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# Evaluating the functional, sexual and seasonal variation in the chemical constituents from feces of adult Iberian wolves (Canis lupus signatus)

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# Abstract

Chemical signals play an important role in intraspecific and interspecific communication of many mammals, We described the chemicals found in fresh feces of adult wolves by means of analyses using gas chromatography-mass spectrometry (GC-MS) of samples collected from wild breeding groups. We identified 56 compounds in the feces, mainly heterocyclic aromatic organic compounds such as indole or phenol, but also steroids, such as cholesterol, carboxylic acids and their esters between n-$C_4$ and n-$C_{18}$, aldehydes, alcohols and significant quantities of squalene and α-tocopherol, which would increase the chemical stability of feces on humid substrates. All samples visually identified as wolves were subsequently identified to species level by sequencing a small fragment of mtDNA and sexed typing DBX6 and DBY7 sex markers. There is variability in the number and proportions of compounds between sexes, which could be indicative of their function as chemical signals. We also found variability in different reproductive states, especially in odorous compounds, steroids and α-tocopherol. Feces with a presumed marking function had higher proportions of α-tocopherol and steroids than feces with non-marking function. Therefore, these compounds could be involved in intragroup and intergroup communication of wolves and their levels could be directly related with the wolf's physiological status.


# Introduction

Chemical signals play a very important role in intraspecific and interspecific communication of many animal species [1–5]. In many mammals, different semiochemicals are incorporated into feces, urine or other scent marks, so that they remain on the substrate with the aim of scent-marking the boundaries of their territory or attracting potential mates for reproduction [6, 7]. For a carnivore mammal, placing scent marks in its environment is very important for several reasons; in this way they can delimit their territory and, thus maintain the separation between individuals and groups [8], reaffirm the possession of resources [9, 10], and signal their social status [11, 12] or a certain physiological, reproductive or emotional state [13, 14]. In addition, these odorous signals may help individuals to orient themselves within their own territories or simply make them feel safe therein [15]. In many carnivores, there is a close association between dominance and scent marking, and in some species, such as the wolf (*Canis lupus*), only high-ranking individuals, that is, the alpha pair, show marking behavior with urine [16, 17] and feces [12], through which they continuously signal their social status to the rest of the group members [18].

Chemical communication plays an important role in the social organization and spatial distribution of wolves [12, 15, 19–21]. Indeed, they largely depend on smell, which allows them to acquire information about their environment and communicate within the group and with other groups [22, 23]. Wolves invest their time and energy in an odorous marking strategy that guarantees the maximum probability of detection of fecal signals [19, 24]. They deposit their feces and urine on visually conspicuous and elevated substrates, which improve their function as marks [15–17, 20, 24, 25]. In addition, feces are accumulated in strategic sites, such as crossroads where the probability of being detected by other congeners is greater [19]. In wild populations, it has been documented that the Iberian wolf selects plants as a substrate to deposit the

feces according to their species and size (diameter and height) [24]. The plants marked with greater diameter and height make the deposited feces more effective as visual signals, and also, when they are higher they increase their active space, which facilitates the dispersion of the smell by the wind and the increase of the surface of evaporation [24].

The studies in which the fecal volatile compounds were analyzed have reaffirmed the function of feces as chemical signals, due to the large number of chemical compounds found in them. These include a high proportion of aromatic organic heterocyclic compounds (ie, with benzene rings), aldehydes, low-weight fatty acids and alcohols, which are strongly odoriferous compounds [26]. These results confirmed the important role that feces play in chemical communication in the wolf. Previous studies have also identified similar volatile compounds in the feces of domestic dogs [27].

Many of the volatile compounds found in the feces originate in the secretions of the anal sacs and are later incorporated into the stool during defecation [18], and these chemical compounds have also been identified in the secretions of the anal sacs of wolves [28, 29]. However, there are many other compounds that have been identified in the secretions of the anal sacs of the wolf but not in the feces. Martín et al. [26] indicated that this may be due to the highly volatile nature of these compounds, which could have evaporated from the feces before the samples were collected, due to their exposure to environmental conditions.

Feces can serve as a substrate to deposit the secretions of the anal sacs, but these are not deposited in all the excrements, being present in less than 10% of the feces. The secretions of the anal sacs can be deposited independently to the defecation [30, 31]. Thus, the functions of the fecal marks and the secretions of the anal sacs can be independent, or be used in different contexts, which suggest a double role in chemical communication. The secretions of the anal sacs deposited independently of the feces could act as a warning signal, while the feces accompanied by secretions from the anal sacs seem to be used in territorial marking [30].

The abundance of some odoriferous compounds present in the feces could be explained as the result of bacterial action on the components of food, instead of being products from the anal sacs [26]. Indole could result from the bacterial degradation of the amino acid tryptophan, phenol from the transformation of the amino acid tyrosine while benzaldehyde from benzoic acid [32]. In addition, too many fatty acids can be derived from microbial fermentation. However, the non-glandular origin of these compounds does not preclude these chemical substances being used with a signaling function. Many mammalian pheromones are produced by the action of bacterial fermentation, such as the secretions of the anal glands of foxes [33] and wolves [28]. In fact, many compounds in the anal sacs of wolves decrease or disappear from secretions after antibiotic treatment [28].

The high molecular weight compounds present in feces are more stable and, therefore, they could play an important role in the long-term chemical communication of many mammals. Thus, the odor signal will

remain longer than in the low molecular weight compounds in which the volatility is greater, such as the present in urine and secretions of the anal sacs [28, 29, 34].

The feces of young wolves and adults are clearly different, not only because the feces of adults have a greater number of volatile compounds, but also the proportions of the shared compounds are very different. Adults have a greater abundance of volatile aromatic compounds and fatty acids, which are absent in the feces of the offspring [26]. These chemical compounds probably come from anal glands that are still undeveloped in pups [28]. For the offspring, it is not necessary to incorporate many volatile compounds into the feces because they do not fulfill a function as chemical signals. Among the compounds shared by adults and pups, indole and phenol, the main components in the feces of all wolves, are more abundant in the feces of pups. These differences could allow wolves to clearly differentiate feces from pups by chemosensory signals, in addition to visual differences in size [26].

Adult males, especially alpha males, deposit anal secretions more frequently when defecating than females or juveniles [30]. Thus, the volatile compounds present in the feces seem to function as very important chemical signal in the intraspecific communication of the wolf, giving information about sex, age, endocrine status and their individual identity [28].

In this study, we aimed to examine several unknown aspects of the function of fecal scent marks as chemical signals in the Iberian wolf (*Canis lupus signatus*). The compounds present in 96 wolf fecal samples were analyzed to evaluate the following hypotheses:

1. There are sexual differences in the presence and abundance of volatile compounds in the feces of adult wolves.
2. The presence and abundance of these compounds varies in the feces depending on the reproductive status of the individuals.
3. Only the excrements with a presumed marking function (on conspicuous substrates, above ground level, at crossroads or as re-marking) play a role in the chemical communication of the wolves.

# Material And Methods

### Study area and collection of fecal samples

The study was carried out in two mountain areas in northwestern Spain, Natural Park Os Montes do Invernadeiro and its surroundings (Ourense prov.; 42°07'52"N / 7°19'09"O; 880-1700 m.a.s.l.) and Sierra de la Culebra (Zamora prov.; 41°53'54"N / 6°20'01"O; 800-1200 m.a.s.l). Vegetation in these areas is mainly composed by predominant mixed scrub with different species of heath (*Erica* spp.) and brooms (*Genista florida*, *Cytisus scoparius*), and other bushes, and small oak forests (*Quercus pyrenaica*) with birch (*Betula celtiberica*) and holly (*Ilex aquafolium*), often replaced by reforestation of conifers (*Pinus pinaster* and *P. sylvestris*). The areas have a high density of wild ungulates (red deer, roe deer, wild boar)

and carnivores, specially holding the highest density of wolves in the Iberian Peninsula and throughout Western Europe.

We collected fecal samples from five wolf-breeding groups; four in the Sierra de la Culebra and one in the Natural Park Os Montes do Invernadeiro. In each group, fresh feces of adult wolves were collected monthly, from May 2007 to December 2008, along forest tracks and firebreaks that were frequently transited by wolves. The fecal samples were collected without handling the animals. To discriminate which group the fecal samples belonged to, the pathways prospected to collect the feces were established in each group in the vicinity of the rendezvous sites, since the wolves defend territorial groups and there is no overlap between them [35, 36]. The rendezvous sites were located at the beginning of the study and were located in the center of the territory where there was a great activity of pups and adults (footprints, excrements, bony remains of prey, trampling of vegetation, tracks, etc.), which facilitated the collection of fresh feces and decreased the likelihood of confusion with the excrements of other carnivores. Nevertheless, to discriminate the feces of wolves from those of other species of sympatric carnivores (fox, wildcat and European marten) their size and shape were taken into account, not collecting samples from feces with a diameter of less than 2.5 cm and a length less than 25 cm. Moreover, despite all these precautions, the feces collected were analyzed by molecular techniques to identify the species and sex (see below).

The transects were inspected in an off-road vehicle twice a day, one at dawn and the other at dusk, so the time from deposition to collection of feces was less than 12 h. In addition, during the study only very fresh feces were collected, those that had a mucosal cuticle (it appears only in the newly deposited excrements and dried quickly after the deposition), a strong smell and showed no signs of dehydration. Moreover, wolves show their peaks of greatest activity at dawn and dusk [36]. Thus, the collection of fecal samples at this time ensured that the time elapsed since the deposition was short, thus minimizing the losses of volatile compounds due to exposure to environmental factors [37].

Feces found were classified into two groups, a) feces deposited with a possible function of marking in intraspecific visual and chemical communication and b) feces deposited by the wolves as simple excretions. Feces were considered to have a marking function when deposited on conspicuous substrates (plants, rocks, trunks, etc.), above ground level, at crossroads and/or over feces of conspecifics (over-marking). We considered that a substrate was conspicuous when it was the most outstanding of all within a 2 m radius circle around the excrement [12, 15, 20, 24]. The rest of the substrates were considered non-conspicuous. In addition, feces were considered as marking cues if they occurred on a substrate >4 cm above ground level [15, 38] and at an intersection where two or more trails crossed [20]. It was considered that there was over-marking or re-marking when the wolves defecated over one or several previous older fecal marks [39]. We collected around 10 g of each fresh excrement and stored it in a portable refrigerator plugged into the lighter of the car and loaded with ice until reaching the laboratory freeze where it was kept at -20ºC until further analyses.

### Specific and sexual identification using molecular techniques

In order to reliably verify that the visually identified samples correspond to wolves, we conducted a species identification step consisting of sequencing mitochondrial DNA (mtDNA) control regionon a subsample of the faecal samples collected in the field. These analyses ensured the origin of the fecal samples collected and avoided confusion with feces of other sympatric carnivores.

We collected samples of each excrement in tubes with 96% ethanol and stored them at -20º C until processed. The extraction of DNA from fecal samples was carried out using an extraction kit based on silica membranes and adapted to non-invasive samples (QIAamp DNA Stool Mini Kit, Qiagen), following the manufacturer's guidelines.

To determine the specific origin of the fecal samples, a 440 bp fragment of the mitochondrial DNA control region was sequenced following the methodology described in Vilá et al. [40]. The experimental part consisted in the amplification of DNA using the PCR technique (Polymerase Chain Reaction) and the use of primers Thr-L 15926 and DL-H 16340 [40] and in its subsequent sequencing through the application of the commercial kit dRhodamine Terminator Cycle Sequencing Ready Reaction (Applied Biosystems), in an automatic sequencer ABI PRISM Model 3130 (Applied Biosystems). The success of the DNA amplification was verified by gel electroforesis. The cleaning and purification of the amplified product was carried out according to the combined method of alkaline phosphatase and exonuclease I (ExoSAP-IT®) developed by Amersham Biosciences, to eliminate the primers and the excess of deoxynucleotides that could interfere in the subsequent sequencing reaction.

Species assignment was made thanks to the comparison of the sequences obtained with reference sequences of dogs and wolves obtained in previous studies [40-45] and with those deposited in the GenBank databases for different mammalian species (http://www.ncbi.nlm.nih.gov/) and using the BLAST 2.0 program (http://www.ncbi.nlm.nih.gov/BLAST/).

To determine the sex of the samples identified as produced by wolves, we used the method described by Seddon [46], designed specifically for sexual determination in fecal samples. To this end, two specific canine markers were amplified using the PCR technique: the DBX intron6 (249 bp), which identifies the X chromosome in males and females, and the DBY intron7 (118 bp) that identifies the Y chromosome in males. The success of the DNA amplification was verified by the electrophoretic migration of the amplified product in 1.5% agarose gels. We identified as males those samples that presented the bands corresponding to the X and Y chromosomes, and as females those samples that exclusively presented the band corresponding to the X chromosome. As there are several problems associated with the low quantity and quality of DNA extracted from scats, all samples were processed in duplicate. Samples whose identification by agarose gel was doubtful and all female samples were also genotyped with two replicates using an automatic sequencer (ABI PRISM 3130, Applied Biosystems). For the visualization and detection of the fragments corresponding to the X and Y chromosomes, the program GENEMAPPER version 4.0 (Applied Biosystems) was used.

## Chemical analyses of volatiles in feces

We transferred a small amount of each fecal sample to a chromatography glass vial to which 250 µl of n-hexane was added (Sigma, capillary GC grade). Each vial was closed with a Teflon-lined stopper before mixing the solution for 1 min using a vortex. Thereafter, the vial was placed in a fridge for 10 min to rest until the solid material that was not dissolved precipitated at the bottom of the vial. We extracted the supernatant clear liquid phase with a glass syringe and transferred it to a clean vial closed with a Teflon-lined stopper. We also made blank control vials using the same procedure, but without adding fecal material, to compare with the wolf samples. Thus, we were able to exclude contaminants from the handling procedure or from the environment and to detect potential impurities in the solvent.

To analyze samples, we used a Finnigan-ThermoQuest Trace 2000 gas chromatograph (GC) fitted with a poly (5% diphenyl/95% dimethylsiloxane) column (Supelco, Equity-5, 30 m length, 0.25 mm ID, 0.25 mm film thickness) and a Finnigan-ThermoQuest Trace 2000 mass spectrometer (MS) as detector. We used helium at 30 cm/s as the carrier gas. We injected 2 µl of each sample in splitless mode with an inlet temperature of 250 ºC. The oven of the GC was programmed so that the temperature was kept initially at 45 ºC for 15 min, and then increased at a rate of 5 ºC/min until a final temperature of 280 ºC, which was kept for 15 min. Ionization by electron impact (70 eV) was carried out at 250 ºC. We did not record mass spectral fragments below m/z = 39.

The initial tentative identification of the volatile compounds in the fecal samples was carried out by comparing the fragmentation patterns (i.e., mass spectra) of the compounds detected in the samples with those available in the NIST/EPA/NIH 2010 mass spectral library. When possible, the identification was confirmed by comparing the spectra and retention times with those obtained under the same conditions of the analysis using authentic standards (from Sigma-Aldrich Chemical Co). Impurities identified in the control vial samples are not reported.

## Statistical analysis

All fecal samples collected were of unknown origin with respect to the individual that had produced. To minimize pseudoreplication and avoid bias in the study due to a small number of different prospective individuals, five wolf breeding groups whose group sizes ranged between 6 and 14 individuals (I. Barja, data unpublished) were followed. The group size was obtained by direct observation of the groups at dusk and dawn. Likewise, the alpha pair of each group is the only one that reproduces, and the rest of the members collaborate in the breeding, providing food for the pups and the female when they are in the den and in the rendezvous sites (cooperative breeding) [36, 47]. Therefore, in the access lanes to these zones, we can often find several fresh excrements belonging to different individuals, thus ensuring that the collection of samples does not distort our results.

The relative amount of each chemical compound was determined as the percentage of the area of its peak in the chromatogram in relation to the total area occupied by all the peaks (TIC area), excluding contaminants. For this, the integration capacity of the peak areas available in the software Xcalibur (Finningan Co.) was used. For statistical analyses, the relative proportions of each compound were

transformed following the formula: log[(proportion)/(1- proportion)], to correct the problem of non-independence between proportions [48].

The software PRIMER V6.1.13 [49] and PERMANOVA + V1.0.3 [50] were used to test for differences between the chemical profiles. We calculated the Euclidean distances between every pair of individual samples and produced a resemblance matrix that was the basis of further analyses. We used permutational multivariate variance analyses (PERMANOVA)[51] based on the Euclidean resemblance matrix, using 999 permutations, to analyse whether chemical profiles of the fecal samples varied between sexes, reproductive status of the individuals (not reproductive vs. reproductive vs. breeding) and in relation to the presumable marking function of feces. Pairwise post-hoc comparisons were made with permutation tests. Differences were further investigated using canonical analyses of principal coordinates (CAP, [52]).To determine which compounds differed between categories (sex, reproductive condition, marking function), we used the transformed areas of the compounds that appeared in at least five samples to make a principal component analysis (PCA) with a varimax normalized rotation. The extracted principal components (PCs) were used as new variables to compare categories using one-way analyses of variance (ANOVA). Post-hoc multiple comparisons were made using Tukey's tests [53]. We further used discriminant analyses to test whether a given sample could be assigned to a given category based on the compounds which PC scores differed significantly between these categories. Statistical analyses were performed using the software Statistica 7.0 (StatSoft Inc., Tulsa, OK).

# Results

### Specific and sexual identification

Out of the 73 fecal samples analyzed by molecular methods, 67 samples were successfully sexed (42 males and 25 females). In addition, after sequencing a 440 bp mtDNA fragment from fecal DNA, we effectively identified 56 samples as wolf. All the sequences obtained in this study matched with sequences published in previousstudies [40-45] and are available from Dryad repository, as well as their correspondence with the published sequences (see Data availabilitysection). Thus, unequivocal species identification was possible in 76.7 % of thesamples, with 17 non genetically identified feces (23.3%).In no case did the genetic analyses indicate that any of the fecal samples came from another species of carnivore found in the study area different to wolves.Also, as all fecal samples were collected in the vicinity of the rendezvous sites and their morphology matched that of wolf feces, we have considered all the 94 samples collected for the chemical analyses.

### Chemicals in feces of adult wolves

We found a total of 56 lipophilic compounds in 94 fresh feces of adult Iberian wolves (Table 1). The main compounds were 11 aromatic heterocyclic compounds (37.6% of the TIC), 24 carboxylic acids and their esters between n-$C_4$ and n-$C_{20}$ (22.3%) and 8 steroids (21.7%). In addition, we also found 5 aldehydes (6.3%), squalene (6.0%),α-tocopherol (3.7%) and other minor compounds, such as two alcohols (0.8%),

two amides (0.8%), cyclic octaatomic sulfur (0.7%) and a ketone (0.02%) (Table 1). On average, the five most abundant compounds were indole (28.5%), cholesterol (11.5%), squalene (6.0%), hexadecanoic acid (5.9%) and phenol (5.0%).

The number of compounds identified in a single fecal sample ranged between 2 and 43 (mean± SE=16±1 compounds/fecal sample). All major compounds (>5%) were detected in most samples, although the presence and relative proportions of some chemicals show a high inter-sample variability.

The PCA analysis of the transformed areas of all the compounds, extracted 6 principal components (PCs) with eigenvalues greater than two, which together accounted for 47.6% of the variance (Table 2). The correlations of the relative proportions of the volatile compounds with the PCs are shown in Table 2.

## Chemicals compounds in feces of males and females

Males and females had similar lipophiliccompounds, although four minor compounds were not found in one of the sexes. The main classes of compounds of males and females were also similar, but proportions were different (Table 1). Males had heterocyclic aromatic organic compounds (39.7%), steroids (24.6%) and carboxylic acids and their esters (19.6%), whereas females had heterocyclic aromatic organic compounds (48.2%), steroids (15.6%) and carboxylic acids and their esters (13.3%). The five most abundant compounds of males were indole (28.1%), cholesterol (12.9%), hexadecanoic acid (6.5%), cholest-4-en-3-one (5.7%) and phenol (5.1%), whereas the main compounds of females were indole (36.5%), squalene (8.0%), cholesterol (6.8%), phenol (5.1%), and α-tocopherol (5.2%) (Table 1).

The PERMANOVA analysis based on the resemblance matrix comparing samples of each sex showed significant differences in the overall proportion of compounds between males and females (pseudo $F_{1,65}$=5.69, P=0.02). Also, results from the PCA showed that there were significant intersexual differences in the compounds described byPC4 (ANOVA, $F_{1,65}$=11.14, P=0.014; Fig. 1), but not in the other PCs (ANOVA, at least P>0.15 in all cases). Thus, according to correlations of the compounds with the PCs, males had higher levels of steroids such as cholesterol and cholesta-4-en-3-one than females (Table 1, Fig. 1). A discriminant analysis based on compounds described by PC4 alone assigned correctly the sex of 79% of feces of males and 31% of feces of females (Wilks'λ=0.85, $F_{1,65}$=11.30, P=0.013).

## Seasonal differences

The PERMANOVA analysis based on the resemblance matrix comparing samples of each season showed that there were significant differences in the overall proportion of compounds among the three seasons (pseudo $F_{2,91}$=1.58, P=0.037). However, pairwise permutational post-hoc tests showed that there were significant differences between the reproductive and the non-reproductive season (P=0.01) and between the reproductive and the breeding seasons (P=0.039), but there were not significant differences between the breeding and the non-reproductive seasons (P=0.51). The CAP analysis assigned 49% of the chemical profiles into the correct season using Euclidean distances between samples (permutational test, $\delta_1^2$=0.49, P=0.043, using leave-one-out cross-validation and m=30 axis).

A further two-way PERMANOVA restricted to samples that could be sexed confirmed that there were significant overall seasonal differences and significant differences between sexes independently of the seasonal variation (season: pseudo $F_{2,61}$=2.15, P=0.002; sex: pseudo $F_{1,61}$=1.89, P=0.027; season x sex: pseudo $F_{2,61}$=0.78, P=0.79).

The analysis of seasonal variation in the PCs resulting from the PCA of compounds showed that there were significant seasonal differences in the compounds described byPC4 (ANOVA, $F_{2,91}$=4.28, P=0.017) and by PC3 (ANOVA, $F_{2,91}$=4.45, P=0.014) (Figs. 2A y B), but not in the other PCs (ANOVA, P>0.25 in all cases). Thus, during the reproductive season there were significant lower relative proportions of indole but significant higher relative proportions of hexanal and several fatty acids (PC3) and of cholesterol andα-tocopherol (PC-4) than during the non-reproductive season (Tukey's tests, P=0.02 for both PCs) and the breeding season (P<0.05 for both PCs) (Table 3). These two seasons did not differ significantly between them (P>0.78 for both PCs). A discriminant analysis based on compounds described by PC3 and PC4 alone classified samples into the correct season for 44% of samples of the reproductive period and 92% of samples of the non-reproductive and breeding seasons (Wilks'λ=0.82, $F_{4,180}$=4.54, P=0.0016).

### Marking function

The PERMANOVA analysis based on the resemblance matrix comparing samples of feces deposited with a presumably marking function(i.e., feces left on conspicuous substrates, above ground level, at crossroads and/or remarking other feces)with those with a non-marking function (i.e., fecesthat were on inconspicuous substrates and/or at ground level, off crossroads, non-remarking)showed that there were not overall significant differences in the overall proportion of compounds between the two functions (pseudo $F_{1,90}$=0.74, P=0.79). Nevertheless, the PCA showed significant differences in the compounds described by the PC4(ANOVA, $F_{1,90}$=8.03, P=0.006; Fig. 3), but not in the other PCs (P>0.12 for all), suggesting that feces with a presumably marking function had higher proportions of α-tocopherol and cholesterol than scats with a no-marking function (Table 4). A discriminant analysis based on these compounds described by PC4 alone classified correctly 95% of samples from feces without a marking function, but only 18% of feces with a marking function (Wilks'λ=0.92, $F_{1,90}$=8.03, P=0.006).

## Discussion

Our results show that Iberian wolf feces (*Canis lupus signatus*) contain a wide variety of chemical compounds, among which there was a large proportion of very odorous compounds such as aromatic heterocyclic organic compounds, aldehydes, low molecular weight fatty acids and alcohols [26], which reaffirm the role that the feces play in the chemical communication of wolves. Among these compounds, we found a significant proportion of squalene and α-tocopherol in the feces. These compounds are lipophilic fixatives and antioxidants [54] that could stabilize the other lipid fractions by decreasing oxidation and, therefore, increasing the chemical stability of feces on wet substrates [37]. In addition, the differences in the proportion of α-tocopherol in feces could be related to the diet, endocrine status or

condition of the individual. The dominant wolves are the ones that feed first on the captured prey and consume the best parts of the prey, being forced the subordinates to consume lower quality food, less rich in proteins, and in scarce circumstances to fast [35]. In addition, dominant wolves are the only individuals that breed within each group and those that are responsible for marking the territory [12, 36]. The α-tocopherol is a chemical compound of dietary origin, and studies carried out with reptiles showed how individuals with a higher quality diet secreted higher proportions of vitamin E in their chemical signals [55]. This suggests that the presence of this compound in the secretions involved in chemical communication is expensive for individuals and therefore depends on the quality of the same. In some insects the nutritional status of males affects the quality of their pheromones in the attraction of a couple [56, 57] and has been seen as in the mountain lizard (*Iberolacerta montícola*) increases the quality of their femoral secretions when supplementing their diet with vitamin D [58].

The fecal marks of males and females were clearly different, feces of males presented a greater number of chemical compounds, and the relative proportions of common compounds also differ from each other. The males presented more volatile aromatic compounds and fatty acids that were absent in the feces of the females. These differences could allow wolves to clearly differentiate the sex of individuals by chemosensory stimuli. Among the shared compounds, α-tocopherol and some steroids such as cholesterol, cholesta-4-en-3-one and cholesta-3,5-diene, are relatively more abundant in feces of males. This suggests that these compounds may be related to reproduction, since males must signal their endocrine status and condition for attracting potential mates and, in addition, it is the dominant male of each group that shows the highest frequency of marking [39].

Variations were also observed in the number and proportion of compounds present in the feces of the wolves in their different reproductive stages. When the adult wolves were not in the reproductive period, the number of volatile compounds present in the feces, such as some aldehydes and fatty acids and the relative proportion of steroids and α-tocopherol, decreased, but that of indole increased. The indole was present in all wolf feces, including those from pups [26]. These results could suggest that outside the reproductive period individuals would not be interested in investing in signalling their physiological status, therefore they would decrease those more expensive compounds that play a major role in the chemical communication.

The results obtained support our hypothesis that not all feces are deposited with a presumed marking function. In those feces that we previously classified as olfactory-visual marks based on the physical characteristics of the substrate and its disposition in the territory, which increase the visibility and detection by residents and intruders wolves [24, 59], we found relative higher proportions of α-tocopherol and steroids, than in the rest of feces without a marking function. Therefore, it seems that when feces were deposited with a signaling function, individuals would invest in secreting more expensive compounds but with a clear function in chemical communication.

The relative proportions of α-tocopherol (= vitamin E) increased during the reproductive period and were also higher in those feces that had a presumed marking function as odor signals and were more

abundant in males than in females. All of these results suggest that α-tocopherol plays an important role in the intragroup and intergroup communication of the wolves and may have three main functions. First, α-tocopherol is a very useful antioxidant, so high levels of it in feces can be indicative of the quality of individuals (good nutrition, good health, etc.). In ocellated lizards (*Timon lepidus*) the release of high levels of vitamin E in the secretions is directly related to the quality of the immune system of males [26]. Second, it may be related to reproduction and partner search, as suggested by the high levels of this compound during the reproductive season. Females could use this feature to assess not only the individual quality of the male, but also to indirectly estimate the quality of the territory occupied by that male, that is, the quality of the available food [60] and might show preference for areas marked with signals that contain high levels of vitamin E. Finally, the α-tocopherol could not really be important as a signaling compound directly in the feces, but its antioxidant nature would increase the duration and intensity of the information provided by other compounds present in the secretions [37]. It is likely that only males with a good individual quality can afford to invest high concentrations of vitamin E in their secretions and be able to maintain their territory and attract females. This could suggest that the secretion of this compound with feces entails a cost for individuals, conferring reliability on the olfactory marks which would allow their evolution as sexual signals.

In summary, the differences between sexes in the relative proportion of certain aromatic compounds, reaffirms the function of feces as olfactory marks in wolves. The greater proportion of certain compounds in those feces deposited on conspicuous substrates, above ground level, at crossroads or re-marking, with respect to those that do not, reaffirm the theory that not all feces are deposited with a possible signaling function. The presence of high levels of α-tocopherol in males, in the breeding season and in those feces deposited with a presumed marking function, suggests its possible function as an indicator of the individual “quality” of wolves or their territories, their function as a sexual signal or as a preservative and amplifier of the signal of other compounds in the secretion. However, all this requires future studies that reveal the role that vitamin E plays in the communication of the wolves.

# Declarations


### Acknowledgements

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### Data availability

The DNA sequences generated in the current study are available in a .fasta file from Dryad repository at: [https://datadryad.org/stash/share/eKtAUkzHwCOV8DabtamcGDRHJpuw4vIK19i4x0-wCRI]. A table listing the faecal samples included in the molecular analyses and their specific and sexual identification is also provided in the same repository.

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# Tables

**Table 1.** Relative proportion (mean ± SE) of lipophilic compounds found in hexane extracts of feces of all adult Iberian wolves (*Canis lupus signatus*), and of males and females separately in fecal samples from which sex was determined.

| RT (min) | Compound | All samples (n=94) | Males (n=42) | Females (n=25) |
|---|---|---|---|---|
| 5.3 | Butanoic acid, ethyl ester | 0.20 ± 0.04 | 0.14 ± 0.06 | 0.19 ± 0.10 |
| 5.4 | Hexanal | 0.08 ± 0.03 | 0.17 ± 0.02 | 0.01 ± 0.01 |
| 6.3 | Butanoic acid | 1.10 ± 0.31 | 1.28 ± 0.49 | 1.60 ± 0.73 |
| 8.9 | 3-Methyl butanoic acid | 0.43 ± 1.12 | 0.74 ± 0.37 | 0.92 ± 0.33 |
| 9.8 | 2-Methyl butanoic acid | 0.33 ± 0.10 | 0.67 ± 0.29 | 0.55 ± 0.27 |
| 10.0 | Pentanoic acid, ethyl ester | 0.06 ± 0.04 | 0.17 ± 0.17 | 0.01 ± 0.01 |
| 13.1 | Pentanoic acid | 0.10 ± 0.05 | - | 0.43 ± 0.28 |
| 16.0 | Benzaldehyde | 0.03 ± 0.02 | 0.08 ± 0.07 | 0.04 ± 0.03 |
| 19.5 | Phenol | 4.98 ± 0.66 | 5.06 ± 1.29 | 5.66 ± 1.39 |
| 24.1 | 4-Methyl phenol (=p-cresol) | 0.68 ± 0.23 | 1.48 ± 0.76 | 0.65 ± 0.30 |
| 28.7 | 2-Piperidinone | 0.53 ± 0.15 | 0.91 ± 0.40 | 1.05 ± 0.58 |
| 29.2 | Quinoline | 2.32 ± 0.47 | 2.83 ± 0.77 | 3.33 ± 1.31 |
| 31.2 | Indole | 28.47 ± 2.27 | 28.12 ± 4.13 | 36.51 ± 6.51 |
| 31.9 | 1,2,3,4-Tetrahydro-quinoline | 0.10 ± 0.06 | 0.09 ± 0.06 | - |
| 32.2 | Benzenepropanoic acid, ethyl ester | 0.14 ± 0.08 | 0.48 ± 0.29 | 0.10 ± 0.05 |
| 33.7 | Benzenepropanoic acid | 0.18 ± 0.07 | 0.43 ± 0.22 | 0.38 ± 0.23 |
| 36.5 | 1,3-Dihydro-2H-indol-2-one | 0.11 ± 0.05 | 0.20 ± 0.12 | 0.36 ± 0.20 |
| 38.2 | Dodecanoic acid | 0.08 ± 0.03 | 0.12 ± 0.11 | 0.16 ± 0.08 |
| 38.6 | Tetradecanal | 0.05 ± 0.02 | 0.03 ± 0.02 | 0.10 ± 0.07 |
| 40.5 | 2-Pentadecanone | 0.02 ± 0.01 | 0.04 ± 0.03 | 0.05 ± 0.02 |
| 40.9 | Pentadecanal | 0.15 ± 0.05 | 0.03 ± 0.02 | 0.26 ± 0.15 |
| 41.8 | 2,4-Dihydroxy-3,6-dimethyl-benzoic acid, methyl ester | 0.09 ± 0.04 | 0.05 ± 0.03 | 0.08 ± 0.05 |
| 42.3 | Tetradecanol | 0.06 ± 0.04 | 0.10 ± 0.08 | 0.03 ± 0.02 |
| 42.4 | Tetradecanoic acid | 1.02 ± 0.31 | 1.73 ± 1.03 | 1.41 ± 0.63 |
| 42.9 | Hexadecanal | 3.38 ± 0.49 | 2.84 ± 0.97 | 2.95 ± 1.31 |
| 43.9 | Pentadecanoic acid, ethyl ester | 0.01 ± 0.01 | - | - |

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| 44.3 | Hexadecanol | 0.73 ± 0.20 | 1.21 ± 0.58 | 1.63 ± 0.53 |
| 44.4 | Pentadecanoic acid | 0.03 ± 0.01 | 0.04 ± 0.03 | - |
| 44.7 | Hexedecenoic acid, methyl ester | 0.01 ± 0.01 | - | - |
| 45.1 | Hexadecanoic acid, methyl ester | 0.02 ± 0.01 | 0.03 ± 0.02 | - |
| 45.9 | Hexadecenoic acid, ethyl ester | 0.61 ± 0.14 | 0.43 ± 0.21 | 0.40 ± 0.29 |
| 46.0 | Hexadecenoic acid | 1.55 ± 0.58 | 0.71 ± 0.27 | 0.21 ± 0.15 |
| 46.3 | Hexadecanoic acid, ethyl ester | 0.64 ± 0.25 | 0.14 ± 0.09 | 0.09 ± 0.06 |
| 46.5 | Hexadecanoic acid | 5.92 ± 1.23 | 6.50 ± 2.76 | 4.90 ± 2.86 |
| 46.9 | Octadecanal | 2.65 ± 0.32 | 2.48 ± 0.59 | 3.31 ± 1.16 |
| 48.2 | Cyclic octaatomic sulfur | 0.67 ± 0.20 | 0.64 ± 0.39 | 1.22 ± 0.47 |
| 48.3 | 9-Octadecenoic acid, methyl ester | 0.57 ± 0.24 | 0.53 ± 0.49 | - |
| 48.8 | Octadecanoic acid, methyl ester | 0.02 ± 0.01 | 0.02 ± 0.02 | - |
| 49.4 | 9,12-Octadecadienoic acid, ethyl ester | 0.95 ± 0.30 | 0.70 ± 0.24 | 0.45 ± 0.30 |
| 49.5 | 9-Octadecenoic acid, ethyl ester | 3.51 ± 0.85 | 3.03 ± 1.31 | 0.56 ± 0.25 |
| 49.9 | Octadecanoic acid, ethyl ester | 0.16 ± 0.05 | 0.11 ± 0.07 | 0.01 ± 0.01 |
| 50.3 | Octadecenamide | 0.01 ± 0.01 | 0.02 ± 0.02 | 0.04 ± 0.02 |
| 49.9 | 9-Octadecenoic acid | 4.79 ± 1.37 | 2.45 ± 2.39 | 1.35 ± 1.24 |
| 52.1 | 5,8,11,14-Eicosatetraenoic acid, ethyl ester | 0.13 ± 0.06 | 0.03 ± 0.03 | 0.08 ± 0.08 |
| 52.6 | 3-Hydroxy-octadecanoic acid, methyl ester | 0.06 ± 0.03 | 0.08 ± 0.08 | 0.01 ± 0.01 |
| 53.1 | Nonadecanamide | 0.81 ± 0.25 | 1.19 ± 0.58 | 0.04 ± 0.02 |
| 59.2 | Squalene | 6.00 ± 1.12 | 3.19 ± 0.59 | 8.03 ± 3.57 |
| 60.4 | Cholesta-4,6-dien-3-ol | 0.89 ± 0.61 | 0.55 ± 0.21 | 0.30 ± 0.12 |
| 60.7 | Cholesta-3,5-diene | 1.06 ± 0.17 | 1.43 ± 0.31 | 2.31 ± 0.64 |
| 63.3 | Cholestanol | 0.26 ± 0.13 | 0.59 ± 0.45 | 0.05 ± 0.05 |
| 63.7 | α-Tocopherol | 3.75 ± 0.47 | 4.05 ± 0.99 | 5.22 ± 1.55 |
| 64.0 | Cholesterol | 11.47 ± 1.22 | 12.92 ± 1.99 | 6.81 ± 2.04 |
| 64.6 | Cholestan-3-one | 1.42 ± 0.36 | 2.11 ± 1.13 | 1.05 ± 0.65 |
| 66.1 | Cholest-4-en-3-one | 4.03 ± 0.56 | 5.67 ± 1.48 | 3.32 ± 1.09 |

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| 66.7 | Cholesta-4,6-dien-3-one | 0.64 ± 0.17 | 0.43 ± 0.21 | 0.15 ± 0.06 |
| 68.8 | Unidentified steroid (189, 203, 218, 313, 409, 424) | 1.94 ± 0.38 | 0.89 ± 0.34 | 1.61 ± 1.16 |

The relative amount of each component was determined as the percent of the total ion current (TIC). Characteristic ions (m/z) are reported for an unidentified compound. RT: retention time.

**Table 2.** Principal components analysis (PCA) for compounds found in feces of Iberian wolves (*Canis lupus signatus*). Correlations between variables (compounds) and the principal components significant at $P < 0.00001$ are marked in bold.

| Compound | PC-1 | PC-2 | PC-3 | PC-4 | PC-5 | PC-6 |
|---|---|---|---|---|---|---|
| Butanoic acid, ethyl ester | 0.28 | 0.32 | -0.01 | -0.02 | 0.01 | 0.01 |
| Hexanal | 0.02 | -0.04 | **0.49** | -0.05 | 0.08 | -0.18 |
| Butanoic acid | **0.75** | 0.10 | 0.43 | 0.02 | 0.01 | 0.09 |
| 3-Methyl-butanoic acid | **0.82** | -0.04 | 0.06 | 0.08 | 0.15 | 0.11 |
| 2-Methyl-butanoic acid | **0.67** | 0.04 | 0.06 | 0.08 | 0.15 | 0.11 |
| Pentanoic acid, ethyl ester | 0.05 | **0.47** | 0.18 | -0.14 | -0.06 | -0.08 |
| Pentanoic acid | 0.30 | -0.03 | 0.09 | -0.12 | 0.16 | 0.36 |
| Benzaldehyde | 0.39 | 0.01 | 0.28 | 0.04 | **0.71** | -0.13 |
| Phenol | 0.23 | **-0.52** | -0.13 | 0.19 | 0.03 | -0.04 |
| 4-Methylphenol (=p-cresol) | **0.61** | -0.15 | -0.08 | -0.01 | 0.08 | 0.15 |
| 2-Piperidinone | **0.59** | 0.01 | **0.49** | 0.05 | 0.25 | 0.12 |
| Quinoline | **0.47** | -0.39 | 0.02 | -0.02 | 0.01 | 0.04 |
| Indole | 0.07 | -0.27 | **-0.75** | -0.08 | -0.20 | 0.02 |
| 1,2,3,4-Tetrahydro-quinoline | **0.58** | -0.03 | 0.01 | 0.11 | -0.11 | -0.35 |
| Benzenepropanoic acid, ethyl ester | **0.65** | -0.03 | -0.12 | 0.07 | 0.14 | 0.10 |
| Benzenepropanoic acid | **0.70** | 0.07 | 0.09 | 0.08 | 0.27 | 0.26 |
| 1,3-Dihydro- 2H-indole-2-one | **0.58** | -0.05 | 0.06 | 0.01 | **0.49** | 0.32 |
| Dodecanoic acid | 0.32 | 0.03 | **0.54** | 0.01 | **0.61** | -0.09 |
| Tetradecanal | 0.36 | 0.06 | 0.33 | -0.01 | 0.16 | **0.68** |
| 2-Pentadecanone | 0.08 | 0.04 | 0.14 | 0.11 | **0.59** | 0.41 |
| Pentadecanal | 0.41 | -0.01 | 0.21 | 0.05 | **0.68** | 0.10 |
| 2,4-Dihydroxy-3,6-dimethyl-benzoic ac. methyl ester | **0.58** | -0.14 | -0.10 | 0.08 | 0.34 | -0.27 |
| Tetradecanol | 0.42 | -0.04 | -0.14 | 0.11 | 0.35 | **0.53** |
| Tetradecanoic acid | 0.19 | 0.07 | **0.72** | 0.07 | 0.27 | 0.24 |
| Hexadecanal | 0.01 | 0.14 | 0.07 | 0.29 | -0.08 | 0.09 |
| Pentadecanoic acid, ethyl ester | -0.01 | 0.23 | 0.06 | -0.1 | -0.23 | 0.02 |
| Hexadecanol | 0.38 | 0.01 | -0.11 | 0.02 | 0.47 | 0.21 |
| Pentadecanoic acid | -0.08 | -0.08 | **0.72** | 0.03 | 0.01 | -0.03 |

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|---|---|---|---|---|---|---|
| Hexadecenoic acid, methyl ester | -0.01 | 0.10 | 0.20 | 0.06 | 0.04 | -0.23 |
| Hexadecanoic acid, methyl ester | 0.03 | 0.36 | 0.15 | 0.08 | 0.22 | -0.24 |
| Hexadecenoic acid, ethyl ester | -0.14 | **0.56** | 0.18 | 0.20 | 0.22 | -0.04 |
| Hexadecenoic acid | 0.34 | -0.02 | **0.53** | 0.17 | -0.06 | 0.02 |
| Hexadecanoic acid, ethyl ester | 0.16 | 0.42 | -0.17 | 0.19 | 0.04 | 0.17 |
| Hexadecanoic acid | 0.03 | 0.11 | **0.73** | 0.01 | 0.25 | 0.15 |
| Octadecanal | 0.01 | 0.03 | -0.05 | 0.28 | -0.23 | 0.10 |
| Cyclic octaatomic sulfur | 0.37 | 0.25 | -0.01 | 0.01 | 0.31 | 0.12 |
| 9-Octadecenoic acid, methyl ester | -0.15 | **0.64** | 0.18 | 0.16 | -0.04 | -0.02 |
| Octadecanoic acid, methyl ester | 0.01 | **0.62** | -0.05 | -0.03 | -0.03 | -0.04 |
| 9,12-Octadecadienoic acid, ethyl ester | -0.07 | **0.46** | 0.23 | 0.22 | 0.21 | 0.04 |
| 9-Octadecenoic acid, ethyl ester | 0.09 | **0.59** | 0.13 | 0.19 | 0.11 | 0.18 |
| Octadecanoic acid, ethyl ester | 0.05 | **0.66** | -0.03 | -0.13 | 0.01 | -0.06 |
| Octadecenamide | 0.24 | 0.07 | 0.19 | -0.01 | **0.73** | 0.09 |
| 9-Octadecenoic acid | 0.11 | 0.19 | **0.61** | 0.06 | -0.09 | 0.38 |
| 5,8,11,14-Eicosatetraenoic acid, ethyl ester | -0.14 | 0.01 | 0.20 | 0.02 | 0.08 | 0.13 |
| 3-Hydroxy-octadecanoic acid, methyl ester | 0.17 | -0.11 | 0.17 | 0.08 | 0.02 | **0.74** |
| Nonadecanamide | -0.04 | 0.27 | 0.13 | **0.46** | -0.05 | -0.02 |
| Squalene | 0.02 | 0.06 | -0.07 | 0.17 | -0.04 | 0.01 |
| Cholesta-4,6-dien-3-ol | 0.27 | 0.09 | -0.07 | 0.26 | 0.23 | 0.15 |
| Cholesta-3,5-diene | 0.27 | -0.16 | -0.05 | **0.56** | 0.15 | 0.21 |
| Cholestanol | -0.07 | 0.04 | -0.13 | 0.14 | 0.20 | -0.03 |
| α-Tocopherol | 0.25 | -0.13 | 0.07 | **0.53** | 0.06 | -0.06 |
| Cholesterol | 0.10 | 0.02 | 0.07 | **0.74** | -0.08 | -0.04 |
| Cholestan-3-one | -0.27 | 0.12 | 0.08 | 0.38 | 0.28 | 0.12 |
| Cholest-4-en-3-one | 0.02 | 0.14 | 0.11 | **0.62** | 0.06 | 0.03 |
| Cholesta-4,6-dien-3-one | -0.13 | 0.19 | 0.39 | 0.42 | 0.27 | 0.23 |
| Unidentif. steroid (189, 203, 218, 313, 409, 424) | -0.04 | 0.08 | 0.01 | -0.01 | 0.26 | -0.05 |
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|---|---|---|---|---|---|---|
| Eigenvalues | 10.39 | 5.42 | 3.47 | 2.78 | 2.36 | 2.21 |
| Explained variance (%) | 18.55 | 9.69 | 6.24 | 4.97 | 4.21 | 3.94 |

**Table 3.** Relative proportions (mean ± SE) of compounds in feces of Iberian wolves (*Canis lupus signatus*) depending on the reproductive status of individuals (B=breeding, NR= non-reproductive, R=reproductive).

| Compounds | B<br>N=41 | NR<br>N=26 | R<br>N=27 |
|---|---|---|---|
| Butanoic acid, ethyl ester | 0.15 ± 0.05 | 0.22 ± 0.08 | 0.24 ± 0.08 |
| Hexanal | 0.04 ± 0.02 | 0.01 ± 0.01 | 0.15 ± 0.09 |
| Butanoic acid | 1.20 ± 0.46 | 1.22 ± 0.82 | 0.67 ± 0.33 |
| 3-Methyl-butanoic acid | 0.55 ± 0.22 | 0.18 ± 0.10 | 0.24 ± 0.14 |
| 2-Methyl-butanoic acid | 0.35 ± 0.14 | 0.26 ± 0.13 | 0.42 ± 0.24 |
| Pentanoic acid, ethyl ester | 0.03 ± 0.02 | 0.00 ± 0.00 | 0.17 ± 0.16 |
| Pentanoic acid | 0.07 ± 0.06 | 0.14 ± 0.14 | - |
| Benzaldehyde | 0.06 ± 0.04 | 0.00 ± 0.00 | 0.01 ± 0.01 |
| Phenol | 5.14 ± 0.89 | 6.92 ± 1.69 | 2.47 ± 0.83 |
| 4-Methylphenol (=p-cresol) | 0.28 ± 0.14 | 1.59 ± 0.76 | 0.22 ± 0.17 |
| 2-Piperidinone | 0.66 ± 0.28 | 0.42 ± 0.30 | 0.48 ± 0.20 |
| Quinoline | 3.23 ± 0.91 | 1.77 ± 0.48 | 1.49 ± 0.84 |
| Indole | 28.83 ± 3.62 | 36.66 ± 4.61 | 19.74 ± 3.53 |
| 1,2,3,4-Tetrahydro-quinoline | 0.21 ± 0.15 | 0.02 ± 0.02 | 0.01 ± 0.01 |
| Benzenepropanoic acid, ethyl ester | 0.05 ± 0.03 | 0.21 ± 0.20 | 0.01 ± 0.01 |
| Benzenepropanoic acid | 0.17 ± 0.10 | 0.09 ± 0.07 | 0.29 ± 0.19 |
| 1,3-Dihydro- 2H-indole-2-one | 0.11 ± 0.08 | 0.03 ± 0.03 | 0.07 ± 0.05 |
| Dodecanoic acid | 0.07 ± 0.04 | - | 0.20 ± 0.11 |
| Tetradecanal | 0.06 ± 0.04 | 0.01 ± 0.01 | 0.06 ± 0.04 |
| 2-Pentadecanone | 0.03 ± 0.02 | - | 0.02 ± 0.01 |
| Pentadecanal | 0.22 ± 0.10 | - | 0.20 ± 0.10 |
| 2,4-Dihydroxy-3,6-dimethyl-benzoic acid methyl ester | 0.19 ± 0.09 | 0.02 ± 0.02 | - |
| Tetradecanol | 0.09 ± 0.08 | 0.07 ± 0.07 | 0.03 ± 0.02 |
| Tetradecanoic acid | 0.66 ± 0.29 | 0.83 ± 0.41 | 1.89 ± 0.92 |
| Hexadecanal | 3.25 ± 0.64 | 2.83 ± 0.84 | 4.48 ± 1.21 |
| Pentadecanoic acid, ethyl ester | 0.02 ± 0.01 | - | 0.01 ± 0.01 |

| | | | |
|---|---|---|---|
| Hexadecanol | 0.73 ± 0.30 | 0.74 ± 0.50 | 0.48 ± 0.27 |
| Pentadecanoic acid | 0.02 ± 0.02 | 0.03 ± 0.03 | 0.05 ± 0.02 |
| Hexadecenoic acid, methyl ester | 0.01 ± 0.01 | - | 0.02 ± 0.01 |
| Hexadecanoic acid, methyl ester | 0.02 ± 0.01 | - | 0.03 ± 0.02 |
| Hexadecenoic acid, ethyl ester | 0.53 ± 0.18 | 0.31 ± 0.19 | 1.09 ± 0.39 |
| Hexadecenoic acid | 0.37 ± 0.21 | 0.20 ± 0.11 | 4.81 ± 1.93 |
| Hexadecanoic acid, ethyl ester | 1.11 ± 0.57 | 0.23 ± 0.14 | 0.39 ± 0.15 |
| Hexadecanoic acid | 4.52 ± 1.66 | 5.87 ± 2.20 | 8.80 ± 2.90 |
| Octadecanal | 2.64 ± 0.49 | 2.44 ± 0.58 | 3.20 ± 0.68 |
| Cyclic octaatomic sulfur | 0.83 ± 0.39 | 0.25 ± 0.15 | 0.91 ± 0.39 |
| 9-Octadecenoic acid, methyl ester | 0.20 ± 0.09 | 1.02 ± 0.59 | 0.76 ± 0.61 |
| Octadecanoic acid, methyl ester | - | 0.05 ± 0.05 | 0.02 ± 0.02 |
| 9,12-Octadecadienoic acid, ethyl ester | 0.58 ± 0.20 | 0.76 ± 0.39 | 1.79 ± 0.98 |
| 9-Octadecenoic acid, ethyl ester | 5.01 ± 1.78 | 1.10 ± 0.53 | 3.91 ± 1.20 |
| Octadecanoic acid, ethyl ester | 0.17 ± 0.07 | 0.13 ± 0.06 | 0.19 ± 0.12 |
| Octadecenamide | 0.01 ± 0.01 | - | 0.03 ± 0.02 |
| 9-Octadecenoic acid | 5.26 ± 2.33 | 3.67 ± 2.36 | 5.70 ± 2.62 |
| 5,8,11,14-Eicosatetraenoic acid, ethyl ester | 0.15 ± 0.07 | - | 0.23 ± 0.18 |
| 3-Hydroxy-octadecanoic acid, methyl ester | 0.04 ± 0.04 | - | 0.15 ± 0.10 |
| Nonadecanamide | 0.37 ± 0.19 | 1.00 ± 0.58 | 1.40 ± 0.61 |
| Squalene | 5.88 ± 1.70 | 6.15 ± 2.43 | 3.46 ± 0.57 |
| Cholesta-4,6-dien-3-ol | 1.74 ± 1.43 | 0.28 ± 0.13 | 0.26 ± 0.15 |
| Cholesta-3,5-diene | 0.92 ± 0.23 | 1.25 ± 0.39 | 0.88 ± 0.26 |
| Cholestanol | 0.49 ± 0.28 | 0.03 ± 0.03 | 0.18 ± 0.18 |
| α-Tocopherol | 2.51 ± 0.54 | 3.91 ± 0.84 | 5.40 ± 1.19 |
| Cholesterol | 11.16 ± 1.64 | 10.57 ± 1.93 | 13.47 ± 3.07 |
| Cholestan-3-one | 1.45 ± 0.72 | 0.77 ± 0.35 | 2.09 ± 0.61 |
| Cholest-4-en-3-one | 3.71 ± 0.93 | 4.64 ± 0.98 | 4.35 ± 1.11 |

| | | | |
|---|---|---|---|
| Cholesta-4,6-dien-3-one | 0.43 ± 0.18 | 0.28 ± 0.14 | 1.37 ± 0.52 |
| Unidentified steroid (189, 203, 218, 313, 409, 424) | 3.44 ± 0.77 | 0.81 ± 0.37 | 0.98 ± 0.35 |

**Table 4.** Relative proportions (mean ± SE) of compounds in feces of Iberian wolves (*Canis lupus signatus*) depending on its presumably marking function in intraspecific communication.

| Compounds | Non-marking function (n=64) | Marking function (n=28) |
|---|---|---|
| Butanoic acid, ethyl ester | 0.20 ± 0.05 | 0.19 ± 0.08 |
| Hexanal | 0.02 ± 0.01 | 0.15 ± 0.09 |
| Butanoic acid | 1.13 ± 0.42 | 0.94 ± 0.44 |
| 3-Methyl-butanoic acid | 0.31 ± 0.13 | 0.49 ± 0.21 |
| 2-Methyl-butanoic acid | 0.29 ± 0.10 | 0.48 ± 0.25 |
| Pentanoic acid, ethyl ester | 0.09 ± 0.07 | 0.01 ± 0.01 |
| Pentanoic acid | 0.10 ± 0.07 | 0.02 ± 0.02 |
| Benzaldehyde | 0.03 ± 0.03 | 0.02 ± 0.02 |
| Phenol | 5.31 ± 0.86 | 3.96 ± 1.07 |
| 4-Methylphenol (=p-cresol) | 0.80 ± 0.33 | 0.28 ± 0.16 |
| 2-Piperidinone | 0.48 ± 0.17 | 0.72 ± 0.37 |
| Quinoline | 2.43 ± 0.61 | 2.10 ± 0.86 |
| Indole | 29.49 ± 2.93 | 27.48 ± 4.00 |
| 1,2,3,4-Tetrahydro-quinoline | 0.14 ± 0.10 | 0.02 ± 0.02 |
| Benzenepropanoic acid, ethyl ester | 0.09 ± 0.08 | 0.08 ± 0.04 |
| Benzenepropanoic acid | 0.14 ± 0.07 | 0.29 ± 0.18 |
| 1,3-Dihydro- 2H-indole-2-one | 0.04 ± 0.02 | 0.18 ± 0.12 |
| Dodecanoic acid | 0.05 ± 0.02 | 0.18 ± 0.11 |
| Tetradecanal | 0.03 ± 0.03 | 0.08 ± 0.05 |
| 2-Pentadecanone | 0.02 ± 0.01 | 0.02 ± 0.01 |
| Pentadecanal | 0.13 ± 0.06 | 0.21 ± 0.09 |
| 2,4-Dihydroxy-3,6-dimethyl-benzoic acid methyl ester | 0.08 ± 0.05 | 0.11 ± 0.06 |
| Tetradecanol | 0.08 ± 0.06 | 0.04 ± 0.02 |
| Tetradecanoic acid | 1.01 ± 0.28 | 1.20 ± 0.85 |
| Hexadecanal | 3.61 ± 0.61 | 3.34 ± 0.95 |
| Pentadecanoic acid, ethyl ester | 0.01 ± 0.01 | 0.02 ± 0.02 |

| | | |
|---|---|---|
| Hexadecanol | 0.75 ± 0.28 | 0.51 ± 0.22 |
| Pentadecanoic acid | 0.03 ± 0.01 | 0.01 ± 0.01 |
| Hexadecenoic acid, methyl ester | 0.01 ± 0.01 | - |
| Hexadecanoic acid, methyl ester | 0.02 ± 0.01 | 0.01 ± 0.01 |
| Hexadecenoic acid, ethyl ester | 0.74 ± 0.19 | 0.42 ± 0.22 |
| Hexadecenoic acid | 2.13 ± 0.86 | 0.41 ± 0.19 |
| Hexadecanoic acid, ethyl ester | 0.76 ± 0.37 | 0.47 ± 0.18 |
| Hexadecanoic acid | 6.46 ± 1.55 | 4.68 ± 2.29 |
| Octadecanal | 2.60 ± 0.40 | 3.03 ± 0.60 |
| Cyclic octaatomic sulfur | 0.39 ± 0.14 | 1.43 ± 0.60 |
| 9-Octadecenoic acid, methyl ester | 0.57 ± 0.32 | 0.64 ± 0.39 |
| Octadecanoic acid, methyl ester | 0.03 ± 0.02 | - |
| 9,12-Octadecadienoic acid, ethyl ester | 1.17 ± 0.43 | 0.60 ± 0.35 |
| 9-Octadecenoic acid, ethyl ester | 3.67 ± 1.12 | 3.30 ± 1.38 |
| Octadecanoic acid, ethyl ester | 0.14 ± 0.05 | 0.20 ± 0.11 |
| Octadecenamide | - | 0.05 ± 0.02 |
| 9-Octadecenoic acid | 5.33 ± 1.76 | 2.37 ± 1.64 |
| 5,8,11,14-Eicosatetraenoic acid, ethyl ester | 0.17 ± 0.08 | 0.07 ± 0.07 |
| 3-Hydroxy-octadecanoic acid, methyl ester | 0.06 ± 0.04 | 0.07 ± 0.07 |
| Nonadecanamide | 0.78 ± 0.29 | 1.01 ± 0.54 |
| Squalene | 4.38 ± 0.97 | 7.49 ± 2.56 |
| Cholesta-4,6-dien-3-ol | 1.15 ± 0.92 | 0.31 ± 0.15 |
| Cholesta-3,5-diene | 1.01 ± 0.21 | 1.03 ± 0.29 |
| Cholestanol | 0.27 ± 0.17 | 0.29 ± 0.20 |
| α-Tocopherol | 3.61 ± 0.58 | 4.15 ± 0.95 |
| Cholesterol | 9.27 ± 1.13 | 16.53 ± 3.07 |
| Cholestan-3-one | 1.68 ± 0.50 | 0.98 ± 0.51 |
| Cholest-4-en-3-one | 4.24 ± 0.71 | 4.17 ± 1.07 |
| Cholesta-4,6-dien-3-one | 0.72 ± 0.24 | 0.55 ± 0.25 |

| | | |
|---|---|---|
| Unidentified steroid (189, 203, 218, 313, 409, 424) | 1.73 ± 0.47 | 2.62 ± 0.72 |

# Figures

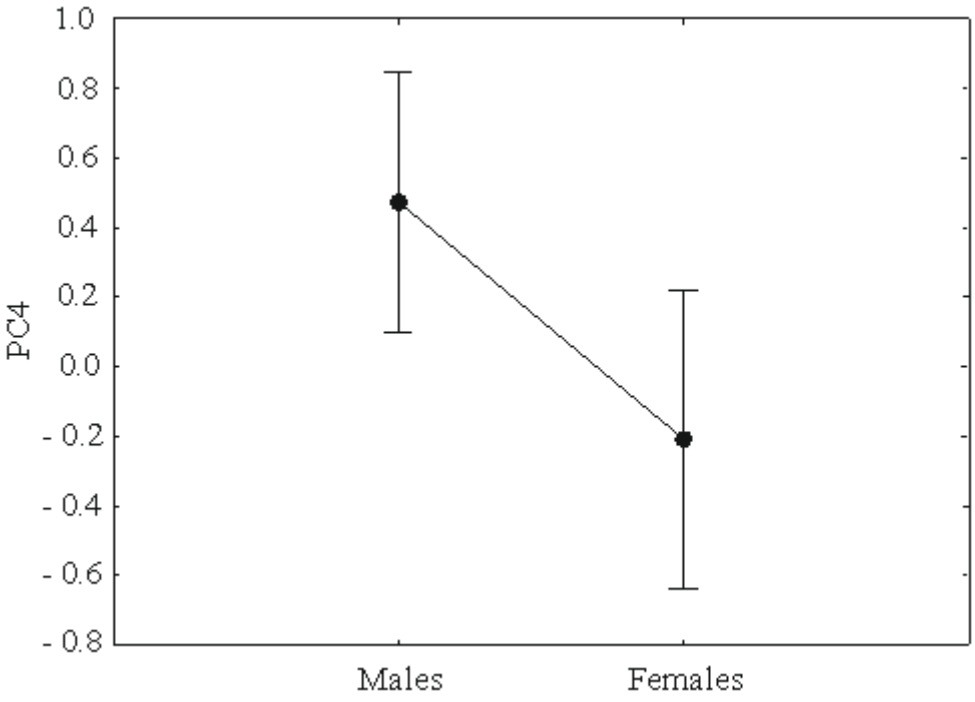
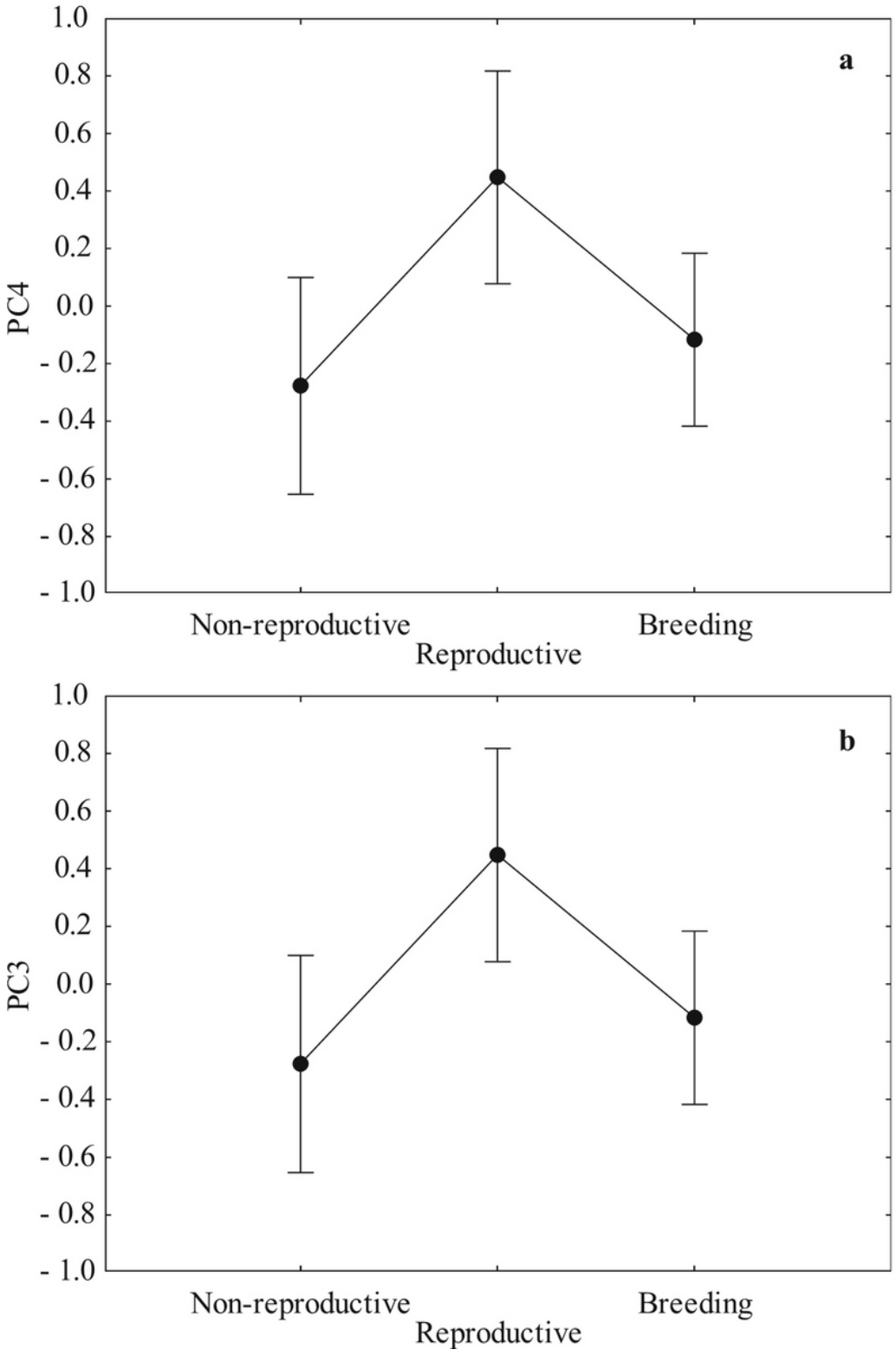



**Figure 1**

Relative proportions of compounds in feces (media ± IC al 95%) depending on sex of individuals of Iberian wolves (*Canis lupus signatus*).




## Figure 2

Relative proportions (media ± IC al 95%) of compounds in feces depending on the reproductive status of individuals (B=breeding, NR= non-reproductive, R=reproductive) of adult Iberian wolves (*Canis lupus signatus*) for A) PC4 and B) PC3.

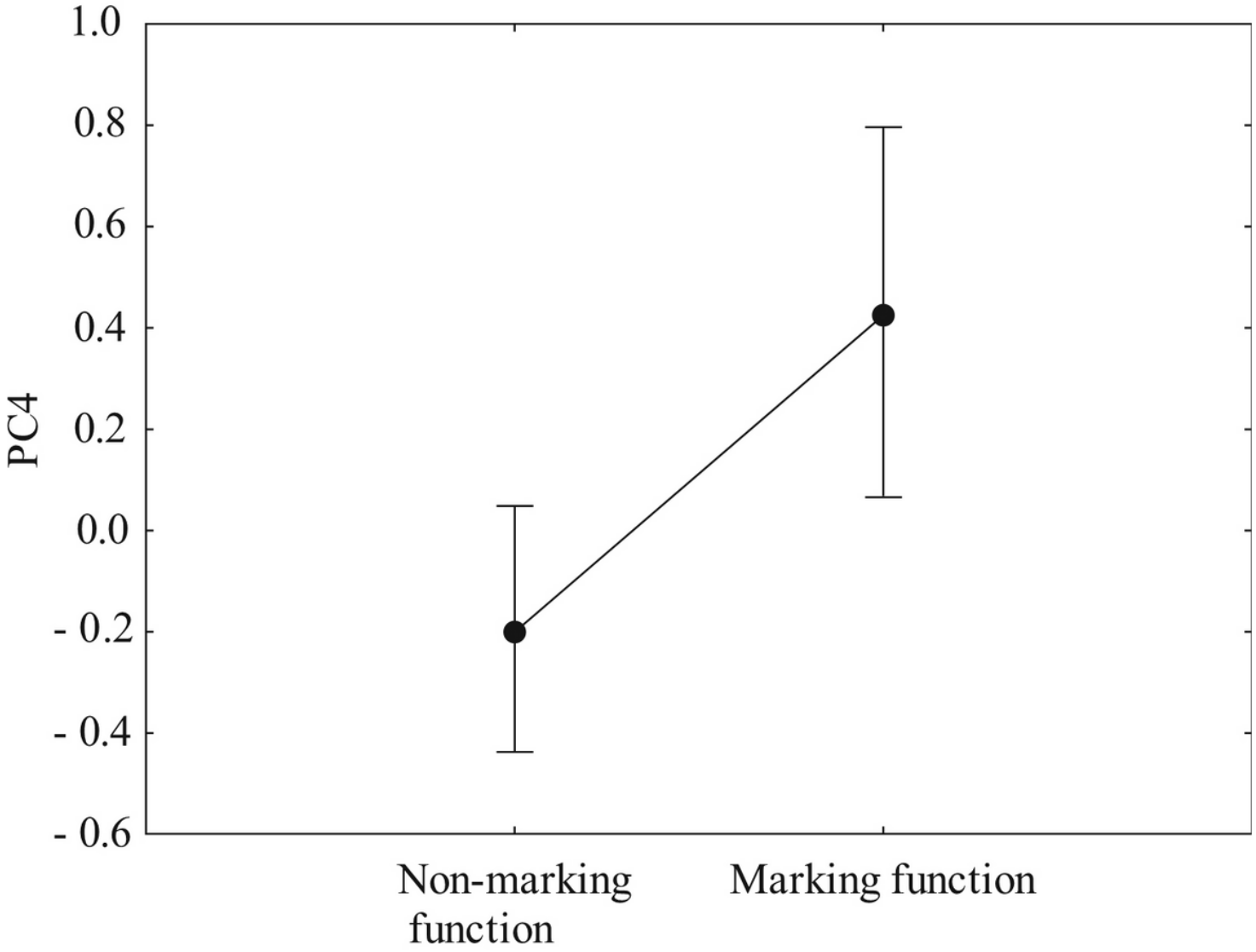



**Figure 3**

Relative proportions (media ± IC al 95%) of compounds in feces of adult Iberian wolves (*Canis lupus signatus*) depending on its presumably marking function in intraspecific communication.