Coding of Group Odor in the Subcaudal Gland Secretion of the European Badger Meles meles: Chemical Composition and Pouch Microbiota

CONFERENCE PAPER · AUGUST 2015

6 AUTHORS, INCLUDING:

Christina D. Buesching
University of Oxford
48 PUBLICATIONS **680** CITATIONS
SEE PROFILE

Veronica Tinnesand
Telemark University College
3 PUBLICATIONS **3** CITATIONS
SEE PROFILE

Frank Rosell
Telemark University College
94 PUBLICATIONS **1,126** CITATIONS
SEE PROFILE

Terry Burke
The University of Sheffield
300 PUBLICATIONS **12,011** CITATIONS
SEE PROFILE

Coding of Group Odor in the Subcaudal Gland Secretion of the European Badger *Meles meles*: Chemical Composition and Pouch Microbiota

Christina D. Buesching [1,*]

Email christina.buesching@zoo.ox.ac.uk

H. Veronica Tinnesand [2]

YungWa Sin [1,3]

Frank Rosell [2]

Terry Burke [3]

David W. Macdonald [1]

[1] Recanati-Kaplan Centre, Wildlife Conservation Research Unit, Department of Zoology, University of Oxford, Tubney House, Abingdon Road, Tubney, OX13 5QL UK

[2] Faculty of Arts and Sciences, Department of Environmental and Health Studies, Telemark University College, 3800 Bø, Telemark, Norway

[3] NERC Biomolecular Analysis Facility – Sheffield, Department of Animal & Plant Sciences, University of Sheffield, Alfred Denny Building, Western Bank, Sheffield, S10 2TN UK

Abstract

The fermentation hypothesis predicts that odor profiles of mammals depend partly on the primary gland products excreted by the animal and partly on the composition of the bacterial flora converting these into secondary metabolites. Some mammalian odors, such as shared group odors, however, need to be consistent yet flexible (e.g., to allow for changes in social-group affiliation), and are thus predisposed for microbial mediation. Using terminal restriction fragment (TRF) length polymorphism analyses we analyzed the microbial community in subcaudal-gland secretions of European badgers (*Meles meles*) in relation to the chemical scent profiles as determined by gas chromatography-mass spectrometry analyses (GCMS) of 66 adults belonging to six different social groups. We found a total of 50 TRFs and 125 different chemical compounds with a subset of four TRFs best explaining the structure in the chemical matrix. Nevertheless, although semiochemical profiles were group specific, microbial profiles were not. In our approach, however, the number of operational taxonomic units exceeded the numbers of TRFs, and thus our analyses were likely limited by the afforded resolution. As it is likely that the variation in metabolic activity is found at the species-, subspecies-, or even strain-level, future high-throughput sequencing can be expected to reveal more subtle differences in the microbial communities between social groups.

5.1. Introduction

To convey discrete olfactory information to their conspecifics, many mammals have developed specialized scent glands as well as adaptive scent-marking behaviors (e.g., Brown and Macdonald 1985; Müller-Schwarze 2006). Diet (e.g., Ferkin et al. 1997) and parasites (e.g., Gangestad and Thornhill 1998), as well as the animal's sex (e.g., Kelliher 2007), age (e.g., Osada et al. 2003), endocrine status (e.g., Woodley and Baum 2003), and genetic makeup (Penn 2002) can affect primary gland products. Often, however, these primary secretions are metabolized by bacteria, and typically it is those secondary metabolites that generate the characteristic odor of the scent mark. The fermentation hypothesis (Albone 1984; Albone et al. 1974; Albone and Perry 1975; Gorman 1976; Archie and Theis 2011) predicts that odor profiles depend partly on the primary gland products excreted by the animal, partly on the composition of the bacterial flora metabolizing these primary gland products, and partly on the age of the scent mark (e.g., Buesching et al. 2002b; Goodwin et al. 2012; Zechman et al. 1984), e.g., the time the bacteria had to metabolize primary gland products. Whereas the management of these zoonotic microbiota is therefore paramount for mammals to ensure consistency and/or meaningful olfactory information content, the potential to vary odor profiles through fluctuating composition of the microbial communities ensures flexibility in body odor and scent marks.

One such example of essential flexibility in odor profiles is scent characteristics encoding group membership. In social mammals, shared group odors have been implicated in social acceptance (e.g., Hurst et al. 1993) and group cohesion (e.g., peccaries: Buyers 1985; badgers: Buesching et al. 2003). However, given that in the majority of species, offspring of one or both sexes disperse at least once in their lives, and in reality may change group affiliation several times even as adults (e.g., to avoid inbreeding: Greenwood 1980), olfactory advertisement of group membership must be reliable yet flexible. Thus, generation of shared group odors has long been suggested to rely predominantly on the assimilation of microbial communities amongst group members (Albone 1984). This has recently been shown to be the case in anal gland secretions of spotted hyenas *Crocuta crocuta* (Theis et al. 2012, 2013; for review of additional species see Wyatt 2010; Ezenwa and Williams 2014; James et al. 2013).

European badgers (*Meles meles*) are macrosmatic, and thus rely heavily on their sense of smell for intra-specific communication and recognition of others (Roper 2010). Their social organization is highly variable and ranges from solitary or pair-living to the formation of large social groups in the South of England (Johnson et al. 2000). In addition to other pathways of olfactory communication common amongst Mustelids, such as urine, feces, anal, and inter-digital glands (Macdonald 1985), badgers have evolved a subcaudal gland which is unique amongst the Carnivora (Macdonald 1985). It consists of several layers of apocrine and sebaceous cells, which secrete a margarine-like paste into a common lumen, the subcaudal pouch (Stübbe 1971), where it is stored until scent marking (Buesching et al. 2002c). The pouch opens at a 20–80 mm wide slit situated between the anus and the base of the tail. Both sexes possess this gland and use the secretion for scent marking by pressing the slit onto the substrate (Őstborn 1976; Buesching and Macdonald 2004) or conspecifics (Buesching et al. 2003). Cubs start to produce traces of subcaudal gland secretion when they are approximately 4 months old (Buesching et al. 2002c). Secretions are highly individual-specific, and encode information about sex, age, body condition, and reproductive status of the donor (Őstborn 1976; Gorman et al. 1984; Buesching et al. 2002a), but change with season and age of the scent mark (Buesching et al. 2002b). In addition, odor profiles are more similar amongst the members of a social group than they are between groups, indicating the existence of a shared group odor (Buesching et al. 2002a).

The subcaudal pouch has been shown to support a rich bacterial flora (Albone et al. 1978; Sin et al. 2012), and semiochemical analyses confirm that subcaudal gland secretions are of high chemical complexity, containing mainly medium- and long-chained carboxylic acids, which are usually of

bacterial rather than mammalian origin (Albone 1984), water and protein (Gorman et al. 1984). A direct empirical link between pouch bacteria and scent profiles, however, remains to be established.

Here, we analyzed the bacterial pouch flora in relation to the chemical scent profile of adults belonging to six different social groups. The aim of this study was threefold: (1) to confirm that group membership is encoded in subcaudal gland secretions, (2) to investigate the overall relation between the bacterial communities and the chemical composition of the subcaudal-gland secretion, and (3) to investigate potential group differences in the bacterial communities of badger subcaudal gland secretions.

5.2. Material and Methods

5.2.1. Study Animals and Collection of Samples

Samples were collected in Wytham Woods, Oxfordshire, England (GPS reference: 51u469260N; 1u199190W; for a detailed description of the study site see Kruuk 1989; for details of the study population see Macdonald and Newman 2002), during two different trapping events: in spring (27th May–9th June 2010, $N = 31$), and in summer (6th Sept.–18th Sept 2010, $N = 35$). As part of an ongoing population study, and following the methodology described by Macdonald and Newman (2002), badgers were trapped overnight in cage traps baited with peanuts. At first capture (usually as cubs), all animals were tattooed with an individual number on the left inguinal region. Thus, after sedation with 0.2 ml ketamine hydrochloride/kg body weight (Thornton et al. 2005), all badgers could be identified individually and linked to their trapping history.

Subcaudal gland secretion (SGS) was scooped out of the subcaudal pouch using a rounded stainless steel spatula, and contact of the spatula with body parts other than the inside of the subcaudal pouch was strictly avoided to exclude bacteriological cross-contamination. Secretions were subdivided into two aliquots. Both were frozen immediately and stored at −20 °C until further analyses. To avoid contact with plasticizing agents, the aliquot for semiochemical analysis was stored first in a glass vial with Teflon lid, and the one for the bacteriological analyses was stored afterwards in a microcentrifuge tube. Between sampling different individuals, the spatula was wiped clean and sterilized three times with 90 % ethanol, which was flamed off to avoid contamination of the semiochemical sample.

All collected samples were used to investigate the correlation between microbiota and chemical composition. To investigate chemical and microbial differences between social groups, however, we used secretions collected from a total of six different social groups ($N_{CH} = 5$, $N_{GAH} = 5$, $N_{LS} = 6$, $N_{MT} = 6$, $N_{PO} = 6$, $N_{RC} = 5$) as established by biannual bait-marking surveys (following the methodology described by Delahay et al. 2000), where we caught a minimum of five adult animals. To avoid pseudoreplication, only one (randomly chosen) sample from each individual was included ($N = 33$, $N_{Males} = 14$, $N_{Females} = 19$).

5.2.2. Chemical Analyses

For each sample, 0.1 g of subcaudal gland secretion was extracted in 1.0 ml of dichloromethane (Pestanal® Grade, Sigma-Aldrich®, Oslo, Norway). Solutions were left at room temperature for 1 h and centrifuged at 3000 rpm through a 0.45 μm filter for 5 min. The resulting particle-free solution was transferred to a 1.5 ml GC-vial (Agilent®, Oslo, Norway).

Chemical composition was analyzed using a Hewlett-Packard (HP, Oslo, Norway) 6890 Series II gas chromatograph (GC) equipped with a nonpolar HP-5 MS 5 % phenyl-methyl-siloxane column (30.0 m

long × 0.25 mm ID × 0.25 μm film thickness), connected to a HP 5973 Series mass spectrometer detector (MS) with a split/splitless inlet in splitless mode using helium as the carrier gas at a constant flow of 0.9 ml/min. Injection port temperature was set at 300 °C. The purge flow to split vent was 49.8 m/min at 1.00 min. An auto-injection system (Agilent 7683 Series Injector, Oslo, Norway) was used to inject 1.0 μl of the SGS solution into the GC-MS. The samples were cold-trapped at 40 °C on the column tip for 2 min and separated using a temperature program of 8 °C/min from 40° to 150 °C, then 6 °C/min from 150 °C to 200 °C, and finally 4 °C/min from 200° to 240 °C (holding for 15 min). A solvent delay of 5 min was set to prevent solvent damage to the detector. Samples were analyzed in random order, and after every five samples, a blank was run to ensure that there was no contamination left in the column. A mixture of unbranched alkanes between C_8 and C_{40} (Sigma-Aldrich, Oslo, Norway) was also run after every five samples to calculate Kováts Retention Indices (KRI), allowing standardization of retention times. The instrument was calibrated every morning to detect possible changes in sensitivity.

Compounds were matched between profiles by their retention times and mass spectra, and given an individual peak number. A tentative identification of the analytes was provided by cross-checking the best suggested matches from the Wiley 275 spectral library (Scientific Instrument Services Inc., Ringoes, USA) with the calculated KRI of the analytes. Identification of 12 compounds (hexanoic acid, octanoic acid, decanoic acid, dodecanoic acid, tetradecanoic acid methyl ester, tetradecanoic acid, hexadecanoic acid methyl ester, hexadecanoic acid, methyl linoleate, arachidic acid methyl ester (eicosanoic acid methyl ester), docosanoic acid methyl ester, tetracosanoic acid methyl ester; chosen to provide a good spread across the profile retention times) was confirmed through injection of commercial standards (Sigma-Aldrich, Oslo, Norway). Where the Wiley library did not provide a good match, analytes were given a name based on retention times and added to a new library.

5.2.3. Bacteriological Analyses

5.2.3.1. DNA Extraction

Following the methodology described in Sin et al. (2012), secretions were transferred to microcentrifuge tubes and re-suspended in 180 μl enzymatic lysis buffer [20 mM Tris–Cl (pH 8.0), 2 mM EDTA, 1.2 % Triton X-100] containing 20 mg/ml lysozyme. Following incubation at 37 °C for at least 1 h in a shaking incubator to lyse cell walls of Gram-positive bacteria before DNA purification, samples were incubated with proteinase K at 56 °C for at least 1 h, followed by the addition of 200 μl of ethanol. DNA isolation was continued by pipetting the mixture into the spin column according to the manufacturer's instructions for animal tissue samples using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany).

5.2.3.2. T-RFLP Analysis

Internal fragments of 16S rRNA genes were amplified from the isolated DNA using universal bacterial primers 341f (5′-CCTACGGGAGGCAGCAG-3′; Muyzer et al. 1993) and 926r (5′-CCGTCAATTCMTTTRAGTTT-3′; Muyzer et al. 1995). Primer 341f was labeled at its 5′ end with the dye 6-carboxyfluorescein (6-FAM) and 926r labeled with hexachloro-6-carboxyfluorescein (HEX). PCR mixtures comprised 1× PCR buffer, 1 mM $MgCl_2$, each dNTP at a concentration of 0.2 mM, each primer at a concentration of 0.6 μM, as well as 0.5 U Taq polymerase (Bioline). Amplification was carried out in two separate 25 μL reaction mixtures under the following conditions: 95 °C for 3 min, amplified for 35 cycles of 95 °C/30s; 55 °C/60s; 72 °C/75 s, and a final extension at 72 °C for 10 min. For each sample, duplicate fluorescently labeled PCR products were pooled after PCR. PCR product sizes were confirmed by agarose gel electrophoresis using 100 bp DNA ladders as size markers and by staining with ethidium bromide. The amplified products (around 586 bp) were purified using the QIA quick gel extraction kit (Qiagen).

AQ1
AQ2

Fluorescently labeled PCR products were digested with MspI restriction enzymes (Applied Biosystems ABI) for 6 h at 37 °C, followed by 20 min at 80 °C for enzyme inactivation. Digested fragments were separated on the ABI 3730 Genetic Analyzer (ABI). Sizes of the fluorescently labeled terminal restriction fragments (TRFs) were determined by comparison with the GeneScan 500 ROX size standard (ABI). Before injection, 0.5 μL of the DNA sample was denatured in the presence of 9.5 μL Hi-Di formamide and 0.05 μL GS 500 ROX size standard (ABI) at 95 °C for 5 min. Injection was performed electrokinetically at 2 kV for 10s, and electrophoresis was run at 15 kV for 30 min. After electrophoresis, terminal restriction fragment length polymorphism (T-RFLP) electropherograms were imaged using the GENEMAPPER software (v. 3.7; ABI). The lengths of TRFs were determined by comparison with the internal standard, within the lower threshold at 50 bp and upper threshold at 500 bp. Only peaks with heights exceeding 50 fluorescence units were evaluated.

T-RFLP profiles were aligned using T-Align (Smith et al. 2005), which identified all fragments unequivocally with ±0.5 bp in all profiles generated, and determined the presence and absence of TRFs and their relative fluorescence in all samples. Log ($n + 1$) transformation was performed in order to normalize the data. The number of the operational taxonomic units (OTUs) and the bacterial identities of the OTUs that contributed to the TRFs were identified by cloning and sequencing of the 16S rRNA genes as detailed in Sin et al. (2012).

5.2.4. Statistical Analyses

All TRFs and chemical compounds that occurred in only one sample were excluded from all analyses.

5.2.4.1. Analyses of Scent Profiles

To account for variation in the total quantity of injected secretion, peak areas of each compound were standardized using the peak area of hexadecanoic acid, which was present in all scent profiles as one of the largest peaks. All data were square-root transformed to reduce the influence of the most abundant variables (Clarke and Warwick 2001), and a Bray–Curtis similarity index calculated between each possible sample pairing.

5.2.4.2. Analyses of Group Differences in Chemical Composition and TRFs

To investigate variation in chemical profiles and microbial communities among social groups, we first used principal coordinate (PCO) analysis based on the Bray–Curtis similarity index in order to visualize the patterns of variation among samples (Gower 1966). If the same animal was caught during both trapping events, only one sample, selected at random, was included in the analysis. Differences between social groups were then compared with a single factor PERMANOVA (Anderson 2001, McArdle and Anderson 2001) using 9999 permutations, applying both main and pairwise tests. PERMANOVA allows distance-based tests of significance for comparing a priori groupings. Significant ($P < 0.05$) and marginally significant ($P < 0.1$) differences between groups were investigated further using canonical analysis of principal coordinates (CAP: Anderson and Willis 2003), which obtains predictive models that search the multivariate data for the best discrimination between a priori groups. To present the reader with all results, we did not correct for multiple testing as suggested by Nakagawa (2004). The number of PCO axes, m, to use in the model and the predictive ability of the model to discriminate between the social groups was assessed by leave-one-out cross-validation (Anderson and Robinson 2003, Mardon et al. 2010). The software PRIMER V6.1.13 (Clarke and Gorley 2006) with the PERMANOVA+ V1.0.3

add-on package (Anderson et al. 2008) was used in all analyses.

5.2.4.3. Analyses of Correlation Between TRFs and Chemical Composition

To investigate any linkage between the TRFs and the chemical composition of the secretion, we performed the BEST procedure (Clarke et al. 2008). The rationale of BEST is to find the "best match" between the multivariate among-sample patterns of two data matrices, one being a fixed resemblance matrix (often biotic variables, or in our case the chemical compounds) and one an explanatory matrix (often environmental variables, in our case the TRF data: Clarke and Gorley 2006). The BVSTEP procedure in BEST selects the combination of explanatory variables that best explains the structure in the fixed matrix, whereas the global BEST test constitutes an overall significance test for the final subset of variables (Clarke et al. 2008).

To investigate correlations between specific TRFs and chemical compounds, we constructed a correlation matrix between all variables that occurred in > 10 % of the samples. All TRF-compound combinations with a correlation > 0.4 were tested for statistical significance using two-tailed tests. Correlation analyses were performed with the software packages JMP 10 (SAS Institute Inc., Cary, NC, USA) and SPSS Statistics 19 (SPSS Inc., New York, USA).

5.3. Results

5.3.1. Group Differences in Chemical Composition

When evaluating chemical variation among social groups including all compounds, an unconstrained 2D PCO explained 49.2 % of the variation in the data, and the third axis explained further 8.7 %, but there was no separation between badger social groups (Fig. 5.1). The main PERMANOVA comparing the 6 groups with ≥ 5 adults sampled was significant (pseudo $F = 1.6692$, $P = 0.0141$), and pairwise tests revealed significant differences between four pairs of groups (Table 5.1). The CAP analysis classified 33.3 % of the chemical profiles into the correct group using leave-one-out cross-validation and $m = 16$ axes ($\delta_1^2 = 0.89814$, $P = 0.028$, Fig. 5.2). The CAP plot, however, is a constrained plot, which views the data cloud through the filter of our hypothesis, and thus should be viewed in conjunction with the results from the cross-validation, which provide information the distinctiveness of each group, and how well the axes discriminate between each of them (Anderson et al. 2008). Although in our analyses the CAP plot suggested that semiochemical differences among social groups were clear, the cross-validation results showed that the predictive ability of the model was low. The cross-validation results, however, were still significant, as, with $n = 6$ social groups, only approx. 16.7 % of samples would have been classified correctly by chance alone (Table 5.2).

Fig. 5.1

Unconstrained 2D PCO including all chemical compounds from adults of six social groups with ≥5 SCG samples

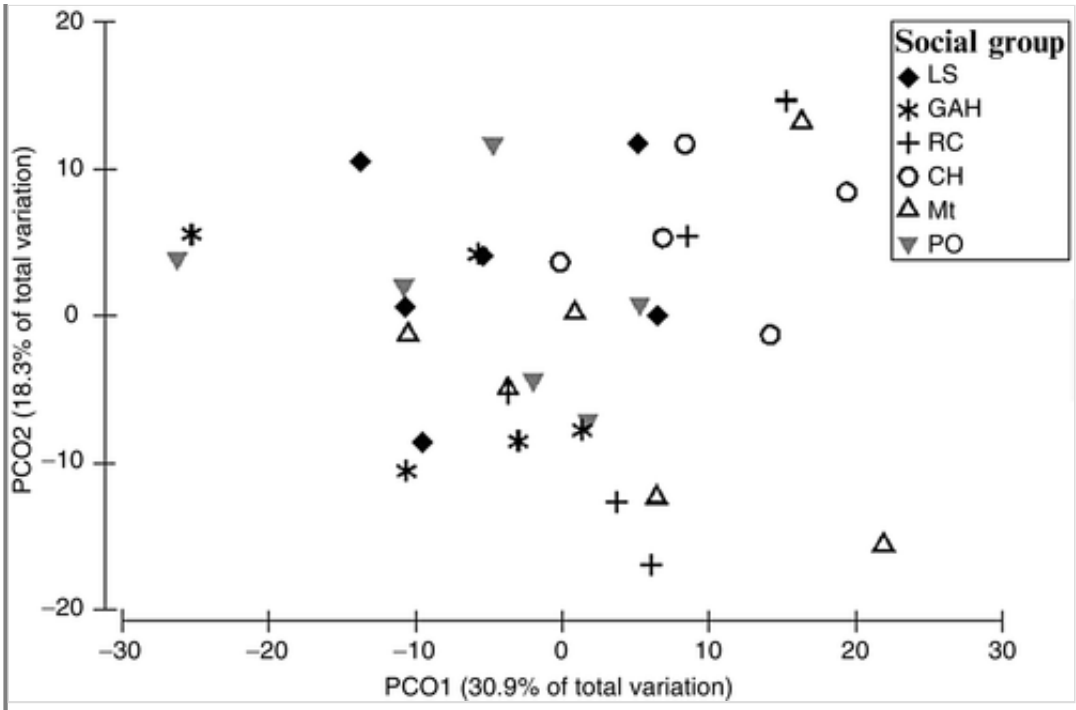


Table 5.1

PERMANOVA results for pairwise comparisons between the chemical profiles and the microbiota of members of six different social groups (significant results highlighted in bold)

	Chemistry				Microbiota	
	All compounds included		Only compounds shared by all group members included		TRFs	
Group	***t***	**P(perm)**	***t***	**P(perm)**	***t***	**P(perm)**
LS vs. GAH	0.9084	0.5719	3.0404	**0.0013**	1.0487	0.3468
LS vs. RC	1.2738	0.1096	3.6784	**0.0035**	0.94561	0.4736
LS vs. CH	1.6147	**0.0281**	4.4595	**0.0025**	0.59353	0.6728
LS vs. Mt	1.3406	0.1041	3.5608	**0.0026**	1.052	0.313
LS vs. PO	0.9924	0.4065	3.4059	**0.0022**	0.64851	0.6137
GAH vs. RC	1.1914	0.1737	3.3558	**0.0018**	0.88187	0.6028
GAH vs. CH	1.5818	**0.0175**	4.2611	**0.0016**	0.7999	0.6341
GAH vs. Mt	1.1415	0.2353	4.5744	**0.0004**	1.6775	**0.0478**
GAH vs. PO	0.99478	0.4149	3.1995	**0.0008**	0.46955	0.8486

RC vs. CH	1.1533	0.1965	3.4795	**0.0094**	0.91271	0.5374
RC vs. Mt	0.91766	0.555	3.2032	**0.002**	1.5689	0.076
RC vs. PO	1.1016	0.2727	2.3542	**0.0036**	0.99276	0.4195
CH vs. Mt	1.1669	0.2369	3.9912	**0.0026**	0.96919	0.4436
CH vs. PO	1.441	**0.0535**	3.3926	**0.0021**	0.75341	0.5956
Mt vs. PO	0.93082	0.4903	3.0985	**0.0029**	1.239	0.1921

Fig. 5.2

CAP analysis including all chemical compounds from adults of six social groups with ≥5 SCG samples. Note that the figure only shows 2 CAP axes of the 15 axes generated in the model

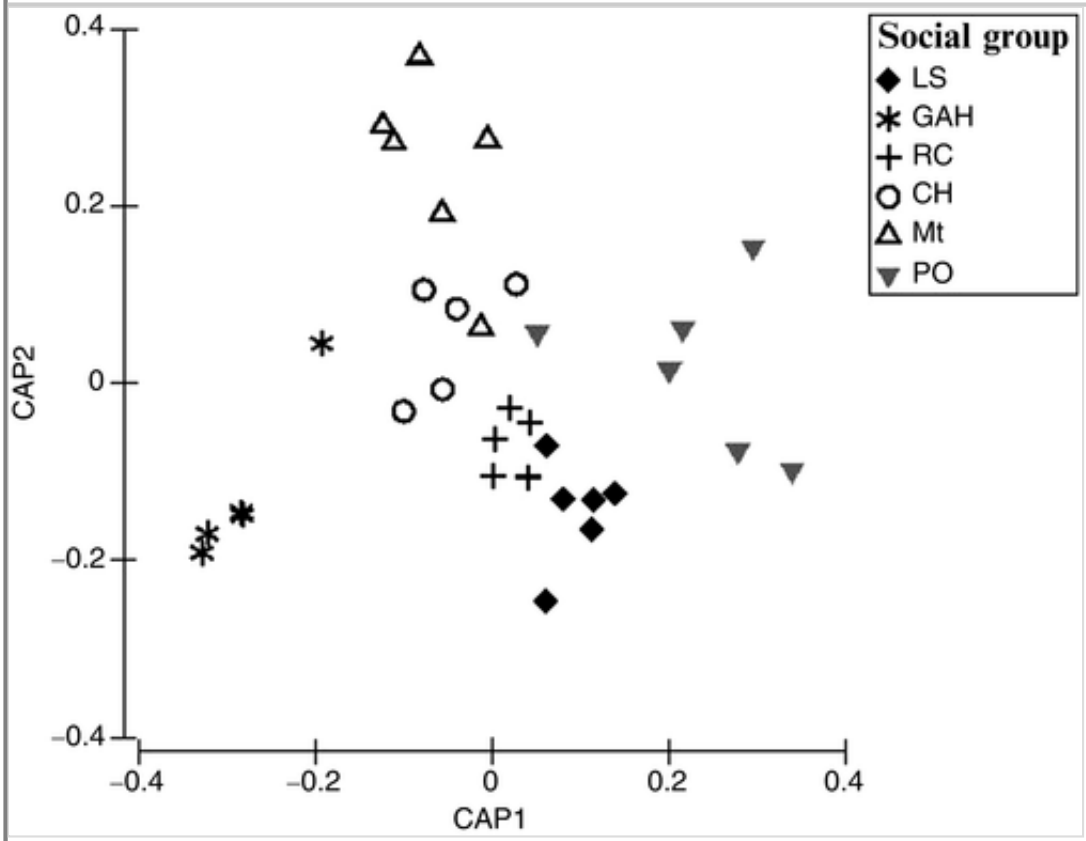


Table 5.2

Cross-validation results for correct classification of samples for each social group

Social group	RC	PO	LS	GAH	CH	Mt	Total	% correctly identified
RC	0	1	1	1	2	0	5	0

PO	0	2	2	0	0	2	6	33.33
LS	1	1	3	0	1	0	6	50
GAH	1	0	2	1	0	1	5	20
CH	1	0	1	0	1	2	5	20
Mt	0	0	1	0	1	4	6	66.67

However, if only compounds shared by all members in each group were included (*sensu* scent signature: Wyatt 2010), the unconstrained 2D PCO explained 54.8 % of the variation in the data (Fig. 5.3), and the third axis explained a further 18.1 %, with separations between social groups. The PERMANOVA comparing the six groups was highly significant (pseudo $F = 12.39$, $P = 0.0001$), as were all pairwise comparisons (Table 5.1). The CAP analysis classified 100 % of the chemical profiles into the correct group using $m = 5$ axes ($\delta_1^2 = 0.98934$, $P = 0.0001$, Fig. 5.4).

Fig. 5.3

Unconstrained 2D PCO including only chemical compounds which were shared by all adults belonging to the same social group (six social groups with ≥5 SCG samples)

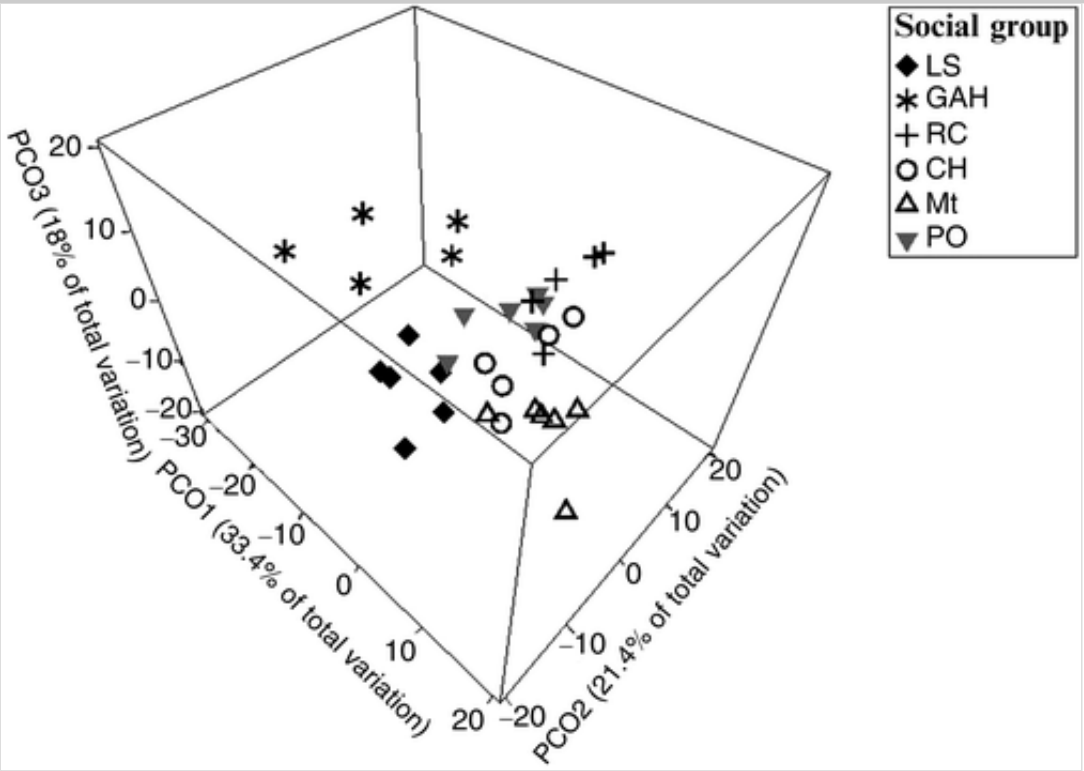
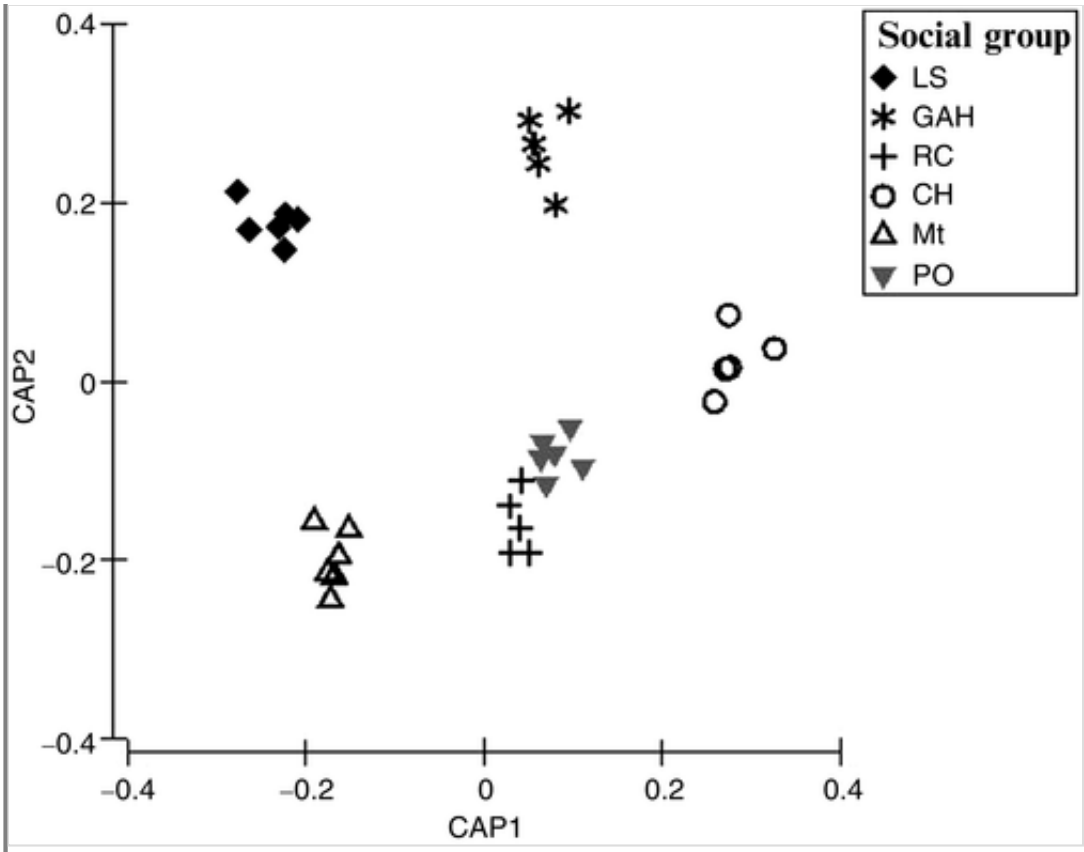


Fig. 5.4

CAP analysis including only chemical compounds which were shared by all adults belonging to the same social group (six social groups with ≥5 SCG samples). Note that the figure only shows 2 CAP axes of the 4 axes generated in the model

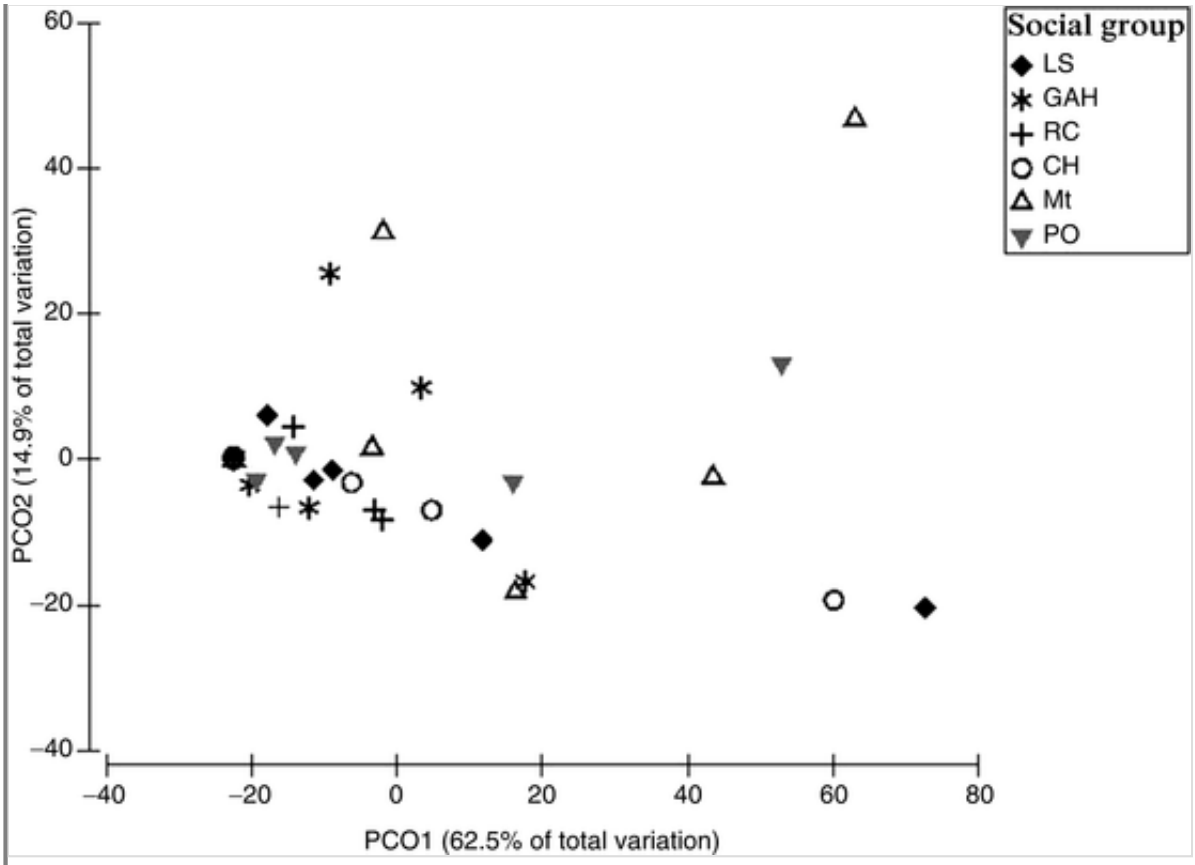


5.3.2. Group Differences in Microbiota

Comparing the TRFs to investigate variation in microbiota among groups, an unconstrained 2D PCO explained 82.3 % of the variation in the data (Fig. 5.5), and the third axis explained further 12.2 %, but there was no separation between social groups, and no significant differences in the TRFs of the 6 social groups were found (PERMANOVA: pseudo $F = 1.007$, $P = 0.4317$, Table 5.1).

Fig. 5.5

Unconstrained 2D PCO comparing the TRFs from 6 social groups with ≥5 adults sampled



5.3.3. Correlation of Microbiota and Chemical Composition of Subcaudal-Gland Secretions

In total, 50 TRFs and 125 different chemical compounds were found in the GC-MS profiles of 66 subcaudal-gland secretions. Using 9999 permutations, the BEST procedure showed an overall significant correlation between microbiota and chemical composition (Rho = 0.228, $P = 0.041$), with a subset of four TRFs (99.2, 138.9, 194.2, and 212.0) best explaining the structure in the chemical matrix (Table 5.3).

Table 5.3

Correlation of microbiota and chemical components in subcaudal gland secretions (BEST procedure using 9999 per

Retention time	Compound	99.16	132.39	133.68	138.94	140.42	141.69	160.58	189.3	194
7.93	Benzaldehyde				**0.60**	**−0.36**				
9.30	Limonene				**0.56**					
10.92	2-Chloroethylbenzene									
10.97	Benzeneethanol	**0.37**			**0.30**					
12.08	Octanoic acid[a]						**0.31**			
14.60	Badger_SGS_14.6	**0.39**								
15.33	Badger_SGS_15.325									

15.40	1,2,3,4-Tetrahydro-1,1,1-trimethylnaphthalene					**−0.39**				
15.45	Decanoic acid[a]	**0.30**					**0.31**			
15.76	Badger_SGS_15.756	**0.46**				**−0.34**				**−0.**
16.03	Badger_SGS_16.032					**0.33**				
16.98	Badger_SGS_16.975									
17.44	Badger_SGS_17.443	**0.49**								
17.77	Badger_SGS_17.77	**0.35**								
18.01	2-Methylundecanoic acid									**−0.**
18.17	Badger_SGS_18.168				**0.36**					
18.74	Dodecanoic acid[a]	**0.41**								
18.92	Badger_SGS_18.922	**0.35**								
19.67	Badger_SGS_19.672									
20.55	Badger_SGS_20.548	**0.45**								
20.77	Badger_SGS_20.765	**0.55**								
20.98	Badger_SGS_20.981	**0.56**								
21.13	Badger_SGS_21.129									
21.27	Badger_SGS_21.267				**0.35**					
21.32	Tetradecanoic acid, methyl ester[a]									
21.56	Badger_SGS_21.556									
21.76	Badger_SGS_21.760			**−0.33**						
21.82	Badger_SGS_21.821		**0.41**						**0.32**	
22.11	Tetradecanoic acid[a]	**0.46**								
22.47	Badger_SGS_22.466									
22.57	Badger_SGS_22.566									
22.70	Badger_SGS_22.701					**0.31**				
23.00	Badger_SGS_23.000									
23.05	Badger_SGS_23.054	**0.43**								
23.06	Badger_SGS_23.060	**0.35**								

23.69	Badger_SGS_23.690				**0.50**					
26.02	Badger_SGS_26.021					**0.33**				
28.57	Linoleic acid									
28.85	Linoleic acid, ethyl ester							**0.32**		
28.96	Oleic acid, ethyl ester							**0.40**		
29.03	Badger_SGS_29.025				**0.56**					
29.40	Octadecanoic acid, ethyl ester							**0.65**		
31.65	4-Octadecanolide									

99.16 = Alphaproteobacteria/Betaproteobacteria; 132.39 Unknown OTU; 133.68 = Firmicutes/Alphaproteobacteria; 13
140.42 = Actinobacteria; 141.69 = Unknown OTU; 160.58 = Gammaproteobacteria/Betaproteobacteria/Actinobacteria;
189.3 = Firmicutes; 194.15 = Unknown OTU; 208.23 = Bacteroidetes; 211.97 = Firmicutes

[a]Marks components validated through co-injection of standards

In the investigation of correlations between specific TRFs and chemical compounds, 5 TRFs and 23 chemical compounds showed a correlation > 0.4. All correlations were statistically significant ($P < 0.01$, Table 5.3).

5.4. Discussion

Most mammals communicate a wide variety of information in their olfactory signals (Brown and Macdonald 1985; Müller-Schwarze 2006). Some species, such as the European badger, encode individual-specific information as well as group membership, seasonality, and age of the scent mark in a single scent mark (Buesching and Macdonald 2001). Whereas some of this information, such as individuality, is static and should thus remain stable over time, other signals are transient (e.g., information pertaining to reproductive receptivity) or need to be pliable (e.g., information pertaining to group membership) to accommodate changes in biology and behavior. As olfactory signals are partly dependent on primary gland products, but also on environmental factors (Wyatt 2010), precise management of the synergistic relationships between endo- and exogenous factors involved in olfactory signal creation is paramount for reliable communication.

The fermentation hypothesis predicts that mammalian scent profiles are heavily affected by bacteria metabolizing the primary gland products excreted by the individual (Albone et al. 1978, Albone and Perry 1975, Gorman 1976). In our population, TRF (Sin et al. 2012) as well as GC-MS (Gorman et al. 1984, Buesching et al. 2002a, b) profiles were indeed individual specific, indicating that many individual advertisement signals might in fact be affected by pouch bacteria (Buesching et al. 2003). Here, we found a significant correlation between the microbiota in the subcaudal pouch and the chemical composition of the subcaudal gland secretion. A subset of four TRFs (99.2, 138.9, 194.2, and 212.0) explained most of the effect. Sin et al. (2012) found TRFs 138.94 to belong to phylum Actinobacteria,

and 97.6 and 211.97 to phylum Firmicutes (no phylum could be assigned from cloning and sequencing for TRF 194.2). Actinobacteria and Firmicutes include several well-known odor producers, and are also abundant in the paste of spotted hyenas (*Crocuta crocuta*: Theis et al. 2012, 2013). Several genera in these phyla produce a diverse array of short- and medium-chain fatty acids, which are prominent in the subcaudal gland secretion of badgers (Buesching et al. 2002a). Some Actinobacteria have been found to play a major role in the transformation of odorless steroids into odorous derivatives (e.g., Gower et al. 1986, Kohl et al. 2001). It is thus highly likely that bacteria belonging to these phyla will affect the chemical composition in the secretion, but further research using advanced 16SrRNA or metagenomic techniques is needed to confirm these associations.

In addition, our GC-MS results confirm the presence of shared group odors in this badger population. Nevertheless, while Buesching et al. (2002a) found clear group differences in samples collected from the same population 15 years prior to the present study also when all components detected by the GCMS were included in the analyses, in the present study group differences were obvious only if analyses were restricted to those components present in all members of the same social group. SGC profiles are chemically highly complex and have been reported to contain up to 58 of a possible ≥110 components, of which many occur only very rarely (Buesching et al. 2002a). By limiting our analyses to those compounds, which occurred at least in all members of one (or several) social group(s), we could increase statistical power considerably while simultaneously ensuring that biologically meaningful components were included. Although scent provision experiments have confirmed in a variety of species that individuals can discriminate between their own scent, scent of other members of their own group and scent from members of other social groups (e.g., badgers: Palphramand and White 2007; Bodin et al. 2006), few studies have tried to determine, what indicators animals utilize in this context, although learning of specific scent signatures appears important (Wyatt 2010). There is some evidence that it might be the case of recognizing components which are present also in their own scent/other members of their own group vs. components which are specific to a given different social group (Natynczuk and Macdonald 1994). Pairwise comparisons revealed significant differences between the groups, but the high number of axes necessary to separate all groups in the statistical analyses indicates that the coding of a specific group scent is complex and multidimensional. Although dispersal rates remain low in this population (Macdonald et al. 2008), and overall population density remained comparatively stable (Macdonald et al. 2009), individuals now move more frequently between social groups (i.e. excursions and visits to neighbors: Macdonald et al. 2008, Noonan et al. 2014) and extra-territorial matings appear to have increased from an estimated 42 % (Dugdale et al. 2007) to 48 % (Annavi et al. 2014). These changes appear to be associated with less pronounced group differences in odor profiles. Similar plasticity in group odors has also been reported in other species (e.g., elephants: Goodwin et al. 2012; meercats: Leclaire et al. 2014). Nevertheless, by restricting group-specific information to the relative amounts and ratios of a subset of components, other chemical compounds can be utilized effectively to encode other information, e.g., relating to individuality.

Interestingly, however, we found no group-specific differences in the microbiota, neither in the composition nor between any pair of social groups as determined by TRFs. Although this could indicate that other factors, such as genetic relationships (e.g., Todrank et al. 1998), are more important in creating the group odor in badgers, the sensitivity of the method applied in this study restricted the sensitivity of our analyses as the number of OTUs exceeded the numbers of TRFs, and a single TRF can comprise several OTUs, thus limiting resolution (Sin et al. 2012). In contrast, Theis et al. (2013) used a high-throughput sequencing approach to analyze the bacterial communities inhabiting the scent organs of spotted hyenas, which afforded a far more detailed analysis and allowed for the most comprehensive view of the bacterial communities inhabiting any specialized mammalian scent gland to date. As it is likely that the variation in metabolic activity is found at the species, subspecies, or even strain level in badgers, high-throughput sequencing can be expected to reveal more subtle differences in the microbial

communities between social groups.

References

Albone E (1984) Mammalian semiochemistry: the investigation of chemical signals between vertebrates. Wiley, Chichester

Albone ES, Perry GC (1975) Anal sac secretion of the red fox, *Vulpes vulpes*; volatile fatty acids and diamines: implications for a fermentation hypothesis of chemical recognition. J Chem Ecol 2:101–111

Albone ES, Eglinton G, Walker JM, Ware GC (1974) The anal sac secretion of red fox (*Vulpes vulpes*); its chemistry and microbiology. A comparison with the anal sac secretion of the lion (*Panthera leo*). Life Sci 14:387–400

Albone ES, Gosden PE, Ware GC, Macdonald DW, Hough NG (1978) Bacterial action and chemical signalling in the red fox (*Vulpes vulpes*) and other mammals. In: Bullard RW (ed) Flavour chemistry of animal foods, vol 67, ACS Symposium Series. American Chemical Society, Washington, DC, pp 78–91

Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. Austral Ecol 26:32–46

Anderson MJ, Robinson J (2003) Generalized discriminant analyses based on distances. Aust N Zeal J Stat 45:301–318

Anderson MJ, Willis TJ (2003) Canonical analysis of principal coordinates: a useful method of constrained ordination for ecology. Ecology 84:511–525

Anderson MJ, Gorley RN, Clarke KR (2008) PERMANOVA+ for PRIMER: guide to software and statistical methods. PRIMER-E, Plymouth

Annavi G, Newman C, Dugdale HL, Buesching CD, Sin YW, Burke T, Macdonald DW (2014) Neighbouring-group composition and within-group relatedness drive extra-group paternity rate in the European badger (*Meles meles*). J Evol Biol 27:2191–2203

Archie EA, Theis KR (2011) Animal behaviour meets microbial ecology. Anim Behav 82:425–436

Bodin C, Benhamou S, Poulle ML (2006) What do European badgers (*Meles meles*) know about the spatial organisation of neighbouring groups? Behav Processes 72:84–90

Brown RE, Macdonald DW (eds) (1985) Social odours in mammals, vol Volume I and Volume II. Clarendon, Oxford

Buesching CD, Macdonald DW (2001) Scent-marking behaviour of the European badger (*Meles meles*): resource defence or individual advertisement. In: Marchlewska-Koj A, Lepri JL, Müller-Schwarze D (eds) Chemical signals in vertebrates 9. Kluwer Academic, New York, pp 321–327

Buesching CD, Macdonald DW (2004) Variations in object-marking activity and over-marking

behaviour of European badgers (*Meles meles*) in the vicinity of their setts. Acta Theriol 49:235–246

Buesching CD, Waterhouse JP, Macdonald DW (2002a) Gas chromatographic analysis of the subcaudal gland secretion of the European badger (*Meles meles*) Part I: Chemical differences related to individual-specific parameters. J Chem Ecol 28:41–56

Buesching CD, Waterhouse JP, Macdonald DW (2002b) Gas chromatographic analysis of the subcaudal gland secretion of the European badger (*Meles meles*) Part II: Time-related variation in the individual-specific composition. J Chem Ecol 28:57–69

Buesching CD, Newman C, Macdonald DW (2002c) Variations in colour and volume of the subcaudal gland secretion of badgers (*Meles meles*) in relation to sex, season and individual-specific parameters. Z Säugetierk 67:1–10

Buesching CD, Stopka P, Macdonald DW (2003) The social function of allo-marking behaviour in the European badger (*Meles meles*). Behaviour 140:965–980

Buyers JA (1985) Olfaction related behaviour in peccaries. Z Tierpsychol 70:201–210

Clarke KR, Gorley RN (2006) PRIMER v6: user manual/tutorial. PRIMER-E, Plymouth

Clarke KR, Warwick RM (2001) Change in marine communities, 2nd edn. PRIMER-E, Plymouth

Clarke KR, Somerfield PJ, Gorley RN (2008) Testing of null hypotheses in exploratory community analyses: similarity profiles and biota-environment linkage. J Exp Mar Biol Ecol 366:56–69

Delahay RJ, Brown JA, Mallinson PJ, Spyvee PD, Handoll D, Rogers LM, Cheeseman CL (2000) The use of marked bait in studies of the territorial organization of the European badger (*Meles meles*). Mam Rev 30:73–87

Dugdale HL, Macdonald DW, Pope LC, Burke T (2007) Polygynandry, extra-group paternity and multiple-paternity litters in European badger (*Meles meles*) social groups. Mol Ecol 16:5294–5306

Ezenwa VO, Williams AE (2014) Microbes and animal olfactory communication: Where do we go from here? Bioessays 36:847–854

Ferkin MH, Sorokin ES, Johnston RE, Lee CJ (1997) Attractiveness of scents varies with protein content of the diet in meadow voles. Anim Behav 53:133–141

Gangestad SW, Thornhill R (1998) Menstrual cycle variation in women's preferences for the scent of symmetrical men. Proc Roy Soc B 265:927–933

Goodwin TE, Broederdorf LJ, Burkert BA, Hirwa IH, Mark DB, Waldrip ZJ, Kopper RA, Sutherland MV, Freeman EW, Hollister-Smith JA, Schulte BA (2012) Chemical signals of elephant musth: temporal aspects of microbially-mediated modifications. J Chem Ecol 38:81–87

Gorman ML (1976) A mechanism for individual recognition by odour in *Herpestes auropunctatus* (Carnivora: Viverridae). Anim Behav 24:141–145

Gorman ML, Kruuk H, Leitch A (1984) Social functions of the sub-caudal scent gland secretion of the European badger (*Meles meles*) (Carnivora: Mustelidae). J Zool Lond 203:549–559

Gower JC (1966) Some distance properties of latent root and vector methods used in multivariate analyses. Biometrika 53:325–338

Gower DB, Nixon A, Jackman PJH, Mallett AI (1986) Transformation of steroids by axillary coryneform bacteria. Int J Cosmet Sci 8:149–158

Greenwood PJ (1980) Mating systems, philopatry and dispersal in birds and mammals. Anim Behav 28:1140–1162

Hurst JL, Fang J, Barnard CJ (1993) The role of substrate odours in maintaining social tolerance between male house mice, *Mus musculus domesticus*. Anim Behav 45:997–1006

James AG, Austin CJ, Cox DS, Taylor D, Calvert R (2013) Microbiological and biochemical origins of human axillary odour. FEMS Microbiol Ecol 83:527–540

Johnson DD, Macdonald DW, Dickman AJ (2000) An analysis and review of models of the sociobiology of the Mustelidae. Mamm Rev 30:171–196

Kelliher KR (2007) The combined role of the main olfactory and vomeronasal systems in social communication in mammals. Horm Behav 52:561–570

Kohl JV, Atzmueller M, Fink B, Grammer K (2001) Human pheromones: Integrating neuroendocrinology and ethology. Neuro Endocrinol Lett 22:309–321

Kruuk H (1989) The social badger: ecology and behaviour of a group-living carnivore (*Meles meles*). Oxford University Press, Oxford

Leclaire S, Nielsen JF, Drea CM (2014) Bacterial communities in meerkat anal scent secretions vary with host sex, age, and group membership. Behav Ecol 25:996–1004

Macdonald DW (1985) The carnivores: order Carnivora. In: Brown M, Macdonald DW (eds) Social odours in mammals, vol 2. Clarendon, Oxford, pp 619–722

Macdonald DW, Newman C (2002) Badger (*Meles meles*) population dynamics in Oxfordshire, UK, Numbers, Density and Cohort life histories, and a possible role of climate change in population growth. J Zool 256:121–138

Macdonald DW, Newman C, Buesching CD, Johnson PJ (2008) Male-biased movement in a high-density population of the Eurasian badger (*Meles meles*). J Mammal 89:1077–1086

Macdonald DW, Newman C, Nouvellet PM, Buesching CD (2009) An analysis of Eurasian badger (*Meles meles*) population dynamics: Implications for regulatory mechanisms. J Mammal 90:1392–1403

Mardon J, Saunders SM, Anderson MJ, Cochoux C, Bonadonna F (2010) Species, gender, and identity: cracking petrels' sociochemical code. Chem Senses 35:309–321

McArdle BH, Anderson MJ (2001) Fitting multivariate models to community data: a comment on distance-based redundancy analysis. Ecology 82:290–297

Müller-Schwarze D (2006) Chemical ecology of vertebrates. Cambridge University Press, Cambridge

Muyzer G, De Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59: 695-700

Muyzer G, Teske A, Wirsen CO, Jannasch HW (1995) Phylogenetic relationships ofThiomicrospira species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arc Microbiol 164: 165-172

Nakagawa S (2004) A farewell to Bonferroni: the problems of low statistical power and publication bias. Behav Ecol 15:1044–1045

Natynczuk SE, Macdonald DW (1994) Scent, sex, and the self-calibrating rat. J Chem Ecol 20:1843–1857

Noonan MJ, Markham A, Newman C, Trigoni N, Buesching CD, Ellwood SA, Macdonald DW (2014) Climate and the individual: inter-annual variation in the autumnal activity of the European badger (*Meles meles*). PLoS One 9, e83156

Osada K, Yamazaki K, Curran M, Bard J, Smith BP, Beauchamp GK (2003) The scent of age. Proc Roy Soc Lond 270:929–933

Őstborn H (1976) Doftmarkering hos graveling. Zool Revy 38:103–112

Palphramand KL, White PC (2007) Badgers, *Meles meles*, discriminate between neighbour, alien and self scent. Anim Behav 74:429–436

Penn DJ (2002) The scent of genetic compatibility: sexual selection and the major histocompatibility complex. Ethology 108:1–21

Roper TJ (2010) Badger. Collins, London

Sin YW, Buesching CD, Burke T, Macdonald DW (2012) Molecular characterization of the microbial communities in the subcaudal gland secretion of the European badger (*Meles meles*). FEMS Microbiol Ecol 81:648–659

Smith CJ, Danilowicz BS, Clear AK, Costello FJ, Wilson B, Meijer WG (2005) T-Align, a web-based tool for comparison of multiple terminal restriction fragment length polymorphism profiles. FEMS Microbiol Ecol 54:375–380

Stübbe M (1971) Die analen Markierungsorgane des Dachses (*Meles meles)*. Zool Garten NF 40:125–135

Theis KR, Schmidt TM, Holekamp KE (2012) Evidence for a bacterial mechanism for group-specific

social odors among hyenas. Sci Rep 2:615

Theis KR, Venkataraman A, Dycus JA, Koonter KD, Schmitt-Matzen EN, Wagner AP, Holekamp KE, Schmidt TM (2013) Symbiotic bacteria appear to mediate hyena social odors. Proc Natl Acad Sci U S A 110:19832–19837

Thornton PD, Newman C, Johnson PJ, Buesching CD, Baker SE, Slater D, Johnson DDP, Macdonald DW (2005) Preliminary comparison of four anaesthetic regimes in badgers (*Meles meles*). Vet Anaesth Analg 32:40–47

Todrank J, Heth G, Johnston RE (1998) Kin recognition in golden hamsters: evidence for kinship odours. Anim Behav 55:377–386

Woodley SK, Baum MJ (2003) Effects of sex hormones and gender on attraction thresholds for volatile anal scent gland odors in ferrets. Horm Behav 44:110–118

Wyatt TD (2010) Pheromones and signature mixtures: defining species-wide signals and variable cues for identity in both invertebrates and vertebrates. J Comp Physiol 196:685–700

Zechman JM, Martin IG, Wellington JL, Beauchamp GK (1984) Perineal scent gland of wild and domestic cavies: bacterial activity and urine as sources of biologically significant odors. Physiol Behav 32:269–274