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Original Investigation

A new sexual signal in rutting male red deer: Age related chemical scent constituents in the belly black spot

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ABSTRACT

Rutting behaviour of red deer stags (*Cervus elaphus*) includes an extensive repertoire of visual and acoustic signals directed either to rival males or to females. As in other mammals, olfactory communication is expected to play a central role in these rutting interactions too, but this has rarely been investigated. Only during the rutting season, red deer males show a conspicuous black spot area throughout most of their underbelly produced by the impregnation of substances with a strong scent. Here, we examined the origin of these compounds and their potential role as chemical signals. By using gas chromatography–mass spectrometry (GC–MS), we identified 67 compounds in the hair from the belly black spot of red deer stags, mainly heterocyclic aromatic organic compounds, such as *m*-cresol, benzoic acid, cyclohexanecarboxylic acid and ethylphenol, but we also found steroids, such as cholesterol and androstane-3,17-dione, carboxylic acids and their esters between *n*-C_6 and *n*-C_{22}, alcohols, squalene and other minor compounds. Many of these compounds are found in the belly black spot but not in other hair areas, and may have originated from several sources, such as the urine or the sebaceous glands of the skin, which impregnated the belly. Moreover, we found differences in chemical profiles depending on age, with older males having higher proportions of benzoic acid and androstane-3,17-dione, but lower proportions of *m*-cresol. Because most of these compounds are strongly odoriferous, and appear related to male characteristics, our data indicate that scent from the hairs forming the black spot of the belly may be regarded as an overlooked new sexual chemical signal in red deer in the context of competition for mates during the rutting season.

Introduction

Chemical signals (pheromones) play an important role in intraspecific communication of many animals (Müller-Schwarze, 2006; Wyatt, 2014). In mammals, pheromones or semiochemicals are produced by a wide variety of glands (e.g. anal, orbital, tail, etc.) and are very often incorporated into faeces, urine or other scent marks with the purpose of marking territory boundaries or attracting mates (reviewed in Burger, 2005; Brennan and Kendrick, 2006; Müller-Schwarze, 2006; Apps, 2013; Wyatt, 2014). Artiodactyls, and deer in particular, produced secretions with a variety of organic compounds (reviewed in Burger, 2005), which are used in olfactory communication. For example, in white-tailed deer, *Odocoileus virginianus*, the sebaceous and apocrine glands in the forehead region and the interdigital glands produce secretions with many volatile compounds that are transferred to the hair for both lubrication and scent communication via rubbing (Gassett et al., 1996, 1997). These secretions may be important in identifying individuals, establishing dominance, and signalling sexual readiness. In several Eurasian deer species, including red deer, secretions from preorbital, metatarsal and interdigital glands have been shown to contain individual differences that would be useful for individual recognition (Lawson et al., 2000) as well as for signalling individual attributes such as sex or age (Lawson et al., 2001).

Urine also may be important in intraspecific communication in deer. For example, in white-tailed deer, the presence and concentration of some urinary compounds depend on the season, sex, reproductive status and social rank of the animals (Jemiolo et al., 1995; Miller et al., 1998). Adult male white-tailed deer often urinate on their tarsal glands during the breeding season, which may

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allow deer to scent-mark territories and also to carry chemical cues indicative of social status (Miller et al., 1998).

The Iberian red deer (*Cervus elaphus hispanicus*) is a polygynous species, and adult males usually defend harems or mating territories at places used by females (Carranza et al., 1990, 1996; Carranza and Valencia, 1999). Rutting behaviour of male red deer includes an extensive repertoire of communicative behaviours directed either to rival males or to females. The most widely known signal is the highly evident roaring acoustic call (Clutton-Brock and Albon 1979; Passilongo et al. 2013). Also highly evident are the visual signals composed by the whole body and antler size, displayed in lateral view to rivals during the parallel walk preceding antler fighting (Clutton-Brock et al., 1982).

Olfactory communication is expected to play a central role in these rutting interactions too, but this possibility has rarely been investigated. Red deer produce secretions from the tail gland with several aromatic volatile compounds, such as *m*-cresol, benzoic acid and cyclohexanecarboxylic acid (Bakke and Figenschou, 1983). Other type of volatile compounds, mainly carboxylic acids and aromatic compounds are incorporated in the urine (Bakke and Figenschou, 1990). There are large variations in the compositions of the tail gland secretions between sex and individuals and among seasons for each individual (Bakke and Figenschou, 1983). Also, during the rutting season there are changes in the compounds excreted in the urine by adult males, but not by females (Bakke and Figenschou, 1990). These observations suggest that all these secreted compounds may have an important role in intraspecific chemical communication in this species, for example signalling individual characteristics.

During the rutting season, red deer males show a conspicuous dark area throughout most of their underbelly, from the penile opening up to the ventral base of the neck (Carranza et al., unpubl. data). This black spot on the belly of rutting stags is not a permanent trait, as their size increases during the rutting season, reaching up to 70 cm. long in mature males (Carranza et al., unpubl. data). The black coloration is not produced by melanin in the hair, but rather by the impregnation of some substances, which produces a characteristic strong scent even for human olfaction. We hypothesized that this black coloration is probably produced by chemical secretions from specialized glands (e.g. skin, tail, preputial) and/or from the urine. The deposition of chemical compounds on the hair of the belly might increase the volatilizing surface, improving the detectability of the chemical signal, as reported in other mammals (MacDonald et al., 1984). In fact, the maximal surface occupied by this black belly spot increases with age, until they reach six years old, after which it stabilizes in older males, following the same pattern than other traits related to intrasexual competition between males, such as antler size or body weight (Carranza et al., unpubl. data).

We report here the results of an analysis by gas chromatography-mass spectrometry (GC–MS) of the lipophilic fraction of the compounds that impregnated the hair from the belly black spot of male Iberian red deer (*Cervus elaphus hispanicus*) collected in the wild during the rutting season. We described chemical constituents found in underbelly hair of red deer stags, and specifically compared these compounds with those previously described in the tail gland, urine and in the hair from other parts of the body in this and other deer species in order to elucidate the origin of the compounds that form the belly black spot. We also compared proportions of these compounds in relation to the age, and hence competitive ability of the males (Clutton-Brock et al., 1982, 1988). We hypothesized that if compounds in black belly have a role in communication in the context of competition for mates, we should find differences in chemical profiles of males related to their age or social status.

Material and methods

Study site and hair collection

Animals used in the study were Iberian red deer males, harvested during the hunting activities in naturally occurring populations of red deer in Extremadura, South-western Spain. Samples were collected in two hunting states, "Valero" ($N = 17$) and "Matapegas" ($N = 15$) located in Monfragüe and Sierra de San Pedro (Cáceres prov., SW Spain) on 15th October and 6th November 2005, respectively, shortly after the rutting season that takes place in September and early October. Sampled populations included mountain areas covered by Mediterranean shrub (*Cistus* spp., *Erica* spp., *Genista hirsuta*, *Lavandula* spp.) and forest species (*Quercus* spp., *Arbutus unedo*, *Olea europaea*, *Phyllirea* spp.), and lower, flatter land, covered by 'dehesa' open oak woodland (*Quercus* spp.). Within each area, deer typically use the shrub land and forest as refuge and clump in mating areas ('arenas') in open dehesas during the rut (Carranza et al., 1990, 1995; Carranza and Valencia, 1999).

Immediately after deer had been hunted, we examined in the field recently dead animals. We collected hair from the black belly and from the back of each animal by using clean scissors. Hair samples were directly transferred and kept in glass vials with Teflon-lined stoppers and immediately frozen until analyses in the laboratory. We also used the same procedure but without collecting hair, to obtain blank control vials that were treated in the same way to compare with the deer samples to exclude contaminants from the handling procedure or from the environment, and for further examining potential impurities in the solvent or laboratory instruments. Mandibles were removed to estimate age (years) in the laboratory by counting dental cementum layers at the interradicular pad of first molars (see e.g. Carranza et al., 2004 for a more detailed description). Red deer in the sample analysed here had an average (±SE) age of 3 ± 0.3 years (range = 2–8 years).

Chemical analyses

A few weeks after collecting hair, we transferred a small amount of each hair sample to a clean glass vial and added 250 μl of *n*-hexane (Sigma, capillary GC grade, 99.9% purity) and closed the vial with a Teflon-lined stopper. We shook the solution for 1 min using a vortex and left the vial to rest in a fridge for 10 min until solid material (mainly hair) that was not dissolved precipitated at the bottom of the vial. The supernatant clear hexane was extracted with a glass syringe and transferred to a clean vial with Teflon-lined stopper and was immediately analysed.

We analysed samples with 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-μm film thickness) and a Finnigan-ThermoQuest Trace 2000 mass spectrometer (MS) as detector. We used this general technique rather than, for example, head-space analysis, because we aimed to know the compounds found in the black belly spot, irrespective of their volatibility and potential function in communication. This is because the black spot may be formed by non volatile compounds, such as fat, which might not have a role in chemical communication.

The samples, 2 μl of each sample, were injected in splitless mode with an inlet temperature of 250 °C. The program GC was: initial oven temperature of 45 °C for 10 min, then increased at a rate of 5 °C/min to a final temperature of 280 °C maintained for 15 min. The carrier gas was helium at 30 cm/s. Ionization by electron impact (70 eV) was carried out at 250 °C. Mass spectral fragments below $m/z = 39$ were not recorded. Impurities identified in the control vial samples are not reported. Initial identification of compounds was performed by comparison of sample mass spectra with those in