European Journal of Wildlife Research

Use of hair cortisol analysis for comparing population status in wild red deer (Cervus elaphus) living in areas with different characteristics

--Manuscript Draft--

Manuscript Number:	EJWR-D-16-00073R2
Full Title:	Use of hair cortisol analysis for comparing population status in wild red deer (Cervus elaphus) living in areas with different characteristics
Article Type:	Original Article
Keywords:	red deer; Cervus elaphus; Hair; Cortisol; Allostatic load
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Funding Information:	
Abstract:	We tested a method to measure Hair Cortisol Concentration (HCC) in 174 red deer (Cervus elaphus) culled in the hunting season 2011/12 in four areas of Central Italian Alps, with different population densities (SPN: 11.8 deer/km2; HD-AV: 3.6 deer/km2; HD-SO: 2.1 deer/km2; HD-MO: 2.0 deer/km2) and environmental conditions. Our hypothesis was that higher population densities, associated with more difficult environmental conditions, may result in higher allostatic load for these wild ungulates. No significant differences in HCC were detected between sexes (males: 4.77±0.69 pg/mg; females: 5.75±0.63 pg/mg) nor among age classes (calves: 6.17±0.66 pg/mg; yearlings: 4.47±0.83 pg/mg; adults: 5.15±0.74 pg/mg; least square mean±SE), but HCC difference between calves and yearlings was close to statistical significance (P=0.059). HCC showed high individual variation, but on average it was higher in areas with higher deer density (SNP: 7.45±1.01 pg/mg; HD-AV: 6.07±0.89 pg/mg; 4.67±1.14; HD-MO: 2.87±1.56 pg/mg), with significant differences between HD-AV and HD-MO (P=0.01). Carcass weight was significantly lower in SNP (46.74±1.49 kg) than in HD-MO (62.71±4.01 kg), HD-SO (61.73±2.9 kg) and HD-AV (62.07±2.04 kg) (P<0.001). These results seem to confirm our hypothesis that allostatic load is higher in areas with higher density and harder environmental conditions. We suggest that the methodology used in this study to measure HCC provides good information on long-term HPA axis activity and allostatic load and constitutes a highly promising, reliable and non-invasive method in wildlife management for assessing HPA axis activity over extended time periods.

Answer to Reviewers' comments:

Introduction
Line 62: 'Cortisol is the key glucocorticoid hormone of the HPA axis in many animals […]]' or, alternatively, 'Cortisol is a key glucocorticoid hormone of the HPA axis […]'. Just suggestionns in order to be more precise.

AU: corrected

Line 65: or, alternatively, 'reliable physiological indicator'.

AU: replaced “measure” with “indicator”

Lines 85 and 96: The authors said that the citation of Owen et al. (2005) was eliminated, but it still appears twice in the introduction section. Please correct.

AU: the reference has been included

Lines 95-96: This information is exactly the same as that present in the lines 84-86. Please avoid repeating information.

AU: the final part of the sentence at L 84-85 has been deleted.

Line 98: Delete 'b' after 'Sheriff et al. 2011'.

AU: removed

Line 110: […] scarce information is […]

AU: corrected

Line 111: Canada Lynx or Americaan Lynx (*Lynx canadensis*). Please write the complete common name.

AU: the complete common name has been specified.

Material and Methods
Line 125: 'areas' instead of 'area'.

AU: corrected

Line 142-143: Population densities were estimated…

AU: corrected

Line 154: 'warmer' instead of 'wormer'.

AU: corrected

Line 176: 'biometric measures' instead of 'biometrics measures'.

AU: corrected

Line 231: phosphate-buffered saline (PBS). Write the complete term and then the abbreviation.

AU: the complete term has been added

Line 237: I suggest starting the sentence using 'The HCC data…' instead of 'The concentration data…'.

AU: corrected

Line 237-238: 'Statistical analysis was…' or 'Statistical analyses were…'. Please correct.

AU: corrected

Line 249: least square means Â± standard error (SE). Please specify abbreviations when used for the first time.

AU: full name added.

Line 250: Spearman's correlation ranks…

AU: corrected

Line 251: Again, 'biommetric measures' instead of 'biometrics measures'. Please correct.

AU: corrected

Results
Line 260: […] was very close to statistical significance.

AU: corrected

Line 272: Animals from HD-SO showed…

AU: corrected

Line 273: […] individuals were concentrated […]

AU: corrected

Line 277-279: Try to maintain the same nomenclature/symbols when presenting the results for the correlation coefficients. The authors start using 'correlation coefficient', then use the Greek letter 'rho', then use the word 'correlation' only. Please standardise.

AU: sorry, our way to present the results was actually confusing. As we said in M&M, we calculated both Spearman’s correlation coefficients (ρ) and partial correlation coefficients (r). We made it clearer in the sentence at L 270-280.

Discussion and Conclusions
Line 290: Adrenocorticotropic hormone (ACTH). Again, please specify abbreviations when used for the first time.

AU: corrected

Line 308-309: Please enter a reference to support this sentence/statement.

AU: references have been added

Line 316: The authors should try to be more consistent regarding the values/information presented throughout the manuscript. Here it is said that population density in SNP can reach values up to 40 deer per km2, while values up to 30.8 deer per Km2 are referred in the line 147 (M&M section).

AU: corrected at L 146-147.

Line 321: HCC in red deer from HD-MO were […]

AU: corrected

Line 353: Please add also the scientific name of the chamois (*Rupicapra rupicapra*).

AU: added

References
Line 490-492: Koren et al. (2002) is not cited in the manuscript. In the previous version of the ms, this reference was associated with hair cortisol extraction method (in M&M section).

AU: the reference has now been deleted from the reference list.

Use of hair cortisol analysis for comparing population status in wild red deer (*Cervus elaphus*) living in areas with different characteristics

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Acknowledgements

The Authors are grateful to Andrea Cadei and Luciano Rossi (Hunting District of Morbegno, Italy), Jessica Franceschina (Hunting District of Alta Valle, Italy), Alessandro Gugiatti (Hunting District of Sondrio, Italy) and Andrea Zanoli (Stelvio National Park, Italy) for their help in data collection. The Authors declare that they have no conflict of interest.

Abstract

We tested a method to measure Hair Cortisol Concentration (HCC) in 174 red deer (*Cervus elaphus*) culled in the hunting season 2011/12 in four areas of Central Italian Alps, with different population densities (SPN: 11.8 deer/km^2; HD-AV: 3.6 deer/km^2; HD-SO: 2.1 deer/km^2; HD-MO: 2.0 deer/km^2) and environmental conditions. Our hypothesis was that higher population densities, associated with more difficult environmental conditions, may result in higher allostatic load for these wild ungulates. No significant differences in HCC were detected between sexes (males: 4.77±0.69 pg/mg; females: 5.75±0.63 pg/mg) nor among age classes (calves: 6.17±0.66 pg/mg; yearlings: 4.47±0.83 pg/mg; adults: 5.15±0.74 pg/mg; least square mean±SE), but HCC difference between calves and yearlings was close to statistical significance (P=0.059). HCC showed high individual variation, but on average it was higher in areas with higher deer density (SNP: 7.45±1.01 pg/mg; HD-AV: 6.07±0.89 pg/mg; HD-SO: 4.67±1.14 pg/mg; HD-MO: 2.87±1.56 pg/mg), with significant differences between HD-AV and HD-MO (P=0.01). Carcass weight was significantly lower in SNP (46.74±1.49 kg) than in HD-MO (62.71±4.01 kg), HD-SO (61.73±2.9 kg) and HD-AV (62.07±2.04 kg) (P<0.001).

These results seem to confirm our hypothesis that allostatic load is higher in areas with higher density and harder environmental conditions. We suggest that the methodology used in this study to measure HCC provides good information on long-term HPA axis activity and allostatic load and constitutes a highly promising, reliable and non-invasive method in wildlife management for assessing HPA axis activity over extended time periods.

Keywords: Red deer, *Cervus elaphus*, Hair, Cortisol, Allostatic load.

Introduction

The response to a stressor requires the animal to expend energy (biological cost of stress). When the biological cost of stress diverts resources away from physiological functions (immune competence, reproduction, growth), the animal may face a situation of distress (Moberg 2000).

One of the main physiological response to stress is the activation of the Hypothalamus-Pituitary-Adrenal gland axis (HPA). A chronic stimulation of the HPA axis leads to an increase of energetic costs for the animal and it can be especially significant in wildlife living in poor environmental conditions or during particular life stages (e.g. reproductive period, pregnancy, lactation) (Reeder and Kramer 2005). For these reasons, assessing the HPA axis activity could be an important tool in wildlife management, in order to monitor population conditions (McLaren et al. 2007).

Cortisol is a key glucocorticoid hormone of the HPA axis and plays an important role in allostasis, the active process of maintaining and/or reestablishing homeostasis, which helps an animal to adapt to a new situation and/or challenge (McEwen 1998). Cortisol has long been considered a reliable physiological indicator of the allostatic load, the cumulative result of an allostatic state, both in domestic and in wild mammals (McEwen and Wingfield 2003; 2010).

Chronic high concentrations of cortisol and other glucocorticoids deriving from the activity of the HPA axis may lead to a pathological syndrome characterized by metabolic modifications (as increase of gluconeogenesis, lipidic and proteinic catabolism) and depression of reproductive and immune activity, and have direct effects on the central nervous system (Moberg 2000; Charmandari et al. 2005; Macbeth et al. 2010).

Cortisol can be measured in the blood or, non-invasively, in faeces (Dehnhard et al. 2001;

Millspaugh et al. 2002; Huber et al. 2003; Ashley et al. 2011), urine (Rehbinder and Hau 2006), milk (Gygax et al. 2006) and saliva (Negrao et al. 2004). These methods provide a measurement of the cortisol concentration either at a single point in time or within 12-24 hours (Sheriff et al. 2011; Russell et al. 2012). Additionally, cortisol has been evaluated in the claw (Comin et al. 2014). Hair has long been used in toxicology, forensic science, doping control and other fields as a biological matrix for the detection of environmental agents, drugs or toxins because of its unique feature of providing a retrospective calendar of analyte exposure. Hair has been recognised as a matrix that may accumulate glucocorticoids over weeks to months (Davenport et al. 2006; Macbeth et al. 2010), offering potentially new methods of studying the effects of chronic stress, which is accompanied by a hyperactive HPA axis (Manenschijn et al. 2011).

Hair cortisol concentrations (HCC) are unaffected by circadian hormone variations or by factors that induce short-term variations. The collection of hair is simple and non-invasive. Furthermore, the sample does not decompose as rapidly as other body fluids or tissues (Balíková 2005). Hair is a relatively stable medium, known to incorporate blood-borne hormones through passive diffusion from blood capillaries present on the basement membrane during its active growth phase (Pragst and Balíková 2006). These hormones may remain detectable for long periods of time (Kintz et al. 2006; Webb et al. 2010).

Cortisol concentrations in hair provide an integrated rather than a one-time point measure of HPA axis activity (Meyer and Novak 2012), with no need of repeated sampling of individuals, as reported for other matrices by Owen et al. (2005) and by Keay et al. (2006). However there are a number of methodological concerns associated with this method that need to be resolved (Dantzer et al. 2014; Sheriff et al. 2011; Keckeis et al. 2012; Meyer and Novak 2012). Hair concentrations may also partly be the result of a local production

(Keckeis et al. 2012), and hair sampled from different parts of the body that have different rates of hair growth and different time scale show different glucocorticoid levels (Ashley et al. 2011; Terwissen et al. 2013).

In recent years, the quantification of HCC has been used for clinical applications in humans (Gow et al. 2010; Thomson et al. 2010). HCC measurements have been reported as a validated method in both ruminant (Comin et al. 2012a, 2013; Peric et al. 2013) and non-ruminant species (Comin et al. 2012b, Montillo et al. 2012) and in both domestic (Comin et al. 2011) and laboratory animals (Comin et al. 2012c). As to even-toed ungulates, studies on sheep (Stubsjøen et al. 2015), goats (Battini et al. 2015) as well as captive caribou (*Rangifer tarandus granti*) and reindeer (*R. t. tarandus*) have been carried out so far (Ashley et al. 2011). To our knowledge, scarce information is available about HCC in free-ranging large mammals: studies were carried out on Canada lynx (*Lynx canadensis*) (Terwissen et al. 2013), grizzly/brown bear (*Ursus arctos*) (Macbeth et al. 2010; Cattet et al. 2014), Asiatic black bears (*Ursus thibetanus*) (Malcolm et al. 2013), black bear (*Ursus americanus*) (Lafferty et al. 2015) and polar bears (*Ursus maritimus*) (Bechshøft et al. 2011; 2012; 2015; Weisser et al. 2016). Nevertheless, the possibility to investigate on long-term HPA axis activity without causing disturbance to the animals and collecting a large number of samples could be an interesting tool in wildlife management.

In this study, we tested a method to measure HCC in red deer (*Cervus elaphus*) culled in four areas with different population densities and environmental conditions, to check the hypothesis that higher population densities associated with more difficult environmental conditions (i.e. higher altitudes and consequent harsher climatic conditions and poorer vegetation cover) may result in higher allostatic load for these wild ungulates.

Materials and Methods

Study areas and animals

The study was carried out in four areas in Sondrio Province (Central Italian Alps), Lombardy region (Fig. 1). The subjects for this study were 174 red deer: 85 samples were collected from three Hunting Districts (HD) in Sondrio Province (Morbegno=HD-MO, Sondrio=HD-SO and Alta Valle=HD-AV) and 89 from a protected area (Stelvio National Park=SNP) (see Tab. 1 and Tab. 2 for details on sex and age class of samples from each sampling area). Age class estimation was carried out by experienced personnel on the basis of teeth eruption. In non-protected areas (HDs), animals were hunted during the hunting season (September-December 2011), following the rules of the National Law 157/92. In SNP, cullings took place during the biological control (January and February 2012), in the frame of the revised version of the "Plan for conservation and management of red deer in the Lombardy sector of the Stelvio National Park", which received a positive judgment by the National Higher Institute for Environmental Protection and Research (I.S.P.R.A.) and was approved in June 2010 by the Italian Ministry of the Environment. All cullings, both in the Hunting Districts and in the National Park, were achieved with specialized handguns equipped with optical aiming devices, without the aid of hounds.

The main characteristics of the sampling areas are summarized in Tab. 3. Population densities were estimated on the basis of the results of spotlight night counts (Corlatti et al. *in press*). SNP was characterized by particularly high population densities, with a wider spatial distribution of red deer in summer, leading to minimum densities of about 6.9 deer/km^2, while in winter and early-spring animals are concentrated at lower

elevations, where population density can reach values up to 40 deer/km^2 (Pedrotti et al. 2013). HD-MO and HD-SO are characterized by lower population densities, lower mean altitudes (Tab. 3) and consequently by a milder climate than the other areas, which are characterized by higher mean altitudes, which are responsible for an alpine-glacial climate (Tab. 4). In Sondrio Province, climate is characterized by annual average temperatures varying from the isotherm of 12°C (lake, moraine area and lowest altitudes), to that of 2.5°C (alpine area, from 1,700 to 2,400 m a.s.l.), up to the isotherm of 0°C above 2,900 m a.s.l.. Annual thermal excursion (difference between the average temperature in the warmer month and the average temperature in the colder one) is influenced primarily by geomorphology and varies from 21.6°C (low altitudes) to 14°C (alpine environment). Two rainfall regimes are described: the alpine (or continental) one, with one summer peak, and the sublitoral-alpine one, with two peaks (in Spring and Autumn). Snowiness and persistence of snow cover vary significantly with altitude, morphology, and exposure; furthermore, they vary from year to year. The orographic limit of the eternal snows in the sides exposed to North is about 2,700 m a.s.l., one of the lower in the Alps. The permanence time of snow cover increases about 10 days/100 meters of altitude. Substantially, in Sondrio Province we can identify three types of climate: i) sub-alpine (cold season, lasting four months); ii) alpine (above the tree line, with harsh winters, lasting six months); iii) glacial (with temperatures under 0°C, almost exclusively snowy precipitations and almost absent vegetation) (Ferloni 2012). The main tree and herbal species characterizing the different altitudinal levels are summarized in Tab. 4. Climatic conditions and vegetation cover are consequently different in the four areas, as a result of the different altitudinal ranges (Ferloni 2012).

Moreover, anthropic disturbance is higher in SNP and in part of HD-AV, due both to tourism in winter and in summer, and to domestic herds that reach the high pastures during the summer.

Supplementary food was never provided to the animals and no large predators were present in any of the considered areas.

Sample collection procedures

Each animal was identified by a numerical code; sex, age and biometric measures were recorded in a dataset with data relative to culling site, date and time. Biometric measures were collected following the guidelines by Mattioli and De Marinis (2009) and included: body length (BL, in cm), foot length (FL, in cm), height at withers (HW, in cm), jaw length (JL, in mm) and carcass weight of completely eviscerated and not skinned animals (CW, in kg).

Hair samples were stripped from the wither. They were dried if necessary and stored in paper envelopes at room temperature until being analysed.

Hair cortisol assay

Hair strands were washed in 5 ml isopropanol as proposed by Davenport et al. (2006) to minimize the risk of extracting cortisol from outside the hair and also to ensure the removal of any steroids on the surface of the hair due to sweat and sebum. Hair cortisol was extracted with 3 ml of methanol per 40 mg of hair for 18 h at 37°C. Samples were then centrifuged (15 min/1000 rpm) and the supernatant collected and transferred to a 12-mm glass test tube. The supernatant was dried at 37°C under a gentle stream of nitrogen gas and reconstituted with 0.3 ml of phosphate buffer.

HCC were measured using a solid-phase microtiter RIA assay. In brief, a 96-well microtitre plate (OptiPlate, Perkin-Elmer Life Science, Boston, MA, USA) was coated with goat anti-rabbit γ-globulin serum (diluted 1:1000 in 0.15 mM sodium acetate buffer at pH 9) and incubated overnight at 4°C. The plate was then washed twice with RIA buffer (pH 7.4) and incubated overnight at 4°C with 200 μL of the anti-cortisol serum diluted 1:12,000. The rabbit anti-cortisol antibody was obtained from Biogenesis (Poole, UK). After washing the plate with RIA buffer, standards (5–300 pg/well), a quality control extract, the test extracts (10 mg), and tracer (Hydrocortisone (Cortisol, [1,2,6,7-3H (N)]-), Perkin-Elmer Life Sciences, Boston, MA, USA) were added, and the plate was incubated overnight at 4°C. Bound hormone was separated from free hormone by decanting and washing the wells in RIA buffer. After the addition of 200 μL/well scintillation cocktail (Microscint 20, Perkin- Elmer Life Sciences), the plate was counted using a beta-counter (Top-Count, Perkin-Elmer Life Sciences).

Assay validation

To validate the method the sensitivity, specificity, precision, accuracy and parallelisms were investigated. As the biological validation of the assay in a free-living wild species is particularly complicated, it was not possible to carry it out on red deer, and for this experiment we referred to validation carried out in a domestic ungulate species (cow; Peric et al. 2013). The assay sensitivity (defined as the hormone concentration producing a displacement of the labeled hormone at least 2 standard deviations from maximal binding) was 1.23 pg/well. The specificity of the method, estimated by calculating the percentage cross-reaction with different steroids, was: cortisol 100%, corticosterone 1.8%, and aldosterone <0.02%. The precision of the method estimated by repeatedly assaying samples in the same assay and in independent assays was expressed by intra-

assay and inter-assay coefficients of variation (CV%) of hair sample. The intra- and inter-assay coefficients of variation were 3.6% and 9.8%, respectively.

To evaluate assay accuracy, possible interference of components within the extract with antibody binding was analyzed through recovery of exogenous cortisol added to pooled red deer hair extracts. Each of four reconstituted hair extracts were divided in three independent aliquots and spiked with three different known cortisol concentrations, mixed, and assayed. The percentage of recovery was determined as follows: amount observed/amount expected x 100, where the amount observed is the value obtained from the spiked sample and the amount expected is the calculated amount of standard hormone added plus the amount of endogenous hormone in the unspiked sample. Recovery rate was 97.6 ± 1.6% (mean ± SD). The measured hormone concentrations in the spiked samples correlated with the expected concentrations: r was 0.99 and the model was given by the equation y = 0.986 x -0.43. To determine the parallelism between cortisol standards and endogenous cortisol in red deer, hair samples containing high concentrations of endogenous cortisol were serially diluted in phosphate-buffered saline (PBS) 0.05 M, pH 7.5. The relationship between hair cortisol and standard cortisol curve determined through linear regression was linear: the correlation coefficient r was 0.99 and the model was given by the equation y = 1.012 x -1.55.

Statistical analysis

The HCC data, expressed in pg/mg, were stored using MS Excel 2010. Statistical analysis was performed with SPSS (IBM® SPSS® Statistics, vers. 22).

A Generalized Linear Model (GzLM; Distribution=normal; Link=identity) was used to analyse variation in HCC including the effects of sex (2 levels: males and females), age

class (3 levels: calves (< 1 year); yearlings (1-2 years); adults (> 2 years)), culling area (4 levels: SNP, HD-AV, HD-SO and HD-MO) and the interaction sex*age class. Culling date was included in the model as a covariate, in order to correct for this effect. HCC classes were created on the basis of HCC values (8 classes: < 3; 3-3.99; 4-4.99; 5-5.99; 6-6.99; 7-7.99; 8-9.99; > 9.99 pg/mg), in order to allow a better graphical representation of data.

GzLM was used to analyse also variation in CW including the effects of sex, age class, sex*age class and culling area.

Results were expressed as least square means ± standard error (SE).

Spearman's correlation ranks were calculated between HCC and each biometric measure (BL, FL, HW, JL and CW). All correlations between HCC and biometric measures were also tested using a partial correlation, corrected for age, in order to exclude the effect of age class from the results.

A probability value of less than 0.05 was considered as significant.

Results

No significant differences in HCC were detected between sexes (males: 4.77±0.69 pg/mg; females: 5.75±0.63 pg/mg) nor among age classes (calves: 6.17±0.66 pg/mg; yearlings: 4.47±0.83 pg/mg; adults: 5.15±0.74 pg/mg; least square mean±SE), although HCC difference between calves and yearlings was very close to statistical significance (P=0.059).

Although the overall effect of the culling area on HCC only approached statistical significance (P=0.08), pairwise comparisons between areas highlighted lower values in HD-MO than in HD-AV (P=0.01) and SNP (P=0.06) (Fig. 2). No significant differences

among the other areas were found, but HCC was always higher in areas with higher population density (Fig. 2). HCC descriptive statistics (minimum, 25th percentile, median, 75th percentile and maximum) for each sampling area are reported in Tab. 5.

The percentages of calves and yearlings+adults falling in each HCC class are shown in Figg. 3 and 4, respectively. Given the limited number of yearlings and considering that no differences in HCC were evidenced between yearlings and adults, these two age classes were considered together for this graphical representation. HCC classes higher than 8.0 pg/mg were observed only in HD-AV and SNP, where they reached the highest HCC value (43.18 pg/mg). Animals from HD-SO showed the more homogeneous distribution: all the individuals were concentrated in the three central HCC classes, with no extremely high and no extremely low values.

CW was significantly lower in SNP (46.74±1.49 kg) than in HD-MO (62.71±4.01 kg), HD-SO (61.73±2.9 kg) and HD-AV (62.07±2.04 kg) ($P<0.001$).

Significant negative Spearman's correlations were detected between HCC and HW (ρ=-0.179; $P<0.05$), and HCC and CW (ρ=-0.161; $P<0.05$). Partial correlations, corrected for age (HCC-HW: r=-0.048; $P>0.05$; HCC-CW: r=-0.052; $P>0.05$), were not significant. No significant correlations were detected between HCC and other biometric measures (BL, FL and JL; $P>0.05$).

Discussion

Because to our knowledge this is the first study carried out on HCC in free-ranging wild red deer, it was not possible to directly compare the results obtained in the present study with others related to the same species. An overview of the HCC range of values for indirect comparison can be provided by cortisol determinations in hair samples of captive

Alaskan caribou (*Rangifer tarandus granti*) and reindeer (*R. t. tarandus*) populations (Ashley et al. 2011). The mean HCC in shoulder hair of caribou and reindeer before dosing an injection of adrenocorticotropic hormone (ACTH) was 1.5 and 3 pg/mg, respectively. In goats with normal hair, mean HCC was 27.03 ± 0.89 pg/mg (Battini et al. 2015). In cows, HCC values ranged between 0.8 and 41.74 pg/mg (Comin et al. 2011, 2012a, 2013; Peric et al. 2013; Biancucci et al. 2016).

HCC in calves showed a wide range of variation, highlighting a different activation of the HPA axis for each individual. The higher HCC found in calves, compared to other age classes, is in agreement with the results reported by Maiero et al. (2005) in cattle. The weaning period may explain this high HCC: in the Northern hemisphere, red deer calves are born in late spring to early summer (May-June) and they are usually weaned at the age of 5-7 months (Mattiello and Mazzarone 2010). Samples collected in the present study were from 7-8 month-old calves. Therefore, these animals had probably just been weaned or close to weaning: this phase could represent a stressful challenge (Griffin et al. 1988; Pollard et al. 1992; Zavy et al. 1992; Pollard et al. 1998), justifying the observed HCC, that were higher in calves than in other age classes.

In spite of the fact that red deer is a highly sexually dimorphic species, no differences were detected in HCC between males and females. Similar results were observed in their related species, the Alaskan caribou and the reindeer, as reported by Ashley et al. (2011). Cortisol concentrations are closely linked to the activity of the HPA axis and its activation is usually related to an allostatic load. The activity of the HPA axis can be affected by age, biological and mental state (Kudielka and Kirschbaum 2005; Mormède et al. 2007; Jacobson 2014; Gaffey et al. 2016). It is therefore difficult to assess the effect of the sexual dimorphism on cortisol concentrations in the long term, as evaluated by HCC. A

study carried out by punctual sampling as that of blood or saliva or by stimulation test could provide this kind of information.

From a wildlife management point of view, one of the most important differences among culling areas is the red deer density, in particular during the winter. In the whole study area it ranges between 1 and 4 individuals/km^2, but in the HD-AV and in the SNP it can reach higher densities, up to 40 individuals/km^2 in some winter areas in SNP (Pedrotti et al. 2013). High-density populations are usually subject to a series of problems like infertility, decrease of body weight (Vincent et al. 1995; Toïgo et al. 2006), behavioural alterations, and increase of fecal cortisol concentration (Li et al. 2007). Findings from the present study seem to confirm a higher allostatic load in the two areas with higher density and in which environmental conditions are harder: HCC in red deer from HD-MO was significantly lower than in deer from HD-AV and SNP, even though the difference between HD-MO and SNP only approached statistical significance. The difference between the two high-density areas (HD-AV and SNP) and other culling areas is even more noticeable when observing Figg. 3 and 4: only in HD-AV and SNP some individuals reached HCC above 8.0 pg/mg, whereas individuals from HD-MO, in which the lowest HCC in the total sample was detected, were concentrated mainly in the lowest cortisol concentration classes.

The significantly lower CW recorded in SNP compared to that of other culling areas, further supports the hypothesis of a higher allostatic load in the SNP than in the other areas, probably due above all to the high density of red deer in this area. In fact, in wild ungulate populations, body measures can decrease with increasing density (Toïgo et al. 2006), as confirmed by our data.

The negative correlations between HCC and HW and between HCC and CW are actually biased by the effect of age class and cannot be considered reliable: in fact, if age effect is introduced for the calculation of partial correlations, results are no longer significant. Therefore, the apparent negative correlation was most probably due to the presence of calves (with higher HCC and smaller dimensions than other age classes) in the sample.

Findings concerning differences in HCC and CW between culling areas and the distribution of HCC classes in the different sampling areas seem to indicate an increase of allostatic load in SNP and in HD-AV. It seems reasonable to hypothesize that these results are related to the higher population density in these areas, which can lower the availability of trophic resources and can therefore stimulate competitive interactions among individuals. Other possible explanations for these results are related to the different levels of anthropic disturbance in our sampling areas: SNP and neighbouring areas are very popular tourist destinations, both in winter and in summer. This may represent an important source of disturbance especially in winter, when snow cover is deep and makes particularly hard for deer to move and find food, increasing energy expenditure (Jeppesen 1987; Schmidt 1993). This hypothesis seems to be confirmed by the increased fecal glucocorticoid levels recorded in different species in response to various sources of human disturbance, such as snowmobiles (in wolves *Canis lupus* and in the North American elk *Cervus elaphus*; Creel et al. 2002) or other tourist activities (in chamois *Rupicapra rupicapra* and mountain hare *Lepus timidus*; Zwijacz-Kozica et al. 2013 and Rehnus et al. 2014, respectively). Furthermore, several domestic herds (mainly dairy cows) reach the high pastures of SNP during the summer and may cause disturbance to wild red deer, especially during milking procedures (Mattiello et al. 2002; Mattiello et al. 2003). High prevalence of some disease may be another important reason for

increasing allostatic load: in SNP red deer population a high prevalence of paratubercolosis, a chronic disease caused from *Mycobacterium avium subspecies paratuberculosis*, that leads animals to death for dehydration and severe cachexia, has been observed (Garbarino et al. 2014).

Conclusions

The analysis of cortisol concentration in hair provides useful information to evaluate the HPA axis activity over extended time periods in free-ranging red deer and allows to identify animals with high chronic cortisol concentrations, which are difficult to identify otherwise, due to a subclinical stimulation of the HPA axis.

In particular, findings from this study suggest that HCC provides a good index of long-term HPA axis activity and allostatic load in red deer from areas with higher population density, higher anthropic disturbance, and harder environmental conditions.

Hair samples can be easily collected from culled deer, overcoming the problems related to capturing live animals in adverse field conditions (e.g. bad weather conditions, difficulties to reach the capture sites) and reducing both the costs for capturing wild animals and the risks for the animals' and operators' safety during capture and handling. Furthermore, the collection of hair samples from culled animals does not require the presence of experienced operators or specific devices for the collection and storage of the samples. The lack of the necessity to capture and handle animals would enable the researchers to collect a large number of samples from a population and, together with the assessment of substances in hair, would allow wide spectrum investigations on free-ranging red deer population status. These future applications could be further enhanced

by the possibility to collect hair samples from free-ranging living red deer using hair snares (Belant et al. 2007).

The results of the present study could provide a basis for developing similar assays in other free-ranging species, remembering that, for each species, validation of the method and of the results are always required. Unfortunately, in our investigation the impossibility to validate the assay specifically in red deer represents a potential limitation for the interpretation of our results.

In conclusion, the assessment of cortisol concentration in the hair seems to be an interesting tool for future wild red deer management. Such a non-invasive method to assess the population status could play an important role for research and management in free-ranging large mammals, reducing sampling efforts for researches and disturbance and risks for animals. Using the method proposed in this study, associated to a sample collection by hair snares (Belant et al. 2007), wide spectrum investigations on free-ranging population status could be easily achievable.

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Table 1. Absolute number (n) and relative percentages (%) of available samples for each sex in each sampling area.

	Males		Females		
Sampling area	**n**	**%**	**n**	**%**	**Total**
HD-MO	11	39.29	17	60.71	28
HD-SO	8	47.06	9	52.94	17
HD-AV	23	57.5	17	42.5	40
SNP	34	38.2	55	61.8	89
Total	76	43.68	98	56.32	174

Table 2. Absolute number (n) and relative percentages (%) of available samples for each age class in each sampling area (calves: < 1 year; yearlings: 1-2 years; adults > 2 years).

	Calves		Yearlings		Adults		
Sampling area	**n**	**%**	**n**	**%**	**n**	**%**	**Total**
HD-MO	8	28.57	8	28.57	12	42.86	28
HD-SO	9	52.94	4	23.53	4	23.53	17
HD-AV	10	25.00	5	12.50	25	62.50	40
SNP	30	33.71	11	12.36	48	53.93	89
Total	57	32.75	28	16.09	89	51.15	174

Table 3. Characteristics of the four sampling areas (three Hunting Districts - HD - and Stelvio National Park - SNP) in terms of pre-reproductive red deer density and altitude.

Sampling area	Deer density (deer/km^2)[a]	Altitude (meters a.s.l.)			
		Mean	Median	Min	Max
HD-MO	2.0	1431.5	1556.0	197.0	3596.0
HD-SO	2.1	1799.3	1773.0	259.0	3985.0
HD-AV	3.6	2213.9	2150.0	749.0	3444.0
SNP	11.8	2539.6	2614.0	1164.0	3851.0

[a] Data referred to pre-reproductive densities in 2011 (data provided by the Management Committees of the Hunting Districts and Stelvio National Park; Corlatti et al., *in press*).

Table 4. Summary of main vegetal species at different altitudinal levels (Ferloni, 2012).

Altitudinal level	Altitude range (m a.s.l.)	Tree species	Herbal species
Sub-mountain	500-1,000	*Fagus sylvatica* *Abies alba* *Larix decidua* *Picea abies* *Sorbus aucuparia* *Cytisus laburnum* *Calluna vulgaris*	*Trisetum flavescens* *Trifolium montanum* *Ranunculus montanus* *Campanula barbata* *Trolius europaeus*
Mountain	1,000-1,400	*Picea abies* *Larix decidua* *Vaccinium* spp. *Rhododendrum ferrugineum* *Rubus idaeus*	*Festuca ovina* *Melampyrum sylvaticum* *Campanula barbata* *Veronica officinalis* Mosses *(Hylocomium splendens, Rhytidiadelohus triquetus)*
Sub-alpine	1,400-1,800	*Larix decidua* *Pinus cembra* *Pinus montana var. mughus* *Picea abies* *Alnus viridis* *Juniperus communis var. nana*	*Festuca ovina capillata* *Nardus stricta* *Trifolium montanum* *Trifolium alpinum* *Carex* spp. *Juncus* spp.
Lower alpine	1,800-2,400	*Rhododendrum ferrugineum* *Vaccinium* spp. *Pinus montana var. mughus* *Alnus viridis*	*Nardus stricta* *Carex ferruginea* *Salix pentantra* *Salix purpurea*
Alpine	2,400-2,700	-	*Carex curvula* *Carex firma* *Carex elyna*
Nival	> 2,700	-	*Carex curvula* *Carex firma* *Carex elyna* *Saxsifraga panicolata* *Saxifraga aizoon* Mosses and Lichens

Table 5. Hair cortisol concentrations (pg/mg) minimum, 25th percentile, median, 75th percentile, and maximum values in each age class, for each sampling area and in the total sample.

Calves						
Sampling area	**n**	**Min**	**25th percentile**	**Median**	**75th percentile**	**Max**
HD-MO	8	3.97	4.49	4.65	5.8	7.85
HD-SO	9	4.15	4.64	5.78	6.81	6.95
HD-AV	10	3.72	4.76	5.6	6.97	12.53
SNP	30	3.84	5.56	6.48	9.05	29.19
TOTAL	57	3.72	4.77	5.92	7.72	29.19
Yearlings						
Sampling area	**n**	**Min**	**25th percentile**	**Median**	**75th percentile**	**Max**
HD-MO	8	2.09	3.04	3.96	4.25	6.12
HD-SO	4	4.31	4.4	5.48	6.53	6.6
HD-AV	5	5.13	5.24	5.55	6.34	6.64
SNP	11	3.07	3.6	4.66	5.77	7.29
TOTAL	28	2.09	3.92	4.65	5.82	7.29
Adults						
Sampling area	**n**	**Min**	**25th percentile**	**Median**	**75th percentile**	**Max**
HD-MO	12	2.49	2.95	4.59	4.95	6.04
HD-SO	4	5.31	5.36	6.13	6.81	6.83
HD-AV	25	2.72	4.94	6.44	8.49	18.66
SNP	48	2.9	4.33	4.9	7.15	43.18
TOTAL	89	2.49	4.36	5.07	7.07	43.18

FIGURE CAPTIONS

Figure 1. Location of Sondrio Province in Lombardy region (Italy) and of the four sampling areas (three Hunting Districts - HD - and Stelvio National Park - SNP) in Sondrio Province.

Figure 2. Hair Cortisol Concentration (pg/mg) in the four sampling areas (least square means ± SEM).

Figure 3. Percentage of calves from each sampling area in each Hair Cortisol Concentration class.

Figure 4. Percentage of yearlings+adults from each sampling area in each Hair Cortisol Concentration class.

Figure 1

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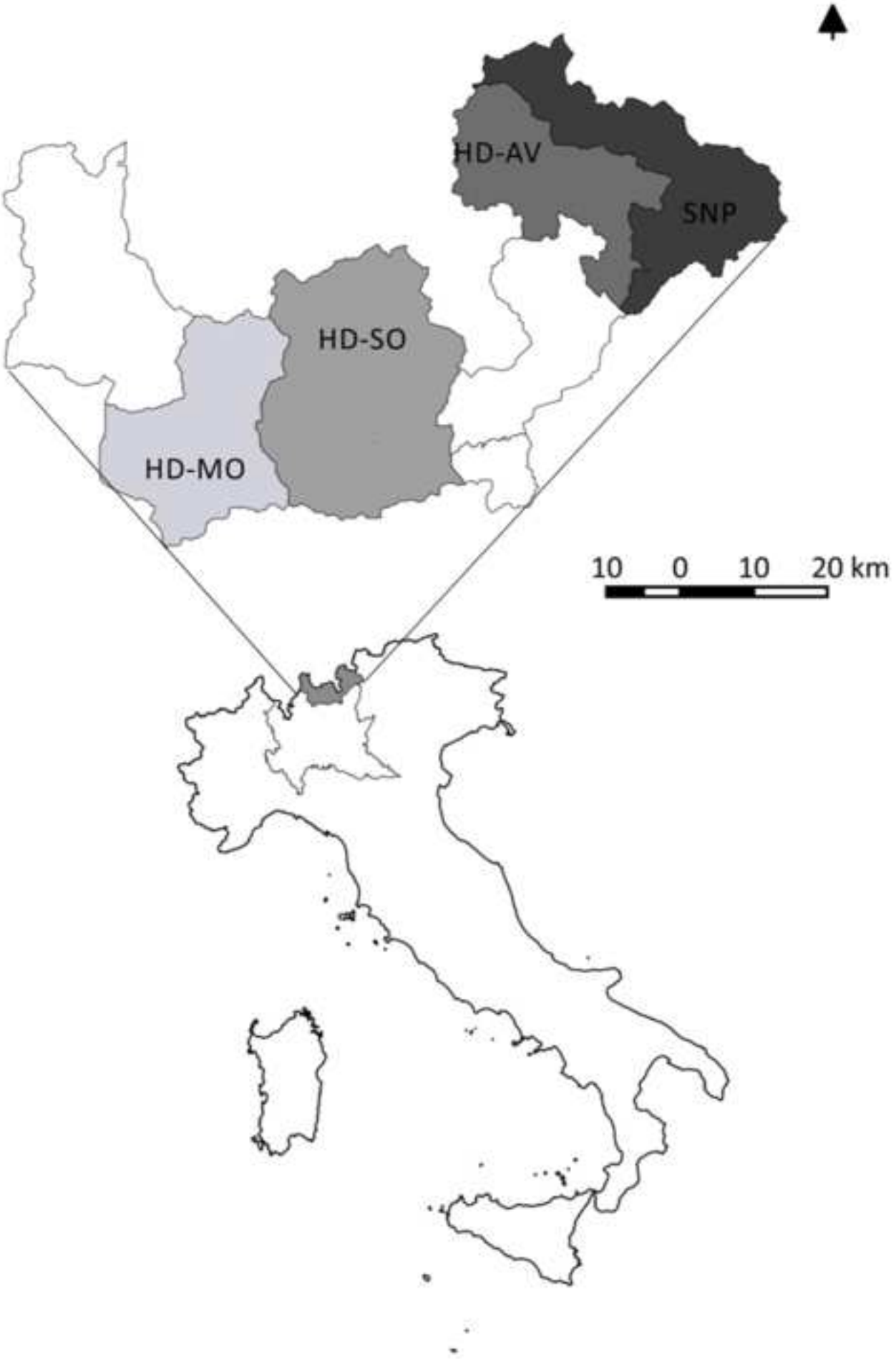

Figure 2

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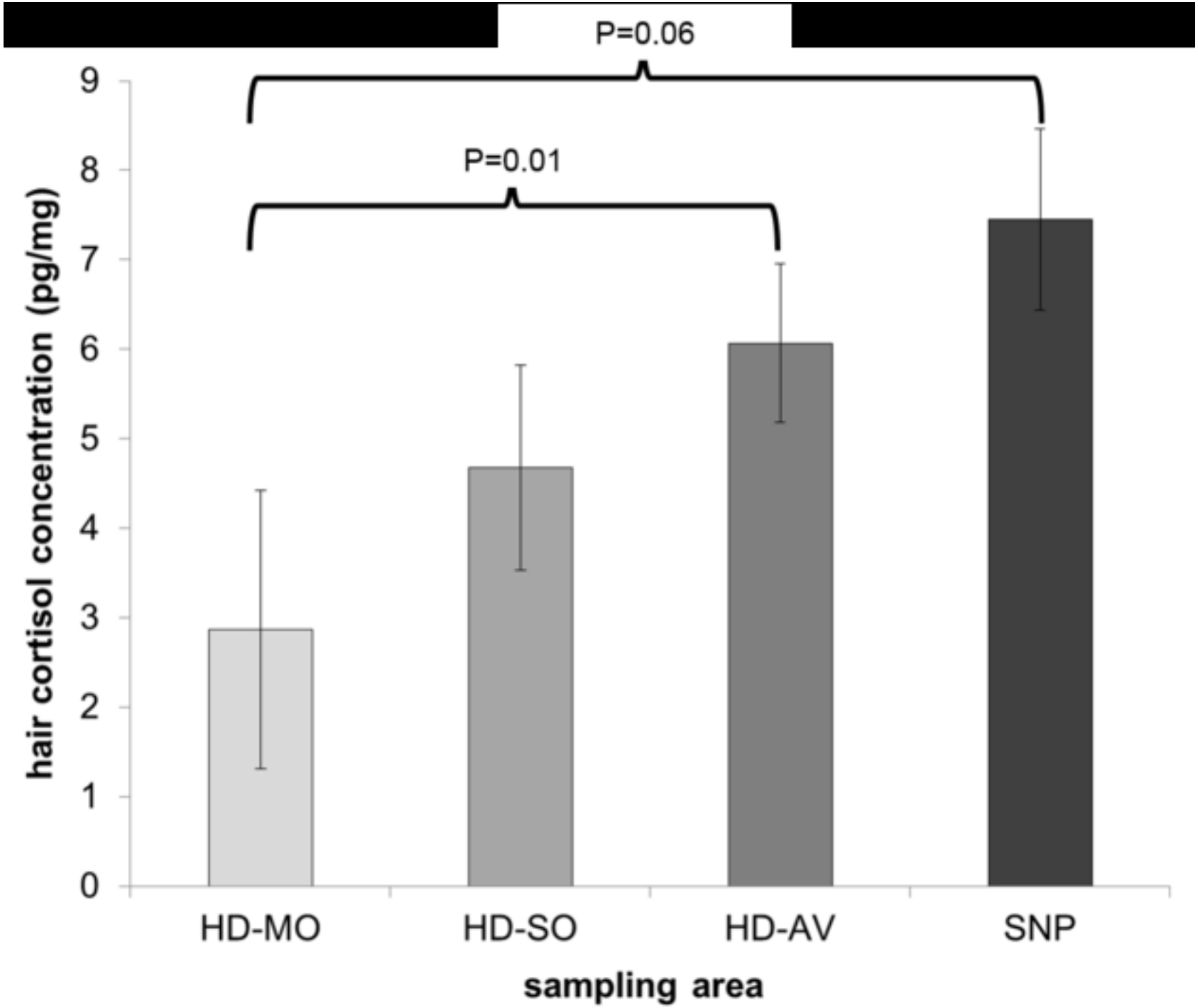

Figure 3

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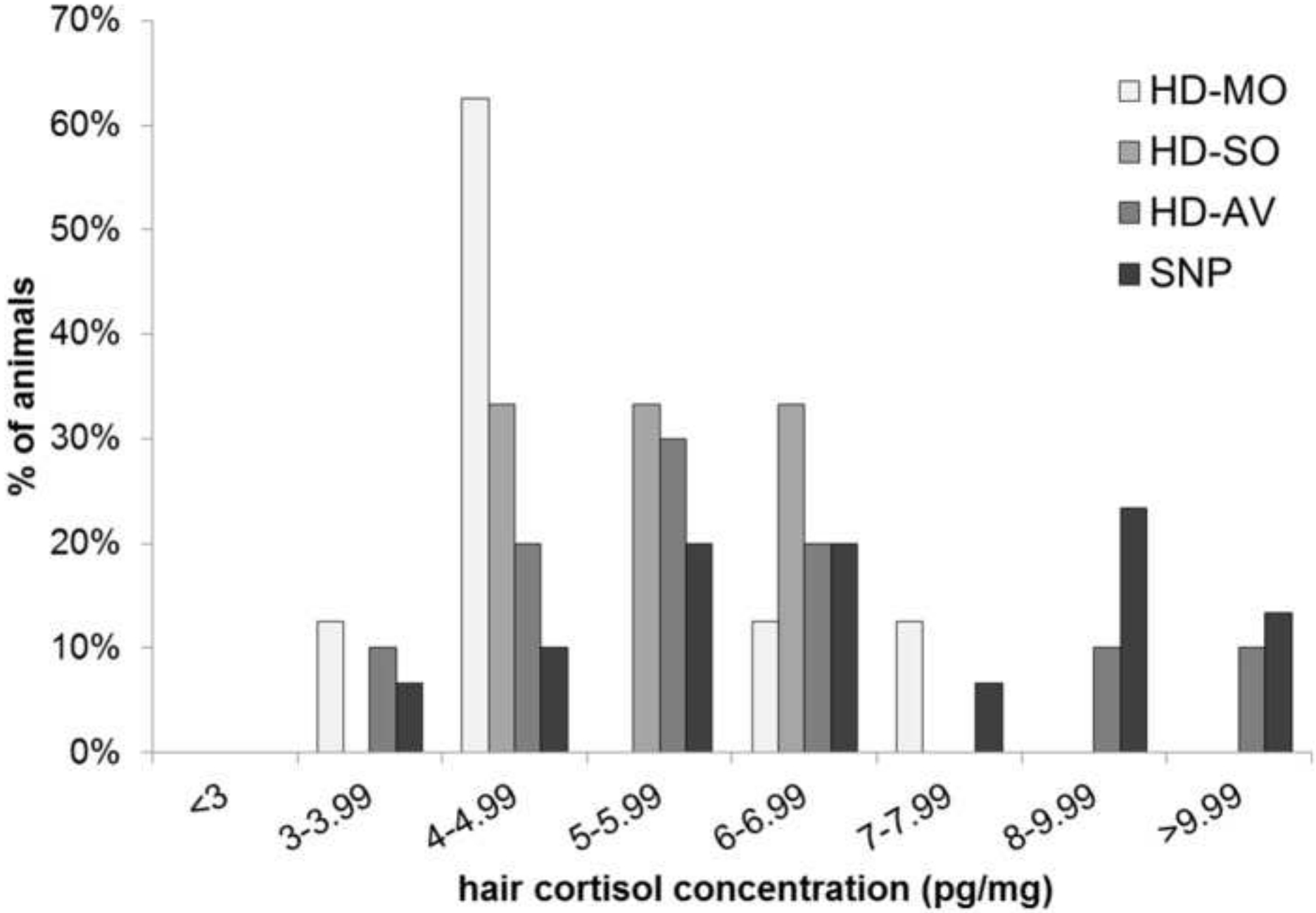

Figure 4

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