NON-INVASIVE MONITORING OF THE ADRENOCORTICAL RESPONSE IN RED DEER

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Abstract: To facilitate easy and non-invasive assessment of the stress experienced by red deer (*Cervus elaphus*), we tested whether adrenocortical function can be monitored by measuring fecal corticoid metabolites. We measured concentrations of a group of cortisol metabolites (3α,11-oxo CM) in the feces of 6 captive red deer hinds before and after an adrenocorticotropic hormone (ACTH) challenge or a control saline injection by enzyme immunoassay (EIA). Results revealed that analysis of fecal cortisol metabolites enabled monitoring of adrenocortical activity. Administration of ACTH resulted in a 6.5 to 20-fold increase in fecal cortisol metabolite levels after approximately 18 hr. Following a planned disturbance, fecal cortisol metabolite concentrations increased 3 to 10-fold in 4 out of 6 individuals. Concentrations of 3α,11-oxo CM were not significantly different in feces collected within approximately 6 hr from defecation as compared to feces sampled immediately after defecation. Thus, fecal corticoid metabolite analysis could be used to evaluate disturbances in farmed red deer, even if immediate sample collection after defecation may not be possible. It also may evaluate stress responses in wild red deer, provided information on the time since defecation is available.

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Key words: ACTH challenge, adrenocortical response, *Cervus elaphus*, feces, glucocorticoids, non-invasive monitoring, red deer, stress.

Environmental disturbances, mainly caused by human impact, are potential stressors of wild red deer (Gossow et al. 1991). Deer farming, which is increasing in countries worldwide, may be stressful to this highly excitable species as well (Drew 1985, Waas et al. 1999). During the stress response, the hypothalamo-pituitary-adrenocortical and the hypothalamo-adrenal medullary system are activated (Sapolsky 1992). Consequently, stress can cause an increased ACTH release, stimulating glucocorticoid production by the adrenal cortex (Axelrod and Reisine 1984). Chronically high glucocorticoid levels in turn detrimentally affect red deer immune system, growth, and reproductive function (Ono et al. 1984, Peristein et al. 1993). Stress-related diseases are well known in red deer (Goddard et al. 1994).

Blood cortisol concentrations and responsiveness to an ACTH challenge have been commonly used as indices of adrenocortical function in several deer species (van Mourik and Stelmasiak 1984, Smith and Bubenik 1990, Bubenik et al. 1991) including red deer (Bubenik and Bartos 1993, Goddard et al. 1994, Hanlon et al. 1994, Ingram et al. 1997). The blood-sampling procedure, however, may in itself influence adrenal activity, thereby interfering with the adrenocortical response under investigation (van Mourik and Stelmasiak 1984, Reinhart et al. 1990, LeMaho et al. 1992). To enable assessment of adrenocortical function in red deer without confounding effects of the sampling technique, researchers developed a remote blood-collection method (Ingram et al. 1997, 1999). The method involves catheterization and fitting of animals with a remote blood-sampling device, which is inappropriate either for wild populations or for everyday use in farmed deer. In these situations, simple non-invasive methods are needed.

Feces have an advantage over blood and urine because they can be collected without handling animals. Miller et al. (1991) showed that measuring cortisol in feces reliably indicated stress responses in Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*). Glucocorticoids are metabolized extensively, however (MacDonald et al. 1983, Brownie 1992), and characterization of fecal metabolites in sheep revealed that cortisol and corticosterone were absent in the feces (Palme and Möstl 1997). Similarly in roe deer, nearly all cortisol is metabolized (Dehnhard et al. 2001). Palme and Möstl (1997) showed that the predominant fecal cortisol metabolites were

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unconjugated steroids, formed from cortisol by side chain cleavage (MacDonald et al. 1983). Möstl et al. (2002) developed an 11-oxoaetiocholanolone EIA for use in fecal samples. In this EIA, Möstl et al. (2002) used an antibody produced against 11-oxoaetiocholanolone-17-carboxymethyloxime coupled with bovine serum albumin, and therefore measuring the group of 3α,11-oxo cortisol metabolites (3α,11-oxo CM). This work was confirmed by liquid chromatography and mass spectrometry (Möstl et al. 2001). Concentrations of 3α,11-oxo CM remained stable in cattle feces stored at room temperature for 4 hr and tended to decrease thereafter (Möstl et al. 2001). This assay seemed promising for determining the adrenocortical response in red deer, where freezing of fecal samples immediately after voiding may not be possible.

We determined whether adrenocortical function in red deer can be assessed by fecal cortisol metabolite analysis. We examined fecal 3α,11-oxo CM levels before and after an ACTH and a disturbance challenge, respectively, as cortisol is the major glucocorticoid in red deer (Monfort et al. 1993). To examine the suitability of this method for free-ranging and wild animals with little information on the interval between defecation and fecal collection, we analyzed to what extent fecal metabolite concentrations were affected by the period of time past defecation.

METHODS

Animals

We performed our study in June 2000 at the Research Institute of Wildlife Ecology in Vienna, Austria. We used 6 healthy red deer hinds as experimental animals. All hinds had been hand-reared in 1998 and lived in a 0.5-ha enclosure. This enclosure was connected via 6 separate stables with 6 paddocks (10 × 10 m). Prior to the experiment, animals could move freely among enclosure, stables, and paddocks. During the experiment, each individual was kept separately in 1 of these stables with a paddock. Paddocks were separated by wire mesh, and hinds could see, hear, and smell each other. Animals were fed a standard red deer diet and water ad libitum. Our experiments were conducted with the permission of the respective national authority (GZ 68.205/45-Pr/4/2000).

Experimental Design

We performed the ACTH challenge test after animals had acclimated for 2 days to their new housing conditions. Each animal received an ACTH injection and a control saline injection: 3 hinds received ACTH first and saline 2 days later, 3 hinds received saline first and ACTH 2 days later. All treatments occurred between 0900 and 1100 hr. After another 2 days, we performed a disturbance experiment on all 6 hinds. Finally, after the ACTH, saline, and disturbance experiments were finished and fecal corticoid levels had reached baseline levels again, we examined the effects of the interval between defecation and sample collection on fecal corticoid levels. For that purpose, the 6 hinds stayed for another 2.5 days in stables with paddocks without any disturbance apart from animal keeping and fecal sampling. Afterward, we released the animals into the enclosure. We chose time intervals of about 48 hr between successive challenges because preliminary data revealed that excretion of cortisol metabolites occurred about 15–20 hr after challenge with baseline levels reached approximately 12 hr later.

ACTH Challenge.—We administered 0.5 mg/100 kg $ACTH_{1-24}$ (Synacthen®, Ciba-Geigy, Switzerland) diluted to a volume of 2 ml with saline by 1 intramuscular (IM) injection to each animal. The control injection to each animal consisted of 2 ml saline IM. All injections were administered by darting hinds at the muscles of the upper hind quarter from a distance of approximately 6 m. We used a blowdart (Telinject, Roemerberg, Germany) with 2 ml darts. For management reasons (to insert an eartag), 2 of 6 animals receiving ACTH and 2 of 6 animals receiving saline were immobilized prior to injection by darting with 3.2 mg etorphine plus 12 mg acepromazine (Large Animal Immobilon® C-Vet Ltd., United Kingdom) and 10 mg xylazine (Rompun® TS, Bayer Leverkusen, Germany). Immobilization was reversed with 6 mg diprenorphine (Large Animal Revivon® C-Vet Ltd., United Kingdom) together with 2 ml yohimbine (Adler Apotheke Wels, Austria).

Disturbance.—We performed the disturbance experiment to evaluate whether fecal cortisol metabolite analysis was sensitive enough to detect the adrenocortical response to an ecologically relevant challenge. Human disturbances are regarded as ecologically meaningful stressors in both wild and farmed red deer (Jeppesen 1987, Wagner 1992). Although the experimental hinds were hand-reared and accustomed to the animal keeper, they reacted nervously to unfamiliar people. We performed the disturbance experiment as follows: a noisy group of 5 unfamiliar humans walked back and forth, talked and gesticulated in

Table 1. Classification of red deer fecal samples to time intervals since defecation (n = 7), Vienna, Austria, 2000.

State of surface	Consistency	Time period past defecation (hr)
Shining and steaming	Moist	0
Shining	Moist	0–3
Slightly begins to dry	Moist	3–6
Predominantly dry	Moist	6–9
Dry	Slightly moist	9–12
Dull and dry	Dry	12–24

front of the paddocks at 0800 hr for 10 min. This resulted in a behavioral response in terms of restless movement in all animals.

Fecal Sampling

We collected all voided feces of each individual 3 times a day (0600, 1200, and 1800 hr) into separate tubes. To enable later analysis, feces were frozen at –20 °C immediately after sampling. For each fecal sample, we noted individual animal, date, and time of sampling. We estimated the time since defecation according to consistency and state of surface of the fecal sample (Table 1). The change of consistency and surface during the respective time intervals was monitored in 2 fecal samples stored in the sun and 2 fecal samples stored in the shade. We also monitored feces of 3 adult red deer collected in a 40-ha enclosure at the institute immediately after defecation and after 12 and 24 hr.

Extraction, Separation, and Enzyme Immunoassay

We extracted 0.5 g of each collected 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 (3α,11-oxo CM) in the supernatant by a group specific EIA. We used 5β-androstane-3α-ol-11,17-dione (11-oxoaetiocholanolone EIA) coupled at the position C17 both as standard and for the production of either the biotinylated label and the antibody (Möstl et al. 2001). 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 6.2% and 16.6%, respectively. Detection limit, based on 90% of maximum binding, was 0.05 μmol/kg. All steroids were purchased from Steraloids (Newport, Rhode Island, USA).

We performed reverse-phase, high-pressure liquid chromatography (RP-HPLC) as described by Teskey-Gerstl et al. (2000). Following extraction, the total supernatant was transferred into another vial, evaporated, redissolved with 50% methanol, and injected directly onto a Novapak C_{18} column (3.9 mm × 150 mm) with a Mini-Guard-column (C18). The flow rate was 1 ml/min and a mixture of methanol and water was utilized as mobile phase. The initial concentration of methanol was 50%. We applied a linear gradient up to 75% within 40 min (and afterwards up to 100% in another 5 min; (Teskey-Gerstl et al. 2000). Following separation, the immunoreactivity of each fraction was tested in a cortisol-, corticosterone-, 11,17-dioxoandrostane- and 3α,11-oxo CM-EIA (Palme and Möstl 1997, Möstl et al. 2001).

Statistical Analyses

We defined baseline levels for each individual as mean 3α,11-oxo CM concentrations of fecal samples collected within 3 hr from defecation after the experimental period the day prior to the release of the animals to the enclosure (n = 3–6 per animal). At this time, animals were neither influenced by new housing conditions nor by any challenge. The cortisol metabolite response to the ACTH, saline, and disturbance challenge was compared to the individual hind baseline values by calculating the percentage increase from the individual baseline value for each hind. To examine whether the interval between defecation and sample collection affected fecal cortisol metabolite levels, we collected fresh (immediately after defecation) and older (various time intervals past defecation) fecal samples. These samples were taken at different times of the day to rule out potential effects of circadian rhythms on cortisol secretion. Concentrations of each fecal sample collected immediately after defecation were considered as 100%, and the values of older fecal samples from the same individual collected at the same time were expressed as a percentage of the respective fresh ones.

Statistical analyses were conducted using SPSS 7.5 for Windows (SPSS 1997). We compared data among time intervals using repeated measures 2-way ANOVA with individual as random factor and time interval as fixed-treatment effect, with least significant difference (LSD) procedure for post-hoc comparisons. In the case that data were not normally distributed, we performed square-root transformation of data prior to ANOVA. We plotted data as mean ± standard error. We used an alpha of 0.05 for all tests.

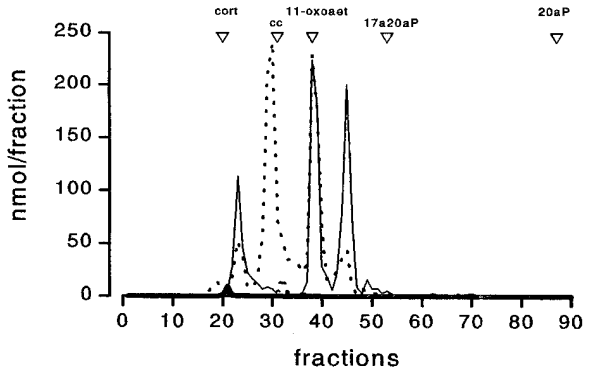

Fig 1. Immunoreactive fecal glucocorticoid metabolites in red deer after RP-HPLC separation, Vienna, Austria, 2000. Immunoreactivity of each fraction (nmol/fraction) was determined by enzyme immunoassays for cortisol (bold solid line near fraction 20), corticosterone (bold dotted line near fraction 30), 11,17-dioxoandrostanes (dotted line) and 3α,11-oxo CM (solid line). Fractions marked with open triangles represent the approximate elution time of respective standards (cort = cortisol, cc = corticosterone, 11-oxoaet = 11-oxoaetiocholanolone, 17a20aP = 17α,20α-dihydroxyprogesterone, 20aP = 20α-hydroxyprogesterone).

RESULTS

Separation of Immunoreactive Metabolites

Analysis of the HPLC fractions of red deer fecal extracts with group specific 11-oxoaetiocholanolone-EIAs measuring 3α,11-oxo CM or 11,17-dioxoandrostanes showed high amounts of several immunoreactive cortisol metabolites that were more detectable in feces than cortisol or corticosterone (Fig. 1). They eluted between cortisol and 17α,20α-dihydroxyprogesterone, and 11-oxoaetiocholanolone was included. Only minor portions of immunoreactive steroids eluting around the respective standards were detected with a cortisol- and a corticosterone-EIA, respectively (Fig. 1).

ACTH Challenge

Baseline fecal 3α,11-oxo CM concentrations ranged from 0.36 to 1.02 (mean 0.64) μmol/kg feces. Administration of ACTH resulted in a 6.5 to 20-fold increase in fecal cortisol metabolite levels after an average of 18.75 hr with individual maximum values ranging from 3.9 to 20.62 μmol/kg (mean 8.73; Fig. 2A). Maximum cortisol metabolite levels represented 668–2,021% (mean 1,178) of each individual hind baseline level.

After control saline administration, concentrations of fecal 3α,11-oxo CM increased 2–9-fold with individual maximum concentrations reaching 0.84 to 7.5 μmol/kg (mean 2.96; Fig. 2B). Maximum cortisol metabolite levels represent

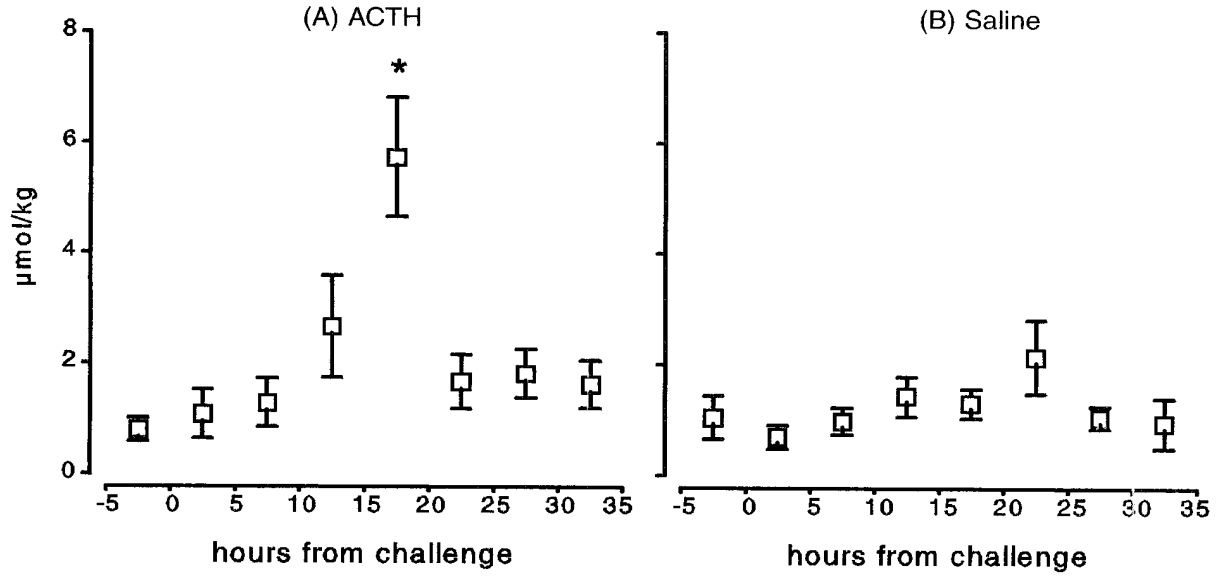

Fig. 2. (A) Fecal 3α,11-oxo CM concentrations (μmol/kg) of 6 red deer hinds in 5-hr intervals before and after ACTH administration (mean and SE; $^*P < 0.01$)[a], Vienna, Austria, 2000. (B) Fecal 3α,11-oxo CM concentrations (μmol/kg) of 6 red deer hinds in 5-hr intervals before and after saline administration (mean and SE)[b].

[a] 2-way ANOVA of square root transformed data: $r^2 = 0.841$; model, $F_{27/56} = 5.472$, $P < 0.001$; factor individual hind, $F_{5/56} = 6.772$, $P < 0.001$; factor 5-hr time interval, $F_{6/56} = 3.368$, $P = 0.013$; interaction, $F_{16/56} = 2.468$, $P = 0.018$; posthoc tests for time interval: 15–20 vs. each single other time interval [–5–0, 0–5, 5–10, 10–15, 20–25, 25–30, 30–35], $P < 0.01$; 0–5 vs. 10–15, $P < 0.05$; each single remaining time interval vs. each other time interval, $P > 0.1$.

[b] 2-way ANOVA of square root transformed data: $r^2 = 0.914$; model, $F_{24/46} = 9.288$, $P < 0.001$; factor individual hind, $F_{5/46} = 23.285$, $P < 0.001$; factor 5-hr time interval, $F_{6/46} = 1.478$, $P = 0.234$; interaction, $F_{13/46} = 2.521$, $P = 0.029$.

Table 2. Percent increase in fecal cortisol metabolite levels from each individual baseline value after adrenocorticotropic hormone (ATCH) and saline treatment in immobilized and not immobilized red deer hinds, Vienna, Austria, 2000.

Individual	Treatment	ACTH	Saline
1	ACTH/immobilized & saline/ not immobilzed	668%	297%
2		936%	461%
3	ACTH/not immobilized & saline/ immobilized	1,181%	753%
4		1,566%	893%
5	ACTH/not immobilized & saline/ not immobilized	1,538%	201%
6		2,021%	213%

201–893% (mean 475) of baseline levels after an average of 24.5 hr.

In immobilized deer, the percent increase in fecal cortisol metabolite levels from baseline values was on average 774% lower after the ACTH challenge (2 deer) and on average 530% higher after saline administration (2 deer) than in deer not immobilized. However, increase in cortisol metabolite levels was higher after ACTH administration then after saline injection in each individual (Table 2).

Disturbance

Following the disturbance challenge, fecal 3α,11-oxo CM levels significantly increased 3 to 10-fold in 4 individuals and nonsignificantly <3-fold in the remaining 2 individuals. Individual peak values of 1.06–5.7 μmol/kg (mean 2.69) were measured 16–21 hr (mean 18.7) after the disturbance challenge (Fig. 3). Peak values represent 178–980% (mean 464) of each individual hind baseline level. The individual profiles of fecal cortisol metabolite concentrations during the ACTH, saline and disturbance challenge of all 6 hinds is shown in Fig. 4.

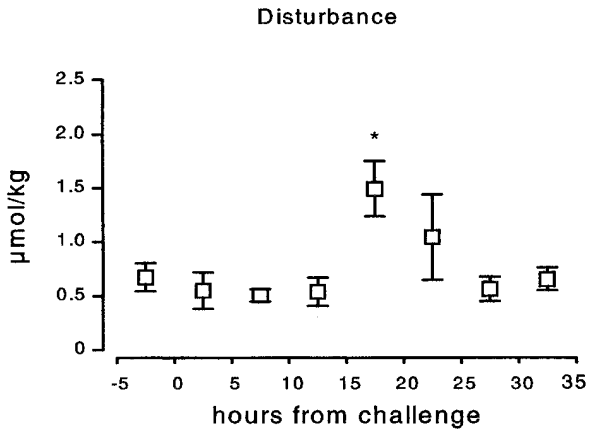

Fig. 3. Fecal 3α,11-oxo CM concentrations (μmol/kg) of 6 red deer hinds in 5-hr intervals before and after a disturbance challenge (mean and SE; *P < 0.05 except 15–20 vs. 20–25)[a], Vienna, Austria, 2000.

[a] 2-way ANOVA of square root transformed data: r^2 = 0.561; model, $F_{28/61}$ = 1.462, P = 0.150; factor individual hind, $F_{5/61}$ = 2.556, P = 0.047; factor 5-hr time interval, $F_{6/61}$ = 2.298, P = 0.059; interaction, $F_{17/61}$ = 0.435, P = 0.964; posthoc tests for time interval: 15–20 vs. each single time interval except 20–25, P < 0.05; each single remaining time interval vs. each other time interval, P > 0.1.

Effect of the Time Period Past Defecation

Fecal samples, collected later than approximately 6 hr past defecation, contained significantly lower 3α,11-oxo CM concentrations than fresh feces, collected and frozen immediately after defecation. In feces sampled within about 6 hr from defecation, a time effect was not detected (Fig. 5). Storage in sun or shade affected condition of the fecal sample. The time past defecation may therefore be overestimated in droppings lying in the sun and underestimated in feces lying in the shade.

DISCUSSION

Separation of HPLC of red deer fecal extracts and analyses with various enzyme immunoassays revealed high amounts of immunoreactive cortisol metabolites measured with the 2 different 11-oxoaetiocholanolone-EIAs, whereas only little cortisol and corticosterone equivalents were detected. A similar situation was found in roe deer, hares, and domestic livestock (Möstl et al. 1999, Teskey-Gerstl et al. 2000, Dehnhard et al. 2001). Consequently, specific cortisol and corticosterone enzyme immunoassays are not suitable for monitoring adrenocortical function in red deer using fecal samples.

In Rocky Mountain bighorn sheep, on the other hand, HPLC confirmed the presence of cortisol in fecal extracts (Miller et al. 1991). Wasser et al. (2000) reported that a corticosterone radioimmunoassay facilitated assessment of immunoreactive glucocorticoid metabolites in the feces of a variety of species including Roosevelt elk (*Cervus elaphus roosevelti*). However, Wasser et al. (2000) did not specify the measured metabolites, and concentrations were low (maximum: 0.056 μmol/kg). Similarly, we detected only small amounts of cortisol and corticosterone equivalents as compared to cortisol metabolites after HPLC separation of fecal extracts. Cortisol metabolites with a 3α,11-oxo configuration have been demonstrated to represent adrenocortical

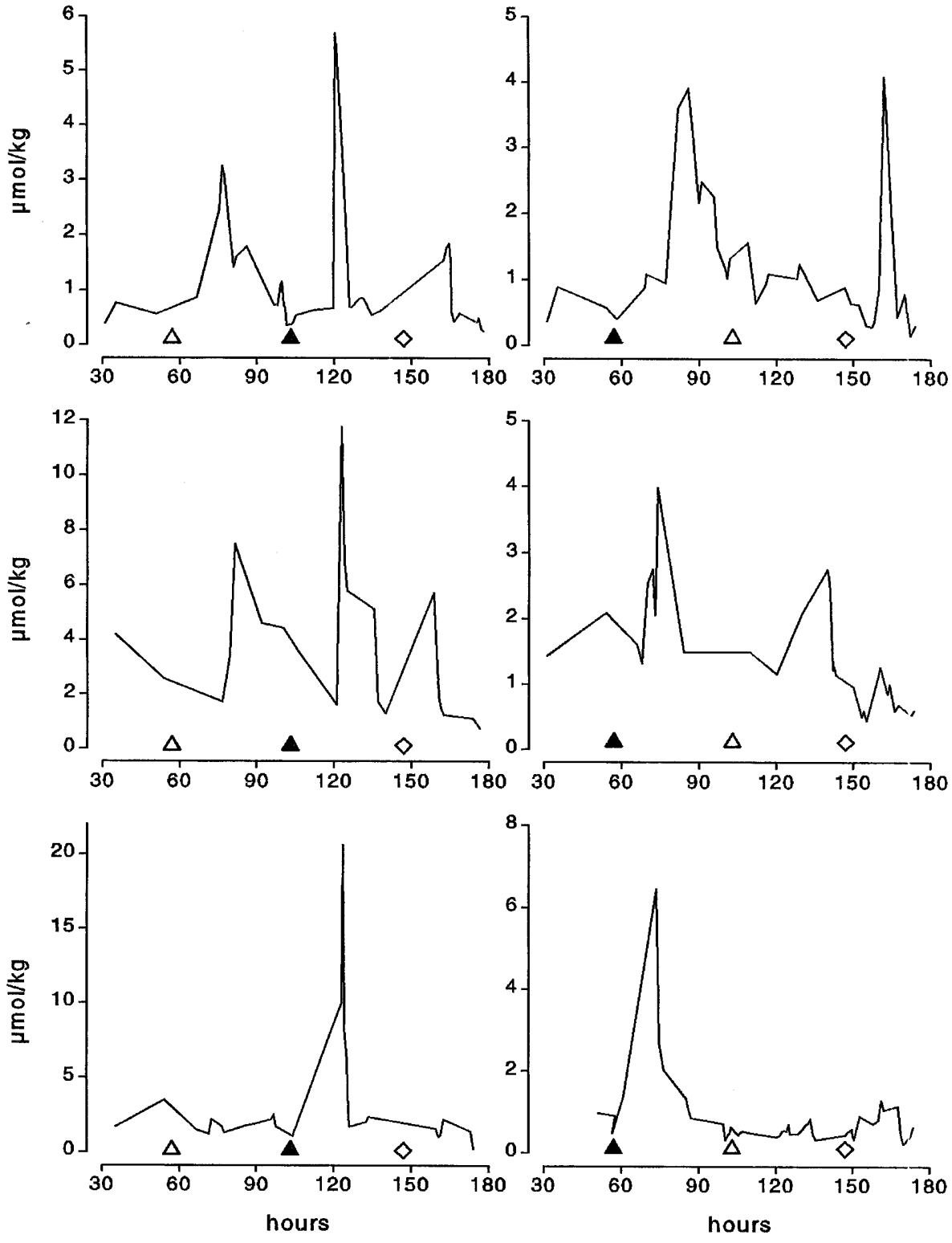

Fig. 4. Fecal 3α,11-oxo CM concentrations (µmol/kg) in all 6 individual red deer during a saline, ACTH, and disturbance challenge (△ = time of saline administration, ▲ = time of ACTH administration, ◇ = time of disturbance challenge; note different y axis scales), Vienna, Austria, 2000.

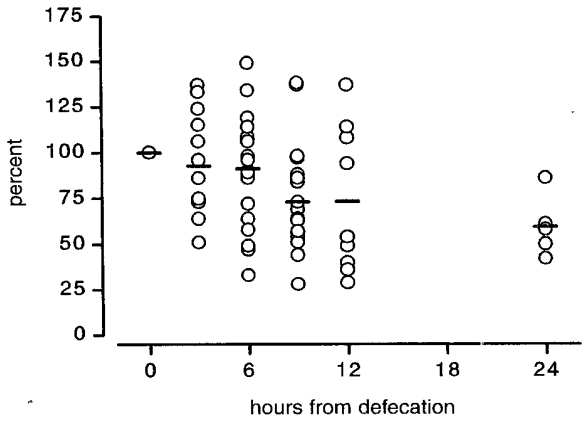

Fig. 5. Concentration of 3α,11-oxo CM measured in older fecal samples (collected at various time intervals past defecation, n = 66), expressed as % levels of fresh samples (collected immediately after defecation, n = 14, range = 0.3–1.8 μmol/kg) of the same individual collected at the same time (dashes represent mean values)[a], Vienna, Austria, 2000.

[a] ANOVA: $F_{5/79} = 3.583$, $P = 0.012$; post hoc tests for time intervals: 0 hr vs. 9, 12, 24 hr: $P < 0.05$; 0 hr vs. 3, 6 hr: $P > 0.1$.

activity in domestic ruminants, showing a higher stability in feces stored at room temperature than the 11,17-dioxoandrostanes analyzed in previous studies (Möstl et al. 2001).

We validated the physiological relevance of cortisol metabolite analysis in red deer feces by stimulating adrenocortical activity with exogenously administered ACTH. The administration of this hormone caused a significant rise in fecal 3α,11-oxo CM levels in each individual hind, indicating that concentrations of these metabolites reflected adrenocortical function in red deer. A control saline injection elicited a lower increase in fecal cortisol metabolite levels, possibly representing the stress experienced by the animals during this procedure. The adrenocortical response to ACTH and saline administration seemed to be influenced by immobilization of deer. This response was somewhat higher after saline treatment and lower after ACTH treatment in immobilized, as compared to not immobilized, animals. Nevertheless, ACTH injection resulted in a higher increase of fecal cortisol metabolite levels than saline injection in each individual.

A complete physiological validation of fecal corticoid analysis requires that the adrenocortical response is elicited to pharmacological challenges and also to an ecologically meaningful stressor that can be detected (Harper and Austad 2000). We found a significant rise in fecal cortisol metabolite concentrations after a disturbance challenge in 4 of 6 individuals. Possibly, 2 hinds failed to react to disturbance because of their pronounced tameness. Non-uniformity and magnitude of the adrenal response to disturbance was similar to adrenocortical activity following saline administration. The inter-individual differences in adrenocortical responsiveness presumably resulted from individual differences in temperament, earlier experiences, perception of stressfulness of the challenge (Friend et al. 1977), and/or from physiologic differences between individuals. Such differences in temperament will also occur in wild deer, adding to variability in cortisol response to disturbance. We can detect the adrenocortical response in association with stressful stimuli, and the technique offers promise to evaluate the degree of stress experienced in red deer.

Möstl et al. (2002) found a decrease in fecal 3α,11-oxo CM concentrations with increasing time interval between defecation and freezing of fecal samples in domestic livestock. Bacterial activity accounted for that decrease. Unstable concentrations of cortisol metabolites in feces may be limiting particularly in free-ranging and wild animals. In these animals, immediate sample collection after defecation may not be possible, and information on time since defecation may be unavailable. We examined whether measured cortisol metabolite levels in red deer feces changed according to the interval between defecation and sample collection and freezing. Cortisol metabolite levels did not differ significantly in feces collected within approximately 6 hr from defecation as compared to feces sampled immediately after voiding. In older feces, however, cortisol metabolite concentrations were significantly lower. Thus, similarly to Möstl et al. (2002), metabolites with a 3α,11-oxo configuration appeared to break down after a few hours.

For future studies, we recommend fecal collection within approximately 6 hr from defecation. This time interval can be ensured by limiting sampling to moist feces with a shining surface unless rain or snow complicates fecal status. We analyzed all fecal samples regardless of interval between defecation and sampling to include any peak of fecal cortisol metabolite levels. Consequently, we possibly underestimated cortisol metabolite concentrations in older feces (<12% of all fecal samples).

MANAGEMENT IMPLICATIONS

Fecal glucocorticoid analysis offers 3 major advantages in assessing behavioral responses of red deer to stressors. First, fecal cortisol metabo-

lite levels provide an integrated measure of circulating blood concentrations that change quickly due to episodic secretion of glucocorticoid pulses from the adrenocortex (Monfort et al. 1998, Palme et al. 1999). Second, these fecal analyses do not require handling the animal. Third, it facilitates examination on a retrospective basis, as fecal cortisol metabolites are excreted with a certain delay time (Palme et al. 1999). We showed that fecal cortisol metabolite analysis enables non-invasive monitoring of adrenocortical responsiveness in red deer. The technique will be most valuable for evaluating stress responses of farmed deer to husbandry practices. It also shows promise for evaluating stress responses of wild deer to environmental disturbances, human impact, or social stress, provided information on approximate time since defecation is available.

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