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Effects of season, sex, and sample collection on concentrations of fecal cortisol metabolites in red deer (*Cervus elaphus*)

S. Huber,[a,*] R. Palme,[b] and W. Arnold[a]

[a] *Research Institute of Wildlife Ecology, University of Veterinary Medicine, Savoyenstr. 1, A-1160 Vienna, Austria*
[b] *Institute of Biochemistry and Ludwig Boltzmann Institute of Veterinary Endocrinology, University of Veterinary Medicine, Veterinärplatz 1, A-1210 Vienna, Austria*

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Abstract

Seasonal variation, sex differences, and invasive sample collection may confound glucocorticoid measures as indices of stress. We investigated the effects of sex and season on glucocorticoid production on a non-invasive basis by measuring concentrations of cortisol metabolites in feces of undisturbed red deer (*Cervus elaphus*). Although feces can be collected easily, assignment to individuals is difficult. Anonymous fecal samples may cause overrepresentation of particular individuals thus introducing a source of error when estimating mean hormone levels within a population. We therefore examined the effects of collecting anonymous fecal samples on mean fecal cortisol metabolite levels. Neither sex nor sample collection mode significantly affected mean fecal cortisol metabolite concentrations in the studied population of red deer. Fecal glucocorticoid excretion varied seasonally with a peak during December and January. Out of several potential predictor variables investigated, minimum ambient temperature and snow proved to be the only factors exerting a significant effect on fecal glucocorticoid excretion. We suggest that high winter glucocorticoid levels may act via catabolic function during adaptation of deer to cold winter month when resources are limited.

Keywords: Red deer (*Cervus elaphus*); Glucocorticoids; Stress; Metabolism; Feces; Non-invasive; Genotyping

1. Introduction

Glucocorticoids are known as stress hormones mobilizing energy reserves needed to cope with environmental challenges (Sapolsky, 1992). Their major endocrine function targets metabolism (Nelson, 2000). However, the adrenocortical response to environmental stressors may be confounded by daily and seasonal variations of basal glucocorticoid production. In addition, sex and reproductive state may have an impact on glucocorticoid secretion as sex specific differences in glucocorticoid concentrations have been described in a variety of species (e.g., Boonstra et al., 2001; Ruis et al., 1997; Wingfield and Farner, 1993).

Using blood glucocorticoid concentrations as indices of adrenocortical activity, sample collection may itself induce corticosteroid secretion, thereby interfering with the adrenocortical response under investigation (LeMaho et al., 1992; Reinhardt et al., 1990). Therefore, non-invasive methods measuring glucocorticoid metabolites in feces have been developed (Dehnhard et al., 2001; Palme and Möstl, 1997; Palme et al., 1999). Fecal glucocorticoid measures can be obtained easily without any need to handle or even observe the animals and provide an integrated measure of fluctuating blood concentrations during the previous 1–2 days (Monfort et al., 1998; Palme et al., 1999). Although feces represent a readily available source of information, assignment to individuals is difficult. With fecal sampling on an anonymous basis, feces from particular individuals might be overrepresented, comprising a source of error. Thus, reliable estimation of mean hormone levels within a population may need information on sex and identity of the defecator.

The present study was aimed at investigating the effects of season, sex, and collection of anonymous fecal

* Corresponding author. Fax: +43-1-4890915-333.
E-mail address: susanne.huber@vu-wien.ac.at (S. Huber).

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samples on fecal glucocorticoid excretion in undisturbed red deer (*Cervus elaphus*), living in a large enclosure under semi-natural conditions to determine whether these factors confounded fecal glucocorticoid metabolites' measures.

2. Methods

2.1. Data collection

We performed the study in 1999 and 2000 on a red deer herd (8 adult females, 3 yearling females, 1 juvenile female, 3 adult males, and 2 yearling males), kept in a 45-ha enclosure at the Research Institute of Wildlife Ecology in Vienna, Austria. Apart from supplemental feeding during November to March and occasional immobilizations, no disturbances occurred in the enclosure. We collected blood samples from 14 deer (12 females and 2 males) during immobilization necessary for transportations or insertion of ear tags. We took blood samples from the jugular vein into EDTA-tubes and froze the whole blood immediately at −20 °C. From three male deer, we collected fecal samples immediately after we had observed the respective animal defecating. We collected additional fresh fecal samples on an anonymous basis once a week for one year opportunistically throughout the enclosure ($n = 344$). Not all 17 deer were kept in the enclosure during the whole collection period. Eight individuals (6 females and 2 males) were gradually transported (after immobilization) to another enclosure towards the end of the sampling period. After disturbance due to an immobilization, we did not collect fecal samples for 1 week. All fecal samples were put into separate plastic tubes and frozen immediately at −20 °C.

We obtained weather data measured at Vienna "Hohe Warte" (<10 km away from the institute) from wetteronline.de/österreich: minimum and maximum ambient temperature (°C), precipitation (mm), snow (mm), and wind (km/h). We included data determined at the day prior to fecal sample collection into analysis, taking into account an approximate 18 h excretion time lag (Huber et al., submitted).

We subdivided the year into reproductive phases using mean dates of beginning and end of these phases following Haigh and Hudson (1993): gestation, 16 September to 31 May; lactation, 1 June to 15 September; rut, 1 September to 31 October.

2.2. Determination of fecal cortisol metabolites

As cortisol is the major glucocorticoid in deer (Smith and Bubenik, 1990; Van Mourik et al., 1985), we previously validated a fecal cortisol metabolite assay measuring a group of cortisol metabolites (3a-11-one cortisol metabolites) for the use in red deer (Huber et al., submitted). Analysis of HPLC fractions of red deer fecal extracts with a group specific 11-oxoetiocholanolone-enzyme immunoassay measuring 3a-11-one cortisol metabolites (Möstl et al., 2002) showed high amounts of immunoreactive cortisol metabolites, whereas only minor portions of immunoreactive steroids eluting around the respective standards were detected with a cortisol- and a corticosterone-EIA, respectively (Huber et al., submitted). An adrenocorticotropic hormone (ACTH) challenge test revealed the biological significance of this assay. ACTH administration resulted in a 6.5- to 20-fold increase in fecal cortisol metabolite levels after approximately 18 h (Huber et al., submitted).

We extracted 0.5 g of each fecal sample with 4.5 ml of methanol (90%). After shaking (40 min) and centrifugation (2.500*g*, 15 min), we determined the amounts of cortisol metabolites (3a-11-one cortisol metabolites) in the supernatant by the 11-oxoetiocholanolone EIA (Möstl et al., 2002). The crossreactions of the EIA were as follows: 5β-androstane-3α-ol-11,17-dione, 100%; 5β-pregnane-3α-ol-11,20-dione, 37%; 5β-androstane-3α, 11β-diol-17-one, 3.3%; and 5β-androstane-3,11,17-trione, 1.2%. Intraassay and interassay coefficients of variation for pooled fecal samples were 10.2 and 17.7%, respectively. Detection limit, based on 90% of maximum binding, was 0.05 μmol/kg. All steroids were purchased from Steraloids (Wilton, USA).

2.3. Genotyping of fecal samples

2.3.1. DNA extraction

We isolated DNA from blood as described by Gustincich et al. (1991). We extracted DNA from feces combining two modified protocols—a silica-based fecal DNA purification method based on the protocols of Boom et al. (1990) and Kohn et al. (1995), and a plant DNA extraction protocol based on Hauser et al. (1998) with modifications following S. Kampfer (pers. comm.)—as described by Huber et al. (2002). Briefly, we added 720 μl CTAB (hexadecyltrimethyl-ammonium bromide) solution (2% CTAB, 100 mM Tris–HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, and 1% polyvinylpyrollidone) to approximately 10 mg of feces. Following overnight incubation at 65 °C, we added 540 μl dichlormethane to extract CTAB. We transferred the supernatant to a fresh tube and added 700 μl lysis buffer (0.1 M Tris–HCl, pH 6.4, 0.02 M EDTA, pH 8.0, 1.3% Triton X-100, and 10 M guanidinethiocyanate [GuSCN]). After incubation for 2 h at 60 °C, we added 450 μl of lysis buffer and 40 μl silica suspension, prepared as described by Boom et al. (1990). We incubated the mixture under constant agitation for 15 min at room temperature and washed the silica pellet twice with 0.8 ml washing buffer (0.1 M Tris–HCl, pH 6.4, 10 M GuSCN), twice with 1 ml of 70% ethanol and once with

1 ml acetone. We eluted DNA with 10 mM Tris–HCl, pH 8.0 and froze the extracts at −20 °C. We included an extraction control (a tube without feces treated identically during extraction and PCR) in the extraction procedure.

2.4. PCR amplification and electrophoresis

We genotyped the 17 red deer using DNA extracted from blood samples of 14 individuals and DNA extracted from fecal samples of 3 individuals. Two primer sets that had been developed for microsatellite loci in cattle (BM203, BM4107; Bishop et al., 1994) and found to be polymorphic in elk (Talbot et al., 1996) proved to be sufficient to identify each individual (allele patterns BM203-BM4107: 218/218-156/156, 216/222-156/156, 216/228-156/156, 216/228-168/168, 216/236-144/168, 216/222-144/168, 220/222-158/174, 220/228-156/166, 220/228-156/168, 222/222-156/156, 222/222-144/168, 222/228-166/166, 222/228-154/162, 222/238-144/156, 222/228-144/144, 228/236-144/156, 214/220-158/168). Genotyping of fecal samples also provided information on the sex of the defecator. We randomly selected 101 fecal samples out of the 344 collected fecal samples for genotyping. We extracted each fecal sample at least twice and performed at least two PCR amplifications from each fecal DNA extraction. Thus, each fecal sample was genotyped at least four times. In case of ambiguous results, we performed additional 1–2 extractions and 1–2 PCR amplifications/extraction. Nevertheless, we did not obtain a reliable result in 13 out of 101 analyzed fecal samples. The remaining 88 genotyped fecal samples were included in the analysis of sex differences of basal fecal cortisol metabolite levels. We calculated monthly means of fecal cortisol metabolite levels per individual whenever it was sampled more than once per month from the genotyped fecal samples, and compared these mean values/individual/month ($n = 60$) to mean values/month derived from all collected fecal samples to examine whether sample collection on an anonymous basis confounded the results.

We performed individual PCR amplifications in 25 μl reactions containing 1.5 μl (~50–100 ng) DNA extract as template, 15 pmol forward primer, 15 pmol reverse primer, 130 μM each dNTP, 10 mM Tris–HCl, pH 8.8, 1.5 mM $MgCl_2$ (BM203), or 1.65 mM $MgCl_2$ (BM4107), respectively, 150 mM KCl, 0.1% Triton X-100, and 0.5 U DyNAzyme II DNA polymerase (Finnzymes, Finland). The 5′-end of the forward primer was fluorescently labeled (MWG Biotech, Germany). We performed hot-start PCR on a RoboCycler 96 with a heating lid (Stratagene, USA). Cycling conditions were: initial denaturation for 20 min at 95 °C for 1 cycle followed by addition of polymerase at room temperature and 40 cycles of 40 s at 95 °C, 40 s at 56 °C (BM203), or 58 °C (BM4107), respectively, 50 s at 72 °C, and a final extension of 10 min at 72 °C. We included positive and negative (reagents without template) DNA controls in all PCR. We avoided contamination by using aerosol-resistant pipette tips and separation of pre- and post-PCR experiment (Taberlet et al., 1999). We concentrated PCR products obtained from fecal extracts prior to gel electrophoresis by evaporation. PCR products were run on 0.25 mm polyacrylamide gels (Long Ranger, FMC) in a Gene ReadIR 4200 automated sequencer (LI-COR). We calculated allele sizes relative to a size standard (LI-COR) using Gene ImagIR version 3.52 (LI-COR).

2.5. Analysis of data

We conducted statistical analyses using SPSS 7.5 for Windows statistical package. As data were not normally distributed, we performed square root transformation of the original data prior to analysis. Data are plotted as medians and quartiles. Comparisons among months were preformed using one-way analysis of variance (ANOVA) with Tukey test for post hoc testing. We performed multiple linear regression to determine the effects of potential predictor variables on fecal cortisol metabolite levels. The relationship between minimum ambient temperature and fecal cortisol metabolite concentrations were analyzed with non-linear regression and the effects of sex and sample collection on fecal cortisol metabolite levels, respectively, were analyzed using two-way ANOVA with month as the second factor. We obtained significance values from two-tailed statistics.

3. Results

3.1. Seasonal pattern of concentrations of fecal cortisol metabolites

Fecal cortisol metabolite levels varied with season. Mean concentrations were significantly higher during December and January than during the remainder of the year (Fig. 1). We examined the effects of the following potential predictor variables on fecal cortisol metabolite levels: reproductive phase, minimum and maximum ambient temperature, snow, precipitation, and wind. The only factors exerting a significant effect proved to be minimum ambient temperature and snow (Fig. 1), while reproductive phase, maximum ambient temperature, precipitation, and wind had no significant effect (Fig. 1; multiple linear regression: $r^2 = 0.596$, $F(6, 307) = 27.601$, $P < 0.001$; minimum ambient temperature, $\beta = -0.547$, $P < 0.001$; snow, $\beta = 0.205$, $P < 0.001$; reproductive phase, $\beta = -0.016$, $P = 0.756$; maximum ambient temperature, $\beta = 0.094$, $P = 0.337$; precipitation, $\beta = -0.032$, $P = 0.546$; wind, $\beta = -0.011$, $P =$

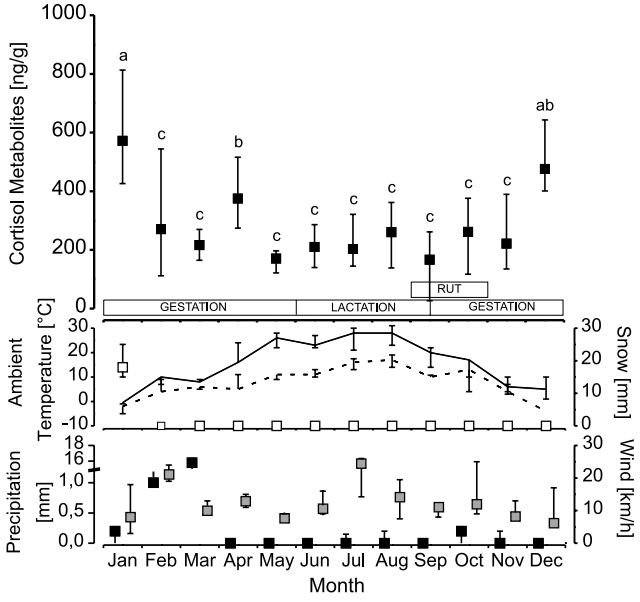

Fig. 1. Seasonal patterns of (a) concentrations of fecal cortisol metabolites (3a-11-one-cortisol metabolites; ng/g feces) in red deer (sample sizes/month, 41/21/24/25/28/11/8/9/14/60/52/51; ANOVA: $F(11, 343) = 22.412$, $P < 0.001$; different letters indicate $P < 0.05$ by post hoc Tukey test), and (b) maximum ambient temperature (°C, solid line), minimum ambient temperature (°C, dashed line), snow (mm, open squares), and (c) precipitation (mm, black squares), and wind (km/h, grey squares) at Vienna "Hohe Warte". Values are medians and quartiles. Horizontal bars in (a) indicate the approximate pattern of reproductive phases.

0.823). There was a significant negative relation between minimum ambient temperature and fecal cortisol metabolite levels that was best fitted by a 3rd order polynominal regression ($r^2 = 0.343$, df $= 3/336$, $F = 58.43$, $P < 0.001$; Fig. 2).

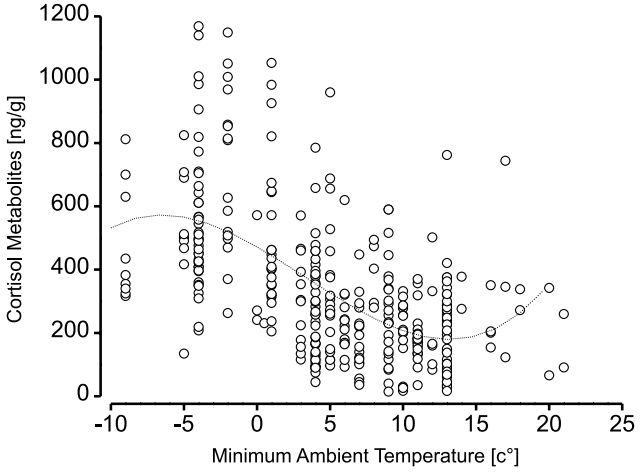

Fig. 2. Minimum ambient temperature (°C) versus concentrations of fecal cortisol metabolites (3a-11-one-cortisol metabolites; ng/g feces) in red deer. Polynominal regression 3rd order, $Y = 471.644 - 26.561x - 1.022x^2 + 0.103x^3$ (dotted line).

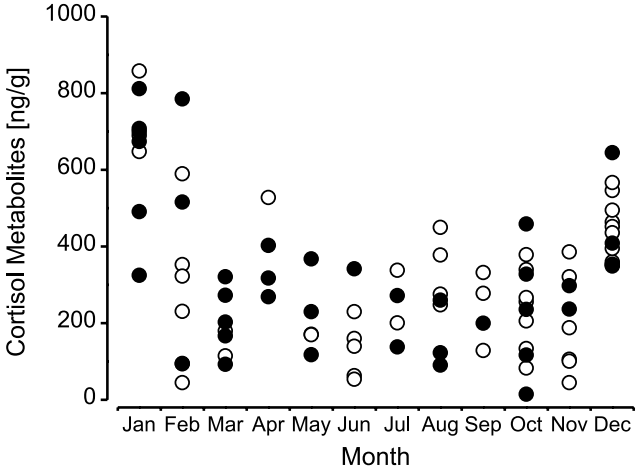

Fig. 3. Seasonal pattern of concentrations of fecal cortisol metabolites (3a-11-one-cortisol metabolites; ng/g feces) in female (open circles; sample sizes/month, 3/6/2/1/2/5/2/4/3/7/6/10) and male red deer (full circles; sample sizes/month, 6/3/5/3/3/1/2/3/1/5/2/3).

3.2. Effects of sex and sample collection

Concentrations of fecal cortisol metabolites did not differ significantly between females and males (two-way ANOVA: model, $F(23, 87) = 3.630$, $P < 0.001$; factor sex, $F = 0.004$, $P = 0.947$; factor month, $F = 5.380$, $P < 0.001$; interaction, $F = 1.244$, $P = 0.278$). There was no obvious sex difference of cortisol metabolite concentrations during any month (Fig. 3).

We found no significant effect of sample collection on an anonymous basis on mean fecal cortisol metabolite concentrations. Mean values/month derived form all fecal samples ($n = 344$) did not differ significantly from mean values/individual/month derived from the genotyped fecal samples ($n = 60$) (Fig. 4; two-way ANOVA: model, $F(23, 403) = 12.814, P < 0.001$; factor mean/month of all samples versus mean/individual/month of genotyped samples, $F = 0.048, P = 0.827$; factor month, $F = 11.907$, $P < 0.001$; interaction, $F = 0.332$, $P = 0.978$). Analyzed separately, not a single significant difference was found during any month (t test: each month, $P > 0.3$).

4. Discussion

4.1. Seasonal pattern of concentrations of fecal cortisol metabolites

We found a seasonal pattern of fecal cortisol metabolite values in red deer with significantly higher concentrations during December and January than during the remainder of the year. A similar variation of cortisol levels with peak levels during winter has been reported in white-tailed deer (*Odocoileus virginianus*) (Bubenik et al., 1983) and mule deer (*Odocoileus hemi-*

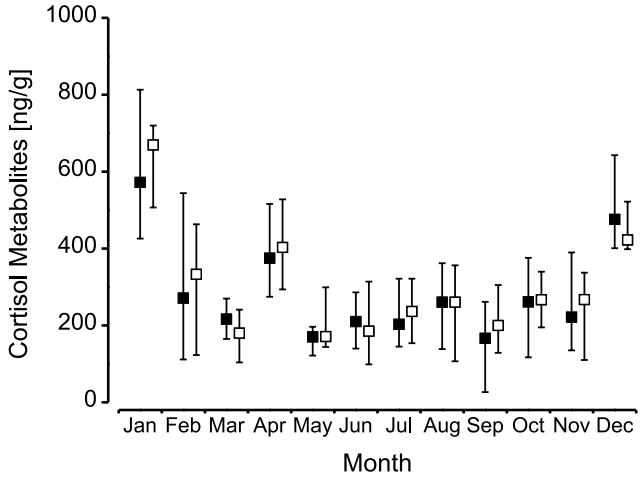

Fig. 4. Seasonal pattern of concentrations of fecal cortisol metabolites (3a-11-one-cortisol metabolites; ng/g feces) in red deer obtained from mean values/month derived from all fecal samples (full squares; sample sizes/month, 41/21/24/25/28/11/8/9/14/60/52/51) as compared to mean values/individual/month derived from the genotyped fecal samples (open squares; sample sizes/month, 6/4/5/3/5/4/4/5/3/7/6/8). Values are medians and quartiles.

onus) (Saltz and White, 1991) but no consistent seasonal changes in adrenocortical activity have been found in other studies on cervids (e.g., Bubenik and Brown, 1989; Bubenik et al., 1998; Monfort et al., 1993; Reyes et al., 1997). In contrast to the presented results, a recent study on North American elk (*Cervus elaphus*) reported maximum concentrations of fecal glucocorticoid metabolites in summer that decreased to minimum levels in winter (Millspaugh et al., 2001). These divergent results may be due to species or climatic differences. The concentrations measured, however, were 10 times lower than those found in the present study. We suspect that Millspaugh et al. (2001) might have measured others than cortisol metabolites as they applied a standard corticosterone RIA kit and did not characterize the detected metabolites. In contrast, we used a group-specific assay for cortisol metabolites (3a-11-one cortisol metabolites; Möstl et al., 2002) that have been characterized with high-pressure liquid chromatography (HPLC) (Huber et al., submitted).

Because we analyzed the feces of captive red deer living undisturbed in a large enclosure, fecal cortisol metabolite levels are likely to represent basal cortisol production affected by season, sex, and reproduction (Ingram et al., 1999; Nelson, 2000; Romero et al., 2000) rather than by stress responses due to disturbances. Accordingly, we examined the effects of reproductive phase, weather conditions (minimum and maximum ambient temperature, snow, precipitation, wind) and sex on fecal cortisol metabolite levels. The only variable exerting a significant effect on cortisol excretion proved to be minimum ambient temperature and snow. Saltz and White (1991) found similar, though only minor effects of ambient temperature and snow depth on urine cortisol excretion in mule deer. Elevated circulating glucocorticoid levels as a response to cold stress were also documented in reindeer (*Rangifer tarandus*) (Yousef et al., 1971) and in a review on farm animals (Dantzer and Mormede, 1983) but not in white-tailed deer (Chao and Brown, 1984). In the present study, we found a significant negative relationship between minimum ambient temperature and fecal cortisol metabolite levels indicating that elevated cortisol production may be an adaptation to harsh environmental conditions. As this negative relation leveled off below approximately −3 °C and above approximately 10 °C, being best fitted with a non-linear regression model, at low temperature extremes, possibly other yet to be examined mechanisms add to the adrenocortical response.

Red deer inhabit boreal and temperate zones which are characterized by strong seasonal variations of environmental conditions and food availability. As an adaptation for energy conservation when resources are reduced, metabolic rate in red deer is reduced during winter (Haigh and Hudson, 1993; Hudson et al., 1985) and deer reduce their food intake even when they are supplied with food ad libtium (Jiang and Hudson, 1994; Worden and Pekins, 1995). Declining nutritional intake, in turn, is associated with elevated cortisol levels in a number of mammalian species (e.g., DelGiudice et al., 1992; Saltz and White, 1991; Tsuma et al., 1996). Increases in cortisol with reduced caloric intake indicate a shift to catabolic metabolism designed to ensure adequate fuels for body tissue in the absence of adequate food (Foster and McGarry, 1988). Glucocorticoids exhibit catabolic functions in terms of mobilizing muscle protein as gluconeogenic precursors and in terms of inducing a linked series of enzymatic reactions converting amino acids to glucose during a process called gluconeogensis (Foster and McGarry, 1988). Accordingly, protein catabolism during winter undernutrition has been reported in red deer (Schmidt and Gutleb, 1997) and in white-tailed and mule deer (DelGiudice et al., 1990; Torbit et al., 1985). Thus, high glucocorticoid production may act via its catabolic function during adaptation of deer to cold months.

Alternatively, the seasonal pattern of fecal cortisol metabolite levels could result from dietary changes without any change in cortisol excretion, as increased fecal weight might dilute fecal cortisol metabolite concentrations. Then the reduced dry matter excretion due to low food intake in winter, as has been reported by Jiang and Hudson (1994) and Worden and Pekins (1995), would have caused the measured high winter cortisol metabolite levels. Two studies considering the effects of dietary changes and fecal weight on hormone excretion, however, did not report any clear relationship among these parameters (Cavigelli, 1999; Foley et al., 2001). In addition, this hypothesis would predict sex

differences in cortisol excretion whenever food intake and, as a result, fecal weight differed between the sexes, such as during lactation (Haigh and Hudson, 1993; Mautz et al., 1992) and rut (Haigh and Hudson, 1993). Although sample sizes were too small for statistical testing, fecal cortisol metabolite levels were not obviously different between sexes during any month. Thus, this alternative hypothesis was not supported.

4.2. Effects of sex and sample collection

As fecal cortisol metabolite levels did not differ between females and males, neither gestation, lactation nor rut seemed to affect fecal cortisol excretion. In reindeer, cortisol concentrations did not differ between adult males, barren and pregnant females, as well (Bubenik et al., 1998). Glucocorticoid concentrations in pregnant cows were reported to be basal throughout gestation except 48 h before parturition (Patel et al., 1996), which was in accordance with our results although we did not have immediately preterm samples. In addition, our results showed that fecal metabolites of placental androgens did not interfere with our EIA, measuring 3a-11-one cortisol metabolites (Möstl et al., 2002).

We collected fecal samples on an anonymous basis. Thus, feces from particular individuals might be overrepresented, comprising a source of error when estimating mean glucocorticoid levels within the population. Kohn et al. (1995) showed that sampling may play a significant role as only one genetic lineage among 13 fecal samples of European brown bears (*Ursus arctos*) was found in one study but six lineages among 17 fecal samples collected in the same area in another. A sampling bias would particularly be becoming a problem if considerable individual differences in glucocorticoid production exist, as has been reported in deer species (Cassirer et al., 1992; Franzmann and LeResche, 1978).

There are several possibilities to avoid this source of error. Fecal sampling on transects, for instance, increases the probability to obtain different individuals but at the cost of reduced overall number of fecal samples. This would be a matter of concern whenever one is looking for particular, e.g. fresh, fecal samples. Alternatively, animals could be observed defecating. However, this would be at the cost of a main benefit of fecal analysis namely sample collection without the need to observe the animals. A third way to eliminate this source of error would be genotyping of the fecal samples. As reliable genotyping of feces requires a lot of time and money (Taberlet et al., 1999), we genotyped only a random sample of all collected fecal samples. Mean values/month derived from all fecal samples did not differ significantly from mean values/individual/month derived from the genotyped fecal samples. Consequently, fecal collection on an anonymous basis did not significantly bias our results. Although this method may not be applicable to each situation, with this method, we ensured that sample collection did not confound our results, making use of the benefits of collecting anonymous fecal samples and saving time and money during genotyping. By determining the effects of season, sex, and collection of anonymous fecal samples on fecal glucocorticoid concentrations in undisturbed animals, this study serves as a basis for measuring stress responses in wild red deer.

Acknowledgments

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