Unprecedented high catecholamine production causing hair pigmentation after urinary excretion in red deer

Ismael Galván[1]* · Francisco Solano[2] · Mohammed Zougagh[3,4] · Fernando de Andrés[4,5] · Khaled Murtada[3,4] · Ángel Ríos[3,4] · Eva de la Peña[6] · Juan Carranza[6]

[1]Department of Evolutionary Ecology, Doñana Biological Station, Consejo Superior de Investigaciones Científicas (CSIC), C/ Américo Vespucio 26, 41092 Sevilla, Spain. ORCID IG: 0000-0002-6523-8592.

[2]Department of Biochemistry and Molecular Biology B and Immunology, Faculty of Medicine, University of Murcia, Campus de Espinardo, 30100 Murcia, Spain. ORCID FS: 0000-0001-9612-761X.

[3]Department of Analytical Chemistry and Food Technology, Faculty of Chemical Science and Technology, University of Castilla-La Mancha, Avda. Camilo José Cela 10, 13071 Ciudad Real, Spain. ORCID MZ: 0000-0002-6395-0172. ORCID KM: 0000-0001-9692-6307. ORCID AR: 0000-0003-1728-3097.

[4]Regional Institute for Applied Scientific Research (IRICA), University of Castilla-La Mancha, Campus Universitario de Ciudad Real s/n, 13071 Ciudad Real, Spain.

[5]Department of Analytical Chemistry and Food Technology, Faculty of Pharmacy, University of Castilla-La Mancha, Avda. Dr. José María Sánchez Ibáñez s/n, 02071 Albacete, Spain. ORCID FDA: 0000-0003-1076-0743.

[6]Ungulate Research Unit, Cátedra de Recursos Cinegéticos y Piscícolas (CRCP), University of Cordoba, Campus de Rabanales, 14071 Córdoba, Spain. ORCID EDP: 0000-0001-9567-1111. ORCID JC: 0000-0002-0368-7173.

*Author for correspondence. E-mail: galvan@ebd.csic.es. Phone: +34954466700.

Acknowledgments Two anonymous reviewers made comments that improved the manuscript. Rafael Palomo Santana kindly allowed us to reproduce his red deer photographs, shown in Figs. 2 and 3. IG benefits from a Ramón y Cajal Fellowship (RYC-2012-10237) from Spain's Ministry of Science, Innovation and Universities, and MZ benefits from an "INCRECYT" research contract. Financial support was obtained from projects CGL2013-48122-P, CGL2015-67796-P and CTQ2016-78793-P from Spain's Ministry of Science, Innovation and Universities, and from project SBPLY/17/180501/000262 from Junta de Comunidades de Castilla-La Mancha.

Abstract

Hormones have not been found in concentrations of orders of magnitude higher than ng/ml. Here we report urine concentrations of a catecholamine (norepinephrine) ranging from 0.05 to 0.5 g/l, and concentrations of its metabolite DL-3,4-dihydroxyphenyl glycol (DOPEG) ranging from 1.0 to 44.5 g/l, in wild male red deer *Cervus elaphus hispanicus* after LC-MS analyses. The dark ventral patch of male red deer, a recently described sexually selected signal, contains high amounts of DOPEG (0.9-266.9 mg/l) stuck in the hairs, while DOPEG is not present in non-darkened hair. The formation of this dark patch is explained by the chemical structure of DOPEG, which is a catecholamine-derived *o*-diphenol susceptible to be oxidized by air and form allomelanins, nitrogen-free pigments similar to cutaneous melanins; by its high concentration in urine; and by the urine spraying behavior of red deer by which urine is spread through the ventral body area. Accordingly, the size of the dark ventral patch was positively correlated with the concentration of DOPEG in urine, which was in turn correlated with DOPEG absorbed in ventral hair. These findings represent catecholamine concentrations about one million higher than those previously reported for any hormone in an organism. This may have favored the evolution of the dark ventral patch of red deer by transferring information on fighting capacity to

rivals and mates. Physiological limits for hormone production in animals are thus considerably higher than previously thought. These results also unveil a novel mechanism of pigmentation based on the self-application of urine over the fur.

Keywords Allomelanins · Catecholamines · Deer · Pigmentation · Urine hormones

Introduction

Hormones are molecules that are secreted into the circulatory system and affect physiology and behavior by binding to specific receptors of cells. This signaling role probably makes that hormones are not produced in large amounts. Although concentration in blood differs between hormone classes, no hormone is found in orders of magnitude higher than ng/ml [1-4]. Catecholamines, particularly epinephrine and norepinephrine, are hormones that are secreted by adrenal glands and are involved in the fight-or-flight response that prepares the cardiovascular and musculoskeletal systems for challenges that require a rapid response [5] by inducing muscle cell contraction [6]. The order of magnitude of circulatory concentrations of epinephrine and norepinephrine, like other hormones, is not higher than ng/ml in mammals [7], the maximum norepinephrine plasma concentration reported in humans being 1.8 ng/ml [8].

Catecholamine hormones and their precursor dopamine are *ortho*-diphenols and as such have the potential to produce nitrogen-free dark pigments similar to cutaneous melanins (i.e., allomelanins) if oxidized to *o*-quinones and polymerized [9]. This hypothetical mechanism of pigmentation, however, has not been explored in any organism. The catecholic structure is shared by other acidic (DL-3,4-dihydroxymandelic acid; DHMA) and alcoholic (DL-3,4-dihydroxyphenyl glycol; DOPEG) compounds produced after monoamine oxidases (MAO) catalyze the deamination of norepinephrine to its aldehyde [10]. These *o*-diphenols are formed after oxidation or reduction in reactions

catalyzed respectively by aldehyde dehydrogenase or aldehyde reductase. These compounds and some of their methoxylated derivatives are then excreted in the urine, thus arising the possibility that urine generates pigmentation (Fig. 1). The reported concentrations of DHMA and DOPEG in urine, although higher than those of their precursor hormones, are still around the order of magnitude of ng/ml in both human [11] and mice [12].

Here we investigated a possible catecholamine-based mechanism of pigmentation in wild Iberian male red deer *Cervus elaphus hispanicus*. During the mating season in which male red deer compete to form harems and maximize the number of copulations, a large and conspicuous dark ventral patch of hair appears associated to a urine spraying behavior in which urine is spread through the ventral body area [13] (Figs. 2-3). Catecholamines and their derived deaminated catechols are the only substances present in the urine with the potential to form dark pigments if oxidized by air after their excretion [9]. We hypothesized that catecholamines in the urine of male red deer are responsible for the generation of the dark ventral patch, as this patch is not composed of newly grown hair (i.e., hair pigmentation in the ventral patch is caused by a exogenous agent). This hypothesis implies, however, predicting unusually high concentrations of catechols in the urine of male red deer. Such high concentrations are probably necessary to generate conspicuous pigmentation, as while urinary excretion of catecholamines occurs in all mammals [14], pigmentation caused by urine has never been reported. It may also be possible that the hair pigmentation is produced by a repeated application of urine containing lower concentrations of catecholamines or deaminated catechols on the same ventral patch, which should led to high accumulation of those melanin precursors absorbed only in hair.

We thus investigated if the concentration of dopamine, epinephrine, norepinephrine, DHMA and DOPEG in urine explains the observed variability in the size of the dark ventral

fur patch among male red deer. We also investigated the composition of catechols in the constituent hair of the dark ventral patch and in non-darkened hair of the lateral part of the body, which served as control.

Materials and methods

Animals

In 2016-2017, 33 adult Iberian male red deer were sampled for urine and hair. The animals were harvested in hunts in natural populations of Iberian red deer in southwestern Spain. Three samples were taken in September, seven samples were taken in October, five samples in November, 10 samples in December, five samples in January and three samples in February. In wild red deer, mating tends to synchronize within a period of about one month, which mostly coincides with September in our study area [15]. Thus, it can be considered that the three samples taken in September were within the mating season of red deer.

The age of male red deer was estimated, as in previous studies on this species in our study area [16], by counting cementum growth marks at the interradicular pad under the first molar [17] and checking by eruption patterns in younger animals. Seventeen males had an age of two years, three an age of three years, six an age of four years, two an age of five years, and four an age of six, seven, eight or nine years. One male could not be aged.

Within 1 h after death, 10 ml of urine was extracted directly from the urinary bladder using a syringe, after which 250 µl of 1 M HCl was immediately added. Urine samples were kept at -4 ºC in the field, and stored at -80 ºC within 4 h of extraction. Twenty hairs from the dark ventral patch (choosing the part with most intense pigmentation) and from brown non-darkened fur of the lateral part of the body (Fig. 2) were cut and stored at -80 ºC until analyses. Lastly, the size of the ventral patch was determined by measuring its

length with a ruler, from the penis to the end of the dark patch at the breast or the base of the neck (Fig. 1).

Analytical methods

Dopamine, norepinephrine, epinephrine, DHMA, DOPEG and heptafluorobutyric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). These reagents were used as analytes. LC-MS grade methanol was used as mobile phase solvent, and purchased from Fisher Scientific (Loughborough, Leics, UK). Acetic acid/sodium acetate buffer and Triton X-100 were used for hair pigment extractions, and purchased from Sigma-Aldrich. Ultrapure water was obtained from a Milli-Q water instrument from Millipore (Merck KGaA, Darmstadt, Germany).

The stock solutions of all analytes were prepared at 1 mg ml^{-1} in 0.5% acetic acid and stored in absence of light at -20°C, and working standard solutions were prepared at 10 µg ml^{-1} by appropriate dilution in water and stored at -20°C as well. The urine real samples were stored at -80°C. The urine samples utilized for calibration were diluted with water (1:10 v/v). The extraction of pigments from pelage was made by trimming 10 hairs per animal and immersing the fragments in tubes containing 20 mM acetic acid/sodium acetate buffer at pH 5 with 0.08% Triton X-100. The tubes were vigorously mixed, left in orbital shaking for 1 h, then centrifuged and the supernatant was analyzed in HPLC-MS.

Instrumentation

We used high performance liquid chromatography (HPLC) in combination with quadrupole mass spectrometry (QMS) to quantify dopamine, epinephrine, norepinephrine, DHMA and DOPEG in the urine of wild male red deer. The equipment was a Chromatography System Agilent series 1200 (Waldbronn, Germany) and an Onyx Monolithic C18 (100 × 4.6 mm) column from Phenomenex® (Torrance, CA, USA) was utilized for the chromatographic

separation of the analytes. This system consists of a degasser (Agilent series 1200), a liquid chromatographic pump (Agilent series 1200), an autosampler (Agilent series 1200), a temperature-controlled column compartment (Agilent series 1200) and a diode array detector (Agilent, 1260 infinity model). The detector is coupled to a system of data acquisition and processing system (Agilent ChemStation HPLC). Detection was carried out with a UV-Vis diode array detector equipped with a 2 µl flow cell coupled in series to an Agilent 6110 series MS detector (Waldbronn, Germany), equipped with an atmospheric pressure ionization source electrospray (API-ES).

Liquid chromatography-MS

DAD detector was set at a wavelength of 210 nm, and MS detection of analytes was carried out in positive ion mode under the following conditions: 12 l min^{-1} of drying gas flow, drying gas temperature at 300°C, a nebulizer pressure of 35 psi and a capillary voltage of 2500 V. Single ion monitoring (SIM) was used to detect and quantify the target analytes using external calibration. Previously, the analytes were qualitatively determined at full scan mode and matching the retention time and mass spectra with standards. The m/z ions used for identification were: 137 (dopamine), 167 (DL-3,4-dihydroxymandelic acid), 153 (DL-3,4-dihydroxyphenyl glycol), 152 (norepinephrine) and 166 (epinephrine).

Chromatographic analyses were carried out using a gradient formed by a solvent A consisted of 0.01% heptafluorobutyric acid in water and a solvent B which was formed by a 0.01% heptafluorobutyric acid in methanol. The gradient was carried out at a flow-rate of 500 µl min^{-1}, starting from 10% B at 0.5 min, 20% B at 3 min, 60% B at 3.5 min, 85% B at 7 min, 90% of B at 8 min and 10% of B over 12 min. Injection volume was 1 µl and the column was maintained at a temperature of 25°C. Re-equilibration of the column was done in 20 min after each run. All solvents were filtered through a 0.45 µm nylon membranes before their use.

The calibration data and validation parameters for this method are shown in Table 1. Calibration curves showed a linear range for catecholamine solutions from 1.0 to 10 µg ml^{-1}. The precision of the method for standard solutions (investigated after analyzing 11 series of 11 replicates) and the relative standard deviation (RSD) was calculated to be 3.03% at the 5 µg ml^{-1} concentration of catecholamines. The limits of detection (LOD) and quantification (LOQ) were found to be in the ranges 0.288 - 0.307 and 0.782 - 0.923, respectively (Table 1). The developed method provides clear and good advantages in terms of precision, accuracy and linearity in relation to a previously published method for the determination of catecholamines in urine by liquid chromatography [18].

Statistical analyses

The dependency of ventral patch size on urine norepinephrine and DOPEG concentrations was tested by means of general linear models (GLM) that included total body size and age as covariates. As norepinephrine and DOPEG concentrations were highly correlated (see Results), a separate GLM's was tested for each response variable. Differences of mean norepinephrine and DOPEG concentrations between mating and non-mating seasons were tested with Student's *t*-tests. Pearson correlation tests were conducted to analyze correlations between pairs of variables. Variables were log_{10}-transformed to achieve the normality assumption of parametric tests.

Results

We did not find the precursor dopamine in the urine of any of the 33 wild male red deer that were sampled. In contrast, unusually high concentrations of norepinephrine were found in the urine of all animals (mean ± SE: 0.18 ± 0.02 g/l; range: 0.05-0.48 g/l; Fig. 4A). In congruence with the known metabolism of catecholamines (Fig. 1), the alcoholic derivative of norepinephrine (DOPEG) was detected in higher concentrations in all animals

(mean ± SE: 8.90 ± 1.42 g/l; range: 0.91-44.52 g/l; Fig. 4A). The acidic derivative (DHMA), however, was only detected in four animals in considerably lower concentrations than DOPEG (mean ± SE: 0.15 ± 0.05 g/l; range: 0.06-0.26 g/l). When the amount of creatinine in urine is considered (mean ± SE: 0.35 ± 0.03 g/l; range: 0.05-1.00 g/l) to express concentrations controlling for the rate of urine production in red deer, the resulting values are even higher (mean ± SE: norepinephrine: 0.69 ± 0.16 g/l; DOPEG: 31.92 ± 8.48 g/l; DHMA: 0.80 ± 0.34). The concentrations of norepinephrine and DOPEG in urine were positively correlated (r = 0.56, p = 0.001).

The size of the dark ventral patch was highly variable among the 33 male red deer (mean ± SE: 40.8 ± 4.4 cm; range: 5-73 cm; Fig. 2B). A high proportion (72.5 %) of this variability was significantly explained by a GLM that included body length as a covariate to control for the size of animals (age was removed from the model as its effect was not significant: $F_{1,28}$ = 1.99, p = 0.169). In this model, the effect of DOPEG urine concentration was significant and positive (regression coefficient = 0.34, $F_{1,30}$ = 5.48, p = 0.026). This indicates a size-independent effect of DOPEG concentration on the size of the dark ventral patch. The positive relationship between urine DOPEG concentration and ventral patch size remained significant when the effect of body size is not considered (r = 0.35, n = 33, p = 0.043) (Fig. 5). In contrast, the effect of norepinephrine urine concentration on ventral patch size was not significant either controlling for body size ($F_{1,30}$ = 3.90, p = 0.057; age was also removed from this model as its affect was not significant: $F_{1,28}$ = 2.38, p = 0.134) or not controlling for it (r = 0.11, n = 33, p = 0.541).

Three out of 33 male red deer included in this study were sampled within the mating season (September), when, according with a sexual signaling role [13], the dark ventral patch is expected to exhibit its maximum expression level (i.e., largest size). We found that the urine concentration of DOPEG in the three males sampled in the mating season were significantly higher (mean ± SE: 20.32 ± 12.38 g/l) than those in the rest of animals

234 sampled outside the mating season (7.75 ± 0.95 g/l; t = 2.80, df = 31, p = 0.009). The
235 same was not observed in norepinephrine levels, which did not differ between the three
236 samples in the mating season (0.13 ± 0.03 g/l) and the others (0.19 ± 0.02 g/l; t = 0.91, df
237 = 31, p = 0.367).

238 Lastly, we found high concentrations of DOPEG stuck in the hairs of the dark
239 ventral patch (mean ± SE: 58.64 ± 10.32 mg/l; range: 0.93-266.93 g/l), while only two
240 animals had detectable concentrations of DOPEG (5.88 and 9.84 g/l) in lateral, non-
241 darkened hair (Fig. 4B). The amount of DOPEG extracted from hair was positively
242 correlated with DOPEG concentration in urine (r = 0.64, n = 33, p < 0.0001).

243

244 Discussion

245 The concentrations of norepinephrine and DOPEG that we found in the urine of wild male
246 red deer constitute the highest amounts of a hormone ever found in an organism. These
247 values are one million higher than those previously reported in any hormone, which had
248 never been in orders of magnitude above ng/ml [1-4, 7, 8]. The presence of
249 norepinephrine and the absence of epinephrine in the urine also denotes an unusual
250 physiological system in red deer, as this suggests that these animals do not express
251 significant amounts of phenylethanolamine N-methyltransferase (PNMT), the enzyme that
252 catalyzes the transfer of a methyl group to norepinephrine to convert it to epinephrine (Fig.
253 1) that is assumed to occur in all vertebrates [19]. It can be speculated that this lack of
254 epinephrine synthesis could be related to the unusually high levels of norepinephrine
255 found in red deer, as this may be the only physiological conditions favoring high
256 concentrations of the hormone, although this will have to be investigated by future studies.
257 The excretion of norepinephrine in red deer, however, apparently follows the expected
258 metabolic mechanism in which the reduction to glycol (DOPEG) represents a more
259 favorable pathway than the oxidation to acid (DHMA) [20], despite the opposite having

been assumed in the last three decades (see however ref. 14). The positive correlation between norepinephrine and DOPEG concentrations in urine actually reinforces the norepinephrine-derived nature of DOPEG. These findings suggest that the activity of aldehyde reductase is considerably higher than that of aldehyde dehydrogenase during the metabolism of catecholamines in red deer, as opposed to what seems to occur in human and model animal species [11, 12].

The fact that the size of the dark ventral patch was positively correlated with the urine DOPEG concentration but not with the norepinephrine urine concentration supports a urine DOPEG-based origin for the dark ventral patch of male red deer. Moreover, we found an increased DOPEG production during the mating season in red deer, which reinforces the role of DOPEG as responsible for the generation of the dark ventral patch. Further evidence of the causative role of DOPEG in pigmentation was obtained after analyzing hair extractions of red deer, as we found high concentrations of DOPEG stuck in the hairs of the dark ventral patch that were positively correlated with the DOPEG concentration in urine.

Our findings have two main implications. First, it is expected that the unprecedented high concentration of a hormone (norepinephrine) in male red deer is associated to an unusually tight control of behavior in these animals. The fight-or-flight response that norepinephrine regulates in vertebrates mainly consists in adjusting cell contraction and, as a consequence, muscular activity as a response to environmental challenges that require fighting or escaping from a cause of risk such as predators [5, 6]. The change in behavior that occurs during the mating season of red deer, when males shift from exhibiting sociality to behave intensively aggressive toward other males, seems to respond to an increase in testosterone levels as shown by castration experiments [21]. We did not find particularly high levels of norepinephrine in the urine of male red deer during the mating season, which suggests that, while the seasonal change in behavior is regulated

286 by testosterone, norepinephrine may regulate a more constitutive (i.e., less labile)
287 characteristic of individual animals. This characteristic may be their intrinsic predisposition
288 to fight, or the permanent vigilance behavior that deer exhibit as because of constant
289 predation risk [22]. As an example of how norepinephrine controls muscular performance,
290 circulating levels of this hormone (but not those of epinephrine) have been observed to
291 peak during combats in human elite wrestlers [23], which is congruent with the apparent
292 absence of epinephrine synthesis in red deer if the hormone also has a role in fighting in
293 the animals. In human wrestlers, norepinephrine effects occur at plasma concentrations in
294 the order of magnitude of ng/l [23], more than one million lower than urine concentrations
295 in male red deer. Thus, irrespectively of the exact behavioral characteristic that
296 norepinephrine regulates in red deer, this might represent the most tightly controlled
297 behavior by a hormone in an animal.

298 On the other hand, our findings unveil a novel mechanism of pigmentation based on
299 the excretion of high amounts of DOPEG in the urine. The *o*-diphenol structure of DOPEG
300 makes it susceptible to be oxidized and form dark compounds similar to cutaneous
301 melanins [9]. The oxidation of DOPEG likely occurs when the urine of male red deer
302 contacts the air, while the spread of the urine through the ventral fur probably leads to the
303 polymerization of the oxidized intermediates and to the formation of relatively stable
304 pigments after generating complexes with the keratin of hairs. These pigments can thus be
305 categorized into the group of allomelanins, a heterogeneous class of nitrogen-free
306 pigments derived from catechols and other polyphenols present in plants and fungi [24].
307 To date, the dark ventral patch of male red deer is therefore the only animal trait produced
308 by allomelanins.

309 The DOPEG-mediated formation of the dark ventral patch may also contribute to
310 understand its evolution. Data on volatile compounds also present in the ventral patch hair
311 suggest that this trait acts as a sexual signal in red deer [13]. Our own preliminary

analyses actually show that males with larger patches achieve higher mating success (unpublished data). As we found that ventral patch size is indicative of DOPEG levels in urine, and DOPEG is a metabolite of norepinephrine, it is likely that patch size can contribute to rival assessment in intrasexual competition and that female red deer may select males with larger patches because this reflects a high fighting capacity or a high ability to escape from predators and thus achieve lifetime fitness. Thus, both components of sexual selection may drive the evolution of the dark ventral patch in males, probably in combination with selection for other male traits such as antler size [25]. Beyond opening the door to exploring these possibilities, this study exemplifies how the study of wild, non-model species widen the knowledge on animal physiology [26].

Ethical standards

All experiments were performed in compliance with the relevant laws and institutional guidelines in Spain.

Conflict interest

The authors declare that they have no conflict of interest.

References

1. Petrie M, Schwabl H, Brande-Lavridsen N, Burke T (2001) Maternal investment: sex differences in avian yolk hormone levels. Nature 412:498.
2. Ganguly S, Weller JL, Ho A, Chemineau P, Malpaux B, Klein DC (2005) Melatonin synthesis: 14-3-3-dependent activation and inhibition of arylalkylamine N-acetyltransferase mediated by phosphoserine-205. Proc Natl Acad Sci USA 102:1222-1227.

337 3. Stafflinger E, Hansen KK, Hauser F, Schneider M, Cazzamali G, Williamson M,
338 Grimmelikhuijzen CJ (2008) Cloning and identification of an oxytocin/vasopressin-like
339 receptor and its ligand from insects. Proc Natl Acad Sci USA 105:3262-3267.
340 4. Vitousek MN, Johnson MA, Donald JW, Francis CD, Fuxjager MJ, Goymann W, Hau
341 M, Husak JF, Kircher BK, Knapp R, Martin LB, Miller ET, Schoenle LA, Uehling JJ,
342 Williams TD (2018) HormoneBase, a population-level database of steroid hormone
343 levels across vertebrates. Sci Data 5:180097.
344 5. Axelrod J, Reisine TD (1984) Stress hormones: their interaction and
345 regulation. Science 224:452-459.
346 6. Fuller MD, Emrick MA, Sadilek M, Scheuer T, Catterall WA (2010) Molecular
347 mechanism of calcium channel regulation in the fight-or-flight response. Sci
348 Signal 3:ra70.
349 7. Künzl C, Kaiser S, Meier E, Sachser N (2003) Is a wild mammal kept and reared in
350 captivity still a wild animal? Horm Behav 43:187-196.
351 8. Silverberg AB, Shah SD, Haymond MW, Cryer PE (1978) Norepinephrine: hormone
352 and neurotransmitter in man. Am J Physiol Endocrinol Metab 234:E252-E256.
353 9. Ito S, Yamanaka Y, Ojika M, Wakamatsu K (2016) The metabolic fate of *ortho*-
354 quinones derived from catecholamine metabolites. Int J Mol Sci 17:164.
355 10. Rang HP, Ritter JM, Flower R, Henderson G (2016) Noradrenergic transmission. In:
356 Rang & Dale's pharmacology, 8th edn. Elsevier Churchill Livingstone, New York,
357 pp177-196.
358 11. Nohta H, Yamaguchi E, Ohkura Y, Watanabe H (1989) Measurement of
359 catecholamines, their precursor and metabolites in human urine and plasma by solid-
360 phase extraction followed by high-performance liquid chromatography with
361 fluorescence derivatization. J Chromatogr 493:15-26.

12. Kanamori T, Funatsu T, Tsunoda M (2016) Determination of catecholamines and related compounds in mouse urine using column-switching HPLC. Analyst 141:2568-2573.
13. Martín J, Carranza J, López P, Alarcos S, Pérez-González J (2014) A new sexual signal in rutting male red deer: Age related chemical scent constituents in the belly black spot. Mamm Biol 79:362-368.
14. Eisenhofer G, Kopin IJ, Goldstein DS (2004) Catecholamine metabolism: a contemporary view with implications for physiology and medicine. Pharmacol Rev 56:331-349.
15. Carranza J, Alvarez F, Redondo T (1990) Territoriality as a mating strategy in red deer. Anim Behav 40:79-88.
16. Carranza J, Alarcos S, Sánchez-Prieto CB, Valencia J, Mateos C (2004) Disposable-soma senescence mediated by sexual selection in an ungulate. Nature 432:215-218.
17. Mitchell B (1967) Growth layers in dental cement for determining the age of red deer (*Cervus elaphus* L.). J Anim Ecol 36:279-293.
18. Lv C, Li Q, Liu X, He B, Sui Z, Xu H, Yin Y, Liu R, Bi K (2015) Determination of catecholamines and their metabolites in rat urine by ultra-performance liquid chromatography-tandem mass spectrometry for the study of identifying potential markers for Alzheimer's disease. J Mass Spectrom 50:354-363.
19. Baetge EE, Behringer RR, Messing A, Brinster RL, Palmiter RD (1988) Transgenic mice express the human phenylethanolamine N-methyltransferase gene in adrenal medulla and retina. Proc Natl Acad Sci USA 85:3648-3652.
20. Mårdh G, Dingley AL, Auld DS, Vallee BL (1986) Human class II (π) alcohol dehydrogenase has a redox-specific function in norepinephrine metabolism. Proc Natl Acad Sci USA 83:8908-8912.

21. Lincoln GA, Guinness F, Short RV (1972) The way in which testosterone controls the social and sexual behavior of the red deer stag (*Cervus elaphus*). Horm Behav 3:375-396.
22. Altendorf KB, Laundré JW, López González CA, Brown JS (2001) Assessing effects of predation risk on foraging behavior of mule deer. J Mammal 82:430-439.
23. Barbas I, Fatouros IG, Douroudos II, Chatzinikolaou A, Michailidis Y, Draganidis D, Jamurtas AZ, Nikolaidis MG, Parotsidis C, Theodorou AA, Katrabasas I, Margonis K, Papassotiriou I, Taxildaris K (2011) Physiological and performance adaptations of elite Greco-Roman wrestlers during a one-day tournament. Eur J Appl Physiol 111:1421-1436.
24. Varga M, Berkesi O, Darula Z, May NV, Palágyi A (2016) Structural characterization of allomelanin from black oat. Phytochemistry 130:313-320.
25. Kruuk LE, Slate J, Pemberton JM, Brotherstone S, Guinness F, Clutton-Brock T (2002) Antler size in red deer: heritability and selection but no evolution. Evolution 56:1683-1695.
26. Galván I, Garrido-Fernández J, Ríos J, Pérez-Gálvez A, Rodríguez-Herrera B, Negro JJ (2016) Tropical bat as mammalian model for skin carotenoid metabolism. Proc Natl Acad Sci USA 113:10932-10937.

Table 1. Calibration data and validation parameters obtained for the determination of catecholamines in the urine of red deer.

Analyte	Linear range ($\mu g\ ml^{-1}$)	$Y = (A \pm S_A^c)X + (B \pm S_B^d)$	R^2	$S_{x/y}^e$	LOD[f] ($\mu g\ ml^{-1}$)	LOQ[g] ($\mu g\ ml^{-1}$)
DHMA[a]	1.0-10	Y = (114571 ± 1595.2)X - (40057.6 ± 9544.6)	0.9994	11722.3	0.307	0.833
DOPEG[b]	1.0-10	Y = (64535.6 ± 995.9)X - (286.7 ± 5959)	0.9993	7318.6	0.340	0.923
Norepinephrine	1.0-10	Y = (1713818.6 ± 23884.5)X - (119729 ± 142908.2)	0.9994	175514.4	0.307	0.834
Epinephrine	1.0-10	Y = (1323143.4 ± 18322.1)X + (389984.7 ± 109627)	0.9994	134639.6	0.305	0.829
Dopamine	1.0-10	Y = (487103.5 ± 6367.6)X - (174575 ± 38099.6)	0.9995	46792.4	0.288	0.782

[a]: DL-3,4-dihydroxymandelic acid.
[b]: DL-3,4-dihydroxyphenyl glycol.
[c]: SD of the slope.
[d]: SD of intercept.
[e]: SD of residuals.
[f]: Limit of detection.
[g]: Limit of quantification.

Legends to figures:

Fig. 1. Metabolic pathway of catecholamines from precursor dopamir pigmentation after urinary excretion. The enzymes catalyzing the processes in italics. Dopamine, norepinephrine and epinephrine are also degraded derivatives by the action of catechol-O-methyl transferases (COMT), but thes are not shown for simplicity. COMT can also catalyze the conversion c DOPEG to vanillylmandelic acid (VMA) and 3-metoxy-4-hydroxyphenylet (MOPEG), respectively, which are also excluded. The high concentration of l urine of male red deer leads to the production of allomelanins when the u through the hairs, thus producing the dark pigmentation of the ventral pela photograph shows the section of a ventral patch of a wild Iberian male r measurement of the length of the patch from penis to neck is exemplified v white arrow.

Fig. 2. Images of dark ventral patches in male red deer. **A** shows a wild Ibe deer from Spain exhibiting the dark ventral patch in the field. **B** shows the section (from penis to base of the neck) of two male red deer that were harve illustrating the variability in the size of the dark patch among males. Photogra Palomo Santana (**A**) and Eva de la Peña (**B**).

Fig. 3. Urine spraying behavior in a wild Iberian male red deer. The image s urine stream is oriented upwards and spreads over the entire belly fr onwards. It can be observed how the area of influence of the urine stream that corresponding to the dark ventral patch. Photograph by Rafael Palomo S

450 **Fig. 4.** Chromatogram traces of catecholamines. The blue curve in **A** corresponds to a 10
451 mg/l standard solution of dopamine, norepinephrine, epinephrine, DHMA and DOPEG.
452 The red curve corresponds to a 1:100 diluted urine sample from a wild Iberian male red
453 deer. **B** shows results of extracts from hair of the dark ventral patch (black curve) and from
454 lateral undarkened hair (brown curve) of the same male red deer. Peaks in **B** different from
455 that of DOPEG are of unknown origin.
456
457 **Fig. 5.** Dependency of dark ventral patch size on DOPEG concentration in the urine of
458 male red deer. The line is the best-fit line.