Differences between chemical profiles of male and female lynx during and outside the mating season. PC1, PC2, PC3 = scores of the first three principal components of the PCA incorporating 10 lynx-specific compounds ($N = 57$). *F* females, *m* males. Mating season = 15th February to 15th April. *Each box* encompasses the 25th through 75th percentiles, with the median represented by an *interior line*. *Whiskers* denote maximum values or in case of outliers 1.5 times the interquartile range. *Circles* denote outliers

communication (Soini et al. 2012) and, possibly, individual recognition (Poddar-Sarkar and Brahmachary 1999). In this study, we were able to relate changes in the relative quantities of the above mentioned compounds to sex and reproductive state. We detected changes in the chemical profiles of male lynx reflected in significantly lower PC3

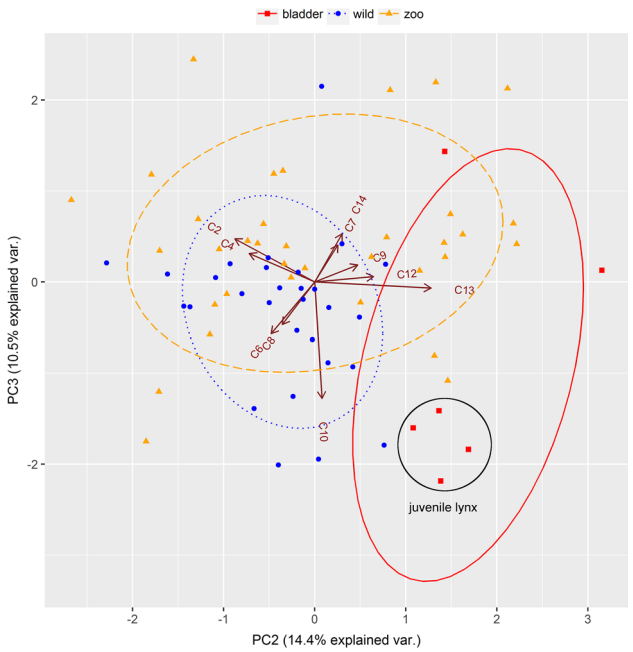


**Fig. 2** PC-biplot of the second and third principal components of the PCA incorporating 10 lynx-specific compounds ($N = 70$). Arrow labels denote identifications of the lynx-specific compounds according to the numeration in Table 2. C2 = Nonanol, 4,8-dimethyl; C4 = 2-Undecanone, 6,10-dimethyl; C6 = unknown aldehyde; C7 = Tetradecanoic acid, 12-methyl, methyl ester; C8 = unknown aldehyde; C9 = Tetradecanoic acid; C10 = unknown aldehyde, C12 = *n*-Hexadecanoic acid; C13 = Cyclic octaatomic sulphur; C14 = Dodecanoic acid, isooctyl ester. *Symbols* and *line* types denote different sampling procedures. *Ellipses* = 68 % normal data ellipses for each group. Bladder (*squares*, *solid line*) bladder urine collected from anaesthetized juvenile and adult lynx, wild (*circles*, *dotted line*) urine collected from adult and subadult wild lynx during snow tracking, zoo (*triangles*, *dashed line*) urine collected from adult zoo animals with a collection device. *Circle* bladder urine samples collected from juvenile lynx

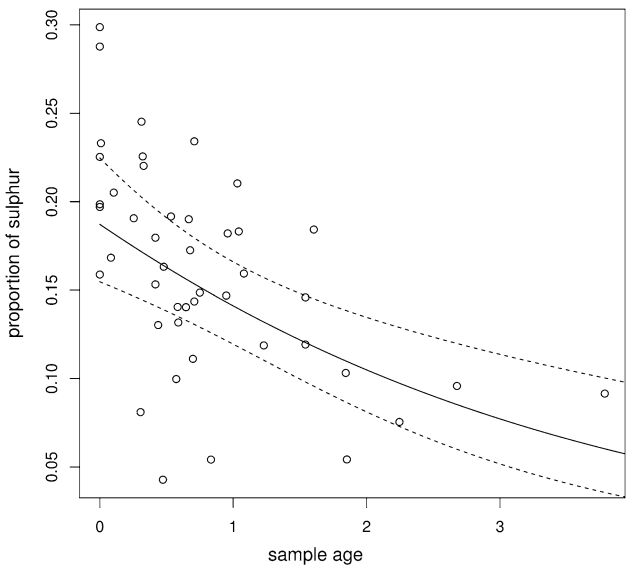


**Fig. 3** Proportion of sulphur in urine decreases with age of the urine sample (in days). *Solid line* fitted values from the LMM, *dashed line* 95 % confidence intervals

values during the mating season. Scent profiles of males could be altered during mating season due to changes of androgen levels or differences in body condition (Petrulis 2013). Many female mammals are attracted to the odours of reproductive male conspecifics, and preferences for individual males are often related to signals of quality or social status (Petrulis 2013; Roberts et al. 2014; Allen et al. 2015b). On the other hand, male mammals have been shown to be attracted to odours of oestrous females (Swaisgood et al. 2000; Scordato et al. 2007; Petrulis 2013), and Eurasian lynx are known to search and mate-guard females during mating season (Breitenmoser and Breitenmoser-Würsten 2008). If urine marks are the means by which female lynx attract males when they are receptive, we would expect that the chemical profile of females should change when they are ready to mate. Our results show some evidence for such changes reflected in PC2 values, although the result is not significant.

PC2 and PC3 were most strongly characterized by different quantitative contributions of an unknown aldehyde (compound 10; Table 2), cyclic octaatomic sulphur, and 4,8-dimethyl nonanol, but no conclusions about the role of these substances in lynx reproductive behaviour can be

**Table 5** Parameter estimates of the linear mixed model (LMM) for relative sulphur content in lynx urine samples

| | *DF* | *F* value | *P* |
|---|---|---|---|
| Intercept | 22 | 429.152 | **<0.001** |
| Time since feeding | 22 | 0.186 | 0.670 |
| (Time since feeding)$^2$ | 22 | 0.235 | 0.632 |
| Sample age | 22 | 15.301 | **<0.001** |

The LMM was fitted to the data assuming a normal error distribution and using Restricted Maximum Likelihood (REML). The relative abundance of sulphur in the urine sample was logit-transformed and set as response variable. *DF* denominator degrees of freedom. $P = p$ values of Wald tests for model terms (values <0.05 are indicated in bold script). The following factors were included as fixed effects: time since last feeding (in days) and age of urine sample (in days). Time since feeding is also entered as quadratic term. The analysis was conducted on data from 45 urine samples for which time since last feeding and age of urine sample could be estimated. Lynx identity ($\sigma = 0.060$) was included as random effect and explained 29 % of the variance in the data

made. Experimental studies are needed to identify the compounds possibly involved in mate attraction in Eurasian lynx. However, pinpointing actual chemosignals involved in mate attraction in mammals is often very difficult (Petrulis 2013), even though many studies, including ours, have found differences in scent profiles related to sex or season (e.g. catta *Lemur catta*, Scordato et al. 2007; owl monkey *Aotus nancymaae*, Macdonald et al. 2008; brown bears *Ursus arctos*, Rosell et al. 2011; short-beaked echidna *Tachyglossus aculeatus*, Harris et al. 2014). Empirical evidence collected until now suggests that it might be subtle shifts in overall scent composition, rather than a single sex pheromone, which are mediating mate attraction in mammals (Petrulis 2013).

Unfortunately, we were not able to explore potential information on social status and test for differences in scent profiles between juvenile, subadult, and adult lynx due to low sample sizes and differences in sampling procedure. However, scent profiles from four juvenile lynx showed great qualitative similarity among each other and tended to cluster together in a PC-biplot. Scent profiles can serve as badges of social status, as shown e.g. in spotted hyenas *Crocuta crocuta* (Burgener et al. 2009), blackbucks *Antelope cervicapra* (Rajagopal et al. 2010), or mice (Mossman and Drickamer 1996), and social status is often related to the age of an animal. In Eurasian lynx, subadult lynx disperse from the home ranges of their mothers in their second year of life in search of a vacant home range. It is the adult resident lynx who occupy stable home ranges and can be considered as dominant resource holders (Breitenmoser and Breitenmoser-Würsten 2008). Wild juvenile lynx show interest in adult's scent-marks, but have not been observed scent-marking themselves (Vogt et al. 2014). Further studies on the ontogenetic changes of scent profiles, and their relationship with the onset of scent-marking behaviour and the acquisition of resident status should be conducted.

### Lynx identity

Our linear mixed models investigated the effects of the fixed factors sex and season on the chemical composition of lynx urine, but also revealed substantial variation in scent profiles between lynx individuals. Lynx identity accounted for 49.5 % of the variance in PC2 and 26.1 % of the variance in PC3, but explained almost no variance in PC1 (<0.1 %). Furthermore, the results of our heuristic approach (the heteroscedastic discriminant analysis on four individuals for which the largest number of urine samples was available) demonstrate that individual variation in PC2 and PC3 could be used for individual recognition, since the percentage of correct classification was higher than expected by chance.

Captive Eurasian lynx have been shown to discriminate between urine of familiar and unfamiliar conspecifics (Sokolov et al. 1996), and a study on scent-marking behaviour of wild lynx has shown that lynx overmark urine marks of other lynx more readily than their own old urine marks (Vogt et al. 2014). While this behaviour alone may not provide sufficient evidence for individual recognition, it shows that lynx can learn to recognize scent profiles. The social organisation of wild lynx populations further suggests possible roles for individual recognition in the contexts of territoriality or mate choice (Allen et al. 2015b): resident lynx may have to respond differently to the scent-marks of strange intruders compared to scent-marks of neighbouring residents (dear enemy phenomenon; Temeles 1994), or they may learn to recognize the individual scent profiles of sexual partners for whom they have developed a mating preference, as has been described in mice (Roberts et al. 2014).

### Dietary cues

Dietary cues are known to act as signals of quality to conspecifics (Ferkin et al. 1997), and may also be exploited by prey species to adjust their anti-predatory behaviours (Murray and Jenkins 1999; Chivers and Mirza 2001; Osada et al. 2013). In wild felids, one possible source of dietary cues in urine is the degradation of sulphur-containing amino acids to volatile organosulphur compounds via production of felinine (Mattina et al. 1991; Hendriks et al. 2001; Miyazaki et al. 2006). Although Eurasian lynx likely produce felinine (Miyazaki et al. 2008), organosulphur compounds have only been detected in very low quantities in lynx urine during an earlier study (Zachariae, pers. comm). In this study, we were unable to detect organosulphur compounds, but we found high amounts of elemental sulphur $S_8$ in almost all lynx urine samples. Estimated concentrations in undiluted bladder urine were quite similar to those in cheetah urine (median = 1.0 µg/ml, lower quantile = 0.5 µg/ml, upper quantile = 1.8 µg/ml), and much larger than those of $S_8$ found in tiger urine (Burger et al. 2008). We found that elemental sulphur was part of the overall signal for reproductive state and sex (see above), but we were also specifically interested in its possible role as a dietary cue or as an indicator of freshness of a scent-mark. We expected the proportion of $S_8$ in lynx urine to be related to food intake, which we measured in terms of time since last feeding. However, we did not find any association. Wild lynx normally fast for several days between kills (Breitenmoser and Breitenmoser-Würsten 2008), and it is possible that our recorded time spans (1-186 h) were not long enough to reflect cysteine depletion. We were also only able to determine when lynx were fed or present at kills, but not for how long they were actually feeding and how much food was

ingested. Thus, our data on food intake may be too imprecise to explain variation in sulphur content in lynx urine, and it remains unclear whether $S_8$ content really conveys information on food condition in Eurasian lynx. On the contrary, we did find a relationship between $S_8$ content and sample age, i.e. the proportion of sulphur in lynx urine decreased with increasing sample age. Elemental sulphur can be converted to sulphates or sulphuric acid by thiobacteria or sulphur bacteria under aerobic conditions (Waksman and Joffe 1922; Dévai et al. 1996). The concentration of derivatives of these products could serve as an indicator of freshness of the scent-mark to other lynx and potentially to prey animals. Felids lack the great variation of major urinary proteins (Miyazaki et al. 2008) which are known to bind volatile chemosignals in mouse urine and extend the longevity of scent-marks (Roberts et al. 2014). In tigers, lipids added to urine in the urinary tract are thought to fulfil this function (Brahmachary and Poddar-Sarkar 2015). Lynx urine does not contain such a lipid fraction (own observation), and it is conceivable that $S_8$ may serve as a source for continuous release of chemosignals from urine. It still remains to be investigated whether this proposed mechanism could also serve to conceal dietary cues in fresh urine marks and release them only with time.

Apart from protein metabolites, compounds produced by lipid metabolism such as carboxylic acids can also hold information on health and metabolic condition (Soini et al. 2012). We detected several carboxylic acids in lynx urine, among them n-hexadecanoic acid which was also found in tiger and bobcat urine and is a constituent of commercial deer repellent (Mattina et al. 1991; Burger et al. 2008). It is a molecule also present in members of other carnivore families (e.g. Iberian wolf *Canis lupus signatus*, Martin et al. 2010; brown bear *Ursus arctos*, Rosell et al. 2011; wild dog *Lycaon pictus* and black-backed jackal *Canis mesomelas*, Apps et al. 2012), and preliminary analysis showed that its proportion was not related to sample age in our study (Spearman's rank correlation, $\rho = -0.029$, $S = 14602.55$, $p = 0.851$), hence, it is available for prey animals to react to in fresh as well as older scent-marks. Many studies on predator urine have so far either been conducted on fresh or old urine samples (when using commercial urine products, as in Mattina et al. 1991). Experimental manipulation of urine age and predator diet may shed more light not only on chemical processes involved in generation of dietary cues, but also on how such cues can be exploited by con- and heterospecifics.

## Conclusions

Our results demonstrate that lynx urine contains sex-specific information on reproductive state, as well as individual variation that may be used for individual recognition. Urine marks are, therefore, well-suited to fulfil a role in reproductive behaviour and social organisation of wild lynx populations. We further found that $S_8$ content in urine was related to sample age. While our study provides first insights into the chemical information contained in lynx urine, the mechanisms involved in the unusually high elemental sulphur excretion in Eurasian lynx and cheetah are still unknown. The influence of diet and body condition on scent profiles should be further investigated by means of experimental studies, and may shed more light on the messages encoded in carnivore scent-marks.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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