A Noninvasive Technique to Evaluate Human-Generated Stress in the Black Grouse

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ABSTRACT: **The continuous development of tourism and related leisure activities is exerting an increasingly intense pressure on wildlife. In this study, a novel noninvasive method for measuring stress in the black grouse, an endangered, emblematic species of European ecosystems that is currently declining in several parts of its European range, is tested and physiologically validated. A radiometabolism study and an ACTH challenge test were performed on four captive black grouse (two of each sex) in order to get basic information about the metabolism and excretion of corticosterone and to find an appropriate enzyme-immunoassay (EIA) to measure its metabolites in the feces. Peak radioactivity in the droppings was detected within 1 to 2 hours. Injected ^{3}H-corticosterone was excreted as polar metabolites and by itself was almost absent. A cortisone-EIA was chosen from among seven tested EIAs for different groups of glucocorticoid metabolites, because it cross-reacted with some of the formed metabolites and best reflected the increase of excreted corticosterone metabolites, after the ACTH challenge test. Concentrations of the metabolites from fecal samples collected from snow burrows of free-ranging black grouse were within the same range as in captive birds. The noninvasive method described may be appropriate for evaluating the stress faced by free-living black grouse populations in the wild, particularly in mountain ecosystems where human disturbance, especially by winter sports, is of increasing conservation concern.**

KEYWORDS: **corticosterone metabolism; noninvasive endocrine monitoring; conservation biology; *Tetrao tetrix*; wildlife management; ecology**

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INTRODUCTION

In addition to habitat degradation, the intensification of human leisure activities exerts a negative pressure on wildlife.[1–5] This is of particular concern as regards species that are otherwise (e.g., through habitat degradation and fragmentation) already threatened and vulnerable. Yet, until the recent development of appropriate analytical tools, it has remained difficult to quantify properly how human-generated disturbances affect animals' constitution, physiological condition and, ultimately, reproductive fitness. Not surprisingly, until recently, most studies have therefore instead focused on variations in time budgets and juvenile survival.[1,3,6,7] Rarely, however, could changes in time allocation to various behaviors and activities be associated with the actual additional physiological costs they entail. The emergence of noninvasive techniques for estimating stress in free-ranging animals opens new avenues for a proper quantification of the impact of human-generated stress onto wildlife.[8–15]

The black grouse (*Tetrao tetrix*), an emblematic game bird with great economic and cultural value, is endangered and declining in several parts of its Palearctic distribution range.[16] Several authors have identified human activities and infrastructures as the probable main cause. This includes habitat loss and fragmentation,[16] hunting,[17,18] collisions with aerial cables and fences,[19,20] as well as increasing disturbance through popular leisure activities such as winter sports and activities.[1,21,22] Despite the knowledge, gathered from research on different species, to the effect that prolonged stress generated either by human or natural factors can have deleterious consequences on an individual,[23–25] physiological constraints imposed by human activities are only poorly explored in the black grouse.[26,27] In order to propose appropriate mitigation measures for this endangered species in those areas where leisure activities are a potential threat, a proper quantification of the impact of human-elicited stress appears a first necessary step. Additionally, however, since black grouse are endangered, a noninvasive method, which does not alter or constrain bird behavior, is another prerequisite in any such study.

The first physiological response of an organism to different stressful stimuli is a cascade of hormone secretions, starting with the release of catecholamines (epinephrine) from the adrenal medulla within seconds after the stimulus, triggering the hypothalamic–adenohypophysal–adrenocortical axis within a few minutes, which is followed by synthesis and secretion of glucocorticosteroids (corticosterone in the case of birds) from the adrenal cortex, as well as cytokines from cells of the immune system.[23] The blood sampling techniques usually applied in stress research are invasive. They are thus not convenient for the study of threatened free-ranging animals such as the black grouse.[28] Instead, noninvasive techniques for monitoring stress on free-living populations have therefore been developed extensively in recent years. In birds, feces are often used for this purpose, as they are easy to collect. However, it must be taken into account that metabolites are excreted with a species-specific time delay of about a few hours in birds.[9–11,29,30]

Until now, noninvasive methods for measuring adrenal activity by measuring fecal glucocorticoid metabolites by group-specific enzyme-immunoassays (EIAs) have been mainly developed on domestic and captive animals for purposes of research on animal welfare[28,29,31] and behavioral ecology.[10,12,32] However, such methods also have a big application potential in conservation biology.[9,13,15,22] Me-

tabolism and excretion of glucocorticoids differ between species, and sometimes even between sexes and individuals within a given species.[30,31] Therefore, it is not possible to draw analogous conclusions from other bird species. Thus, the aims of this study were to get basic information about the metabolism and excretion of corticosterone, to characterize fecal metabolites of black grouse, and to select an EIA for measuring the corticosterone metabolites (CM) in feces of free-ranging individuals. In addition, the stability of the CM was tested within natural settings.

For this purpose, we performed first a radiometabolism study of corticosterone on birds in captivity. We also used the same birds for physiological validation of several EIAs by testing immunoreactivity of the CM excreted after adrenal stimulation with adrenocorticotropic hormone (ACTH). Yet, it should be noted that the stability of glucocorticoid metabolites in feces could be significantly affected by environmental conditions, due, for instance, to bacterial metabolism. This can give misleading results.[33] In order to both define a suitable sampling protocol of black grouse feces in nature and to achieve accuracy in the hormone assay, we first had to find out if the concentration of measured CM significantly changes in feces exposed to a variety of winter environmental conditions, and, second, at which time intervals between defecation and sampling of fecal pellets these changes take place. This was accomplished by incubating feces at different time intervals and at temperatures above 0°C (below this threshold, metabolic activity was assumed to be insignificant), which might occur in winter on sun-exposed snow surfaces. Also, we must stress that our radiometabolism study and the hormonal stimulation experiment for selecting the most appropriate EIA were carried out on captive birds that might have different stress reactions than free-ranging individuals, as well as distinct digestive tract size (ceca) and transit durations due to a different diet.[34–36] Since corticosterone metabolism can be further affected by these factors, we analyzed samples from free-living birds with the selected EIA to finally confirm the suitability of the method for assaying stress in wild black grouse during the winter season.

MATERIALS AND METHODS

Birds and Experimental Setup

Permission for the animal experiment was given by the Department of Veterinary Medicine, canton Lucerne. Two male and two female black grouse, all 7 months old, were used in the experiments. The females came from private raisers, the males from the Bern Zoological Garden. Between experiments, the birds were kept in a large outdoor aviary at the Hasli Ethological Station of the Zoological Institute, University of Bern. Because we intended to apply this method to free-living birds during the cold season, we conducted laboratory experiments by the end of November 2002. At that time of year, the birds had already reached adult plumage and body mass, whereas reproductive mechanisms, which could induce a stress state in displaying males,[10] had not yet matured. During experiments, the birds were placed in individual cages with water and food provided *ad libitum*. Special cages ($80 \times 80 \times 80$ cm in size), with a double bottom, were constructed. The top of the cage as well as the back and one sidewall were made out of green textile mesh (0.8 mm in diameter) to protect the birds from injuries due to the reduced space available. The front wall with the

entrance, as well as one sidewall, consisted of wooden plates that isolated the birds from the researcher during sampling. The bottom of the cage was composed of a wire-mesh floor (1-cm mesh diameter). This enabled droppings to fall through into a removable, exchangeable drawer. Cages and birds were exposed to natural light and temperature conditions, but protected from precipitation.

Habituation of the birds to this setup lasted 4 days. By that time, they were feeding regularly and defecating normal droppings. As they would under natural conditions, birds had at least two main feeding periods during the day[37,38] and defecated almost hourly 1–3 solid feces. To reduce the risk of a possible influence of different dietary compounds on digestion,[34] only homogenous grouse food (article 872.4, Protector SA, CH-1522 Lucens) was provided.

Hormone Administration and Sampling Pattern

Each bird was injected in the ulnar vein with 1.85 MBq (=50 μCi) of radiolabeled corticosterone ([1,2,6,7-^{3}H(N)]-corticosterone, specific activity: 76.5 Ci/mmol, NET-399, Perkin-Elmer Life Sciences, Boston, MA), which was dissolved in 0.5 mL of a physiological solution (0.9% NaCl) containing 10% ethanol. Birds were injected between 8:00 and 8:15 A.M. Manipulation with each individual lasted less than 5 minutes.

Fecal samples were collected one hour before injection, to determine the background radioactivity, and after injection, once per hour during the following 24 hours. The feces were collected from the exchangeable drawers. They were frozen immediately at −22°C until further analyses, and the drawers were cleaned with 70% ethanol and water after each sampling event.

In order to physiologically validate different EIAs, the same birds were injected intravenously with 0.5 mg of adrenocorticotropic hormone (ACTH; Synacthen; Novartis Pharma AG, Basel, Switzerland). On the day before injection, the fecal samples were collected hourly during a total of 24 hours in order to get a control group (pretreatment group). On the following day birds were injected between 8:00 and 8:15 A.M., and the fecal samples were again collected hourly during the next 24 hours (treatment group). During the third and fourth day, feces were collected early in the morning, at midday, and in late afternoon in order to control for posttreatment levels (posttreatment groups I and II, respectively). All samples were immediately stored at −22°C until analyses.

Extraction and Characterization of the Excreted ^{3}H-Corticosterone Metabolites

A total of 0.5 g of each well-homogenized fecal sample was mixed with 3 mL methanol and 2 mL water and vortexed for 30 min. After centrifugation (2500 g; 10 min), aliquots (0.5 mL) of the supernatant (in duplicate) were transferred into scintillation vials (Article 6008117, Packard Instruments, Meriden, CT), each containing 6 mL scintillation fluid (Quicksafe, A, 100800, Zinsser Analytic, Maidenhead, UK). The radioactivity of each sample was measured (5 min) by a liquid scintillation counter (Tri-Carb 2100 TR, Packard Instruments) with a quench compensation program. Radioactivity is expressed as kBq per g of feces.

In order to characterize the CM, samples containing peak radioactivity were extracted, the radioactive substances purified by a Sep-pak C_{18} cartridge and subjected to reverse-phase high-performance liquid chromatography (RP-HPLC), as described by Rettenbacher *et al.* [30] Briefly, steroids were separated on a Novapak C_{18} column (3.9×150 mm, Millipore Corporation, Milford, MA) with a methanol/water solvent. A linear gradient from 20% to 100% methanol with a flow rate of 1 mL/min was applied. A total of 96 fractions were collected (three per min). Radioactivity in an aliquot (50 μL) of each fraction was determined (Top Count; Packard Instruments, Meriden, CT).

Immunoreactivity of ^{3}H-Corticosterone Metabolites and the Physiological Validation of Assays

An array of different, previously established EIAs was tested to select the one best suited for black grouse. Among the seven EIAs were a corticosterone-EIA,[39] a tetrahydrocorticosterone-EIA,[40] a 5α-pregnane-3β,11β,21-triol-20-one-EIA,[31] an 11β-hydroxyetiocholanolone-EIA,[41] an 11-oxoetiocholanolone-EIA,[42] a cortisone-EIA,[30] and a so far unpublished 20β-dihydrocorticosterone-EIA. Aliquots of each HPLC fraction of males were measured using the different EIAs to check if radiolabeled metabolites are recognized. The EIA procedure was described in detail by Palme and Möstl[39] and Touma *et al.*[31] The antibody of the 20β-dihydrocorticosterone-EIA (working dilution 1:80000) was raised against 20β-dihydrocorticosterone-3-CMO:BSA in a rabbit. The label (20β-dihydrocorticosterone-3-CMO-biotinyl-LC; 1:5000000) was produced as described by Möstl *et al.*[42] The standard (20β-dihydrocorticosterone) curve ranged from 0.33 pg/well to 80 pg/well. Only the cortisone-EIA was applied on HPLC fractions of females, but the later three EIAs, which were able to detect significant amounts of immunoreactive substances in the HPLC fractions of males, were used for the analyses of the samples from the stimulation experiment (ACTH) of both sexes. Therefore an aliquot of the supernatant (after diluting 1:10 with assay buffer) was subjected to the EIAs after extraction (5mL of 60% methanol) of the droppings (0.5 g).

Stability of ^{3}H-Corticosterone Metabolites in the Feces

In order to optimize the collection of fecal samples from free-living black grouse, it was important to know whether and how the concentration of metabolites changes with time, especially when ambient temperature increases above zero and allows activation of fecal bacteria. We did this with captive birds, two males and one female, from which we collected feces on the third day (posttreatment control group II, see earlier) after the ACTH injection. All feces excreted by a single bird (ca. 20 g) were pooled, homogenized, and divided into four equal subsamples. A subsample was frozen immediately at −22°C (control), while the three other samples were incubated in a refrigerator at 6–7°C for 24 h, 48 h, and 72 h, respectively. Temperature in the refrigerator was set up as the highest measured temperature in the snow burrow, 6.5°C (unpublished personal data). During the night, as well as during the day, black grouse dig burrows into the snow to protect themselves from the cold during resting periods. These snow burrows or "igloos" are rebuilt anew each time. From each sub-

sample, 10 aliquots of 0.5 g each were extracted and the concentration of the metabolites measured with the cortisone-EIA, as described earlier.

Concentrations of ^{3}H-Corticosterone Metabolites in Feces from Free-Ranging Birds

In a preliminary field experiment, amounts of CM present in free-living birds were measured in order to get information on interindividual and intraindividual variances. We collected samples from the snow burrows of four free-living black grouse males, in February 2003, at Verbier and Les Diablerets (southwestern Swiss Alps). The birds were flushed from their diurnal burrows early in the afternoon and fecal material that had accumulated within burrows (9–15 separate droppings each) was collected. In order to gather information about variance of CM concentrations of an individual bird within the burrow, each dropping was analyzed separately. The cortisone-EIA, which gave the best results in the ACTH challenge test, was selected to further analyze of the feces from the free-living birds. All feces were extracted as described previously (5 mL of 60% methanol), and an aliquot (diluted 1:10) of the supernatant analyzed in the EIA.

Statistical Analyses

Results of the ACTH test (physiological validation) of the three EIAs, which cross-reacted significantly with radioactive metabolites, were analyzed by an ANOVA standard least-square fit model. We tested for the following effects and interaction term: individual bird ($n = 4$), treatment (vs. control day, i.e., data from the day prior to ACTH administration), bird*treatment.

The within-individual (i.e., within-burrow fecal sample) and among-individual (between burrows) variations in CM concentrations of the feces from free-ranging black grouse males were analyzed by one-way ANOVA after controlling for variance homoscedasticity (Levene's test). All statistical analyses were performed with JMP 4.04 (SAS Institute Inc., 1989–2001). Test rejection probability levels were set at 5% throughout.

RESULTS

Excretion and Characterization of the ^{3}H-Corticosterone Metabolites

The main portion of radioactivity was quickly excreted. Peak concentrations (75 to 139 kBq/g feces) were reached after one (one male and one female) or two (the other two animals) hours (FIG. 1). Radioactivity decreased almost continuously afterwards (only one male had a second, somewhat smaller peak after 5 h). When sampling was stopped 24 h after the injection, radioactivity was low, but background levels were not yet reached.

Injected ^{3}H-corticosterone was heavily metabolized, as demonstrated by HPLC separation of CM in the peak radioactivity samples of the four individual birds (FIG. 2). Three to four main metabolites were present, all eluting between fractions 20 and 45, thus resembling conjugated, or polar unconjugated steroids. Males had more polar metabolites if compared with females. In all samples only small amounts,

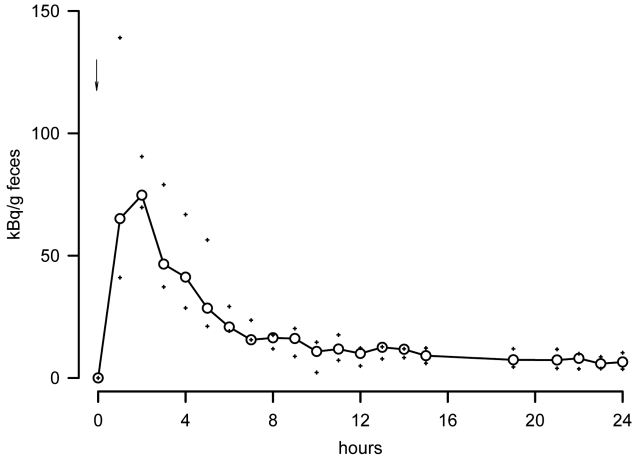

FIGURE 1. Excreted radioactivity (kBq/g feces; min., max., median) in the droppings of four black grouse birds after ^{3}H-corticosterone administration. Animals were injected intravenously between 8:00 and 8:15 A.M. (0 h, *arrow*).

if at all, of unmetabolized corticosterone could be detected. In the HPLC fractions, significant amounts of CM could be measured with three of the seven EIAs tested (FIG. 2), that is, concentrations were higher than the detection limit of the respective EIAs. The cortisone-EIA, measuring metabolites with a common 3,11-dione structure, yielded the highest amounts of immunoreactivity. The most prominent metabolite peaked at fractions 31/32. The 11-oxoetiocholanolone- and the 11β-dihydrocorticosterone-EIA measured only smaller amounts of immunoreactivity.

Physiological Validation of the EIAs–ACTH Challenge

The concentrations measured with the cortisone-EIA (3,11-dioxo-CM) differed significantly between the experimental groups (ANOVA, $df = 1$, $F = 16.41$, $P < .0001$), but no difference among individuals (ANOVA, $df = 3$, $F = 1.933$, $P = .128$) was found, although males tended to have higher basal values. A *post hoc* test showed that this difference was due to differences between the treatment group (i.e., data obtained during the first 24 h after adrenal stimulation) and the pretreatment group, and the posttreatment I and II control groups (Dunnett's test). Note that 24 h after injecting ACTH, the concentration of the CM had returned approximately to

FIGURE 2. Immunoreactivity (nmol per fraction) of ^{3}H-corticosterone metabolites (Bq per fraction) evaluated by reverse-phase high-performance liquid chromatography (RP-HPLC) with three different EIAs (cortisone-, 11-oxoetiocholanolone-, and 11β-dihydrocorticosterone) in two male and two female black grouse. Elution positions of corticosterone, cortisol, 17β-estradiol-disulfate ($E_2\beta$-diSO_4), estrone-glucuronide (E_1G), and estrone-sulfate (E_1S) are marked. A gradient solvent system with a water/methanol ratio changing from 20% to 100% was applied.

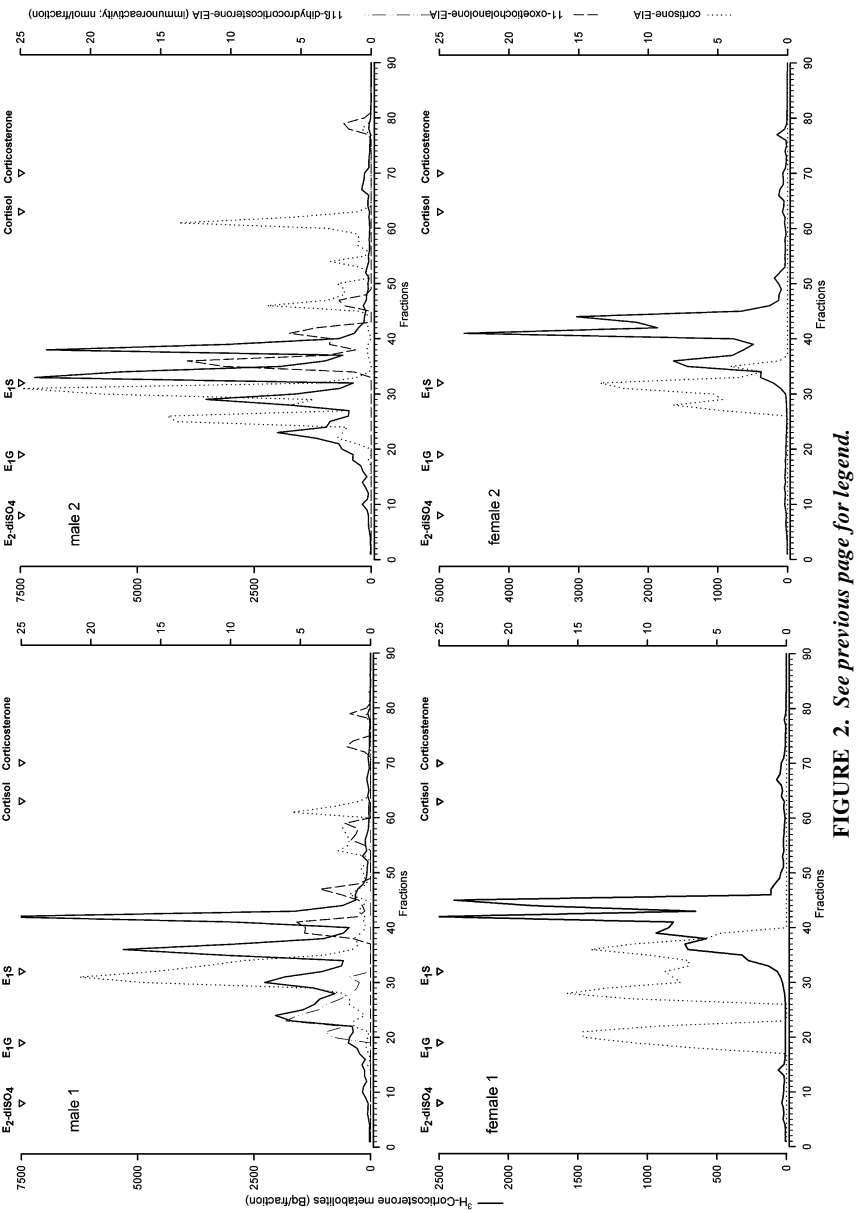

FIGURE 2. *See previous page for legend.*

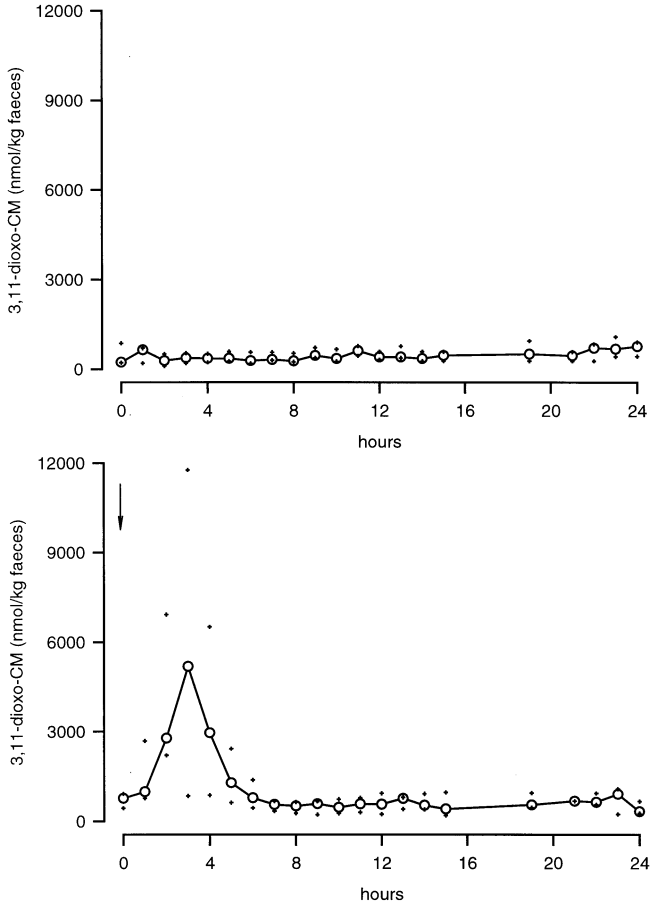

FIGURE 3. Concentrations (min., max., median) of corticosterone metabolites (3,11-dioxo-CM; nmol/kg feces) before (**Upper Panel**: pretreatment group) and after stimulation with ACTH (**Lower Panel**: treatment group), measured by a cortisone-EIA in two male and two female black grouse. Injections took place at 8:00–8:15 A.M.

the levels recorded before the experimental treatment (*post hoc* Dunnett's test, not significant).

During the pretreatment day (pretreatment control group), the mean (±SE) concentration of CM in droppings of all four birds was 454 ± 31 nmol/kg feces (FIG. 3). On the second day, after the ACTH injection at 8:00–8:15 A.M., the CM concentration reached its maximum (3 to 12 µmol/kg) during the first three hours, which represents a 13-fold increase in comparison with the control, pretreatment baseline values. Within the following four hours, the concentration decreased again to 594 ± 25 nmol/kg, a level that remained more or less constant over the next 16 hours. On the third day of the experiment (posttreatment group I), concentrations returned to

levels similar to pretreatment (453 ± 41 nmol/kg feces) and remained similar on the fourth day (posttreatment group II; 438 ± 56 nmol/kg).

The four birds reacted differently to the ACTH injection. The strongest response (24-fold magnitude in comparison with control values) was observed in female 2, but it should be mentioned that this bird showed the lowest mean baseline concentration of CM. The highest peak of metabolites, amounting to 12 μmol/kg feces, was found in the male, which already exhibited the highest average concentration of metabolites in the pretreatment control group.

As with the cortisone-EIA, there was a significant difference in the concentrations of the CM measured with the 11-oxoaetiocholanolone- and the 11β-dihydrocorticosterone-EIA between the pretreatment control group and the treatment group during the 48 hours following the ACTH injection (ANOVA, 11-oxoaetiocholanolone-EIA: $df = 1$, $F = 24.28$, $P < .0001$; 20β-dihydrocorticosterone-EIA: $df = 1$, $F = 57.49$, $P < .0001$), although no prominent peak was recognizable. In addition, concentrations of these CM were 5 to 20 times lower than the 3,11-dioxo-CM measured with the cortisone-EIA and showed statistical differences among individuals (ANOVA, 11-oxoaetiocholanolone-EIA: $df = 3$, $F = 41.13$, $P < .0001$; 20β-dihydrocorticosterone-EIA: $df = 3$, $F = 26.61$, $P < .0001$).

Stability of ^{3}H-Corticosterone Metabolitesin the Feces

After incubation of feces at 6–7°C in a refrigerator, a slight decrease of CM (by 16%) was noticed after 24 hours, which, however, was not statistically significant (360 nmol/kg vs. 430 nmol/kg for the control sample; FIG. 4). Concentrations re-

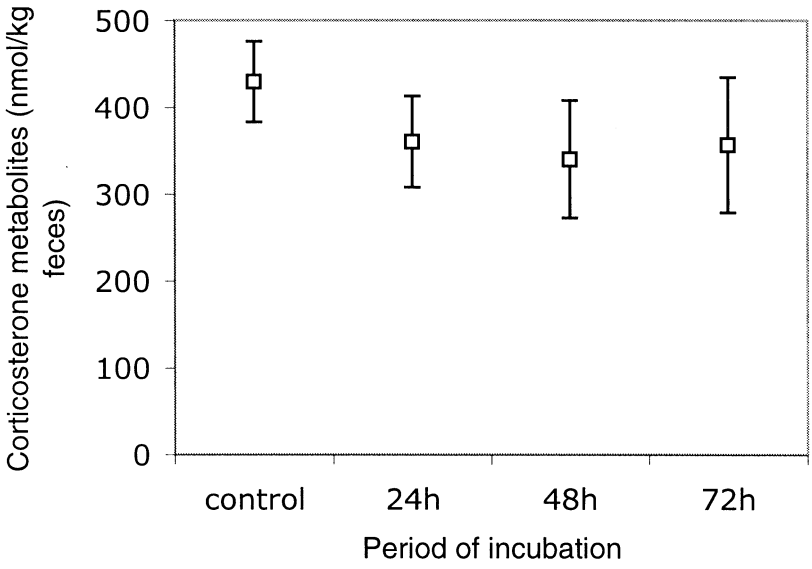

FIGURE 4. Concentrations (mean ± SE; $n = 3$) of corticosterone metabolites (3,11-dioxo-CM; nmol/kg feces) after incubation at 6–7°C for 24, 48, and 72 h, respectively. Controls stem from samples that were immediately frozen at −22°C.

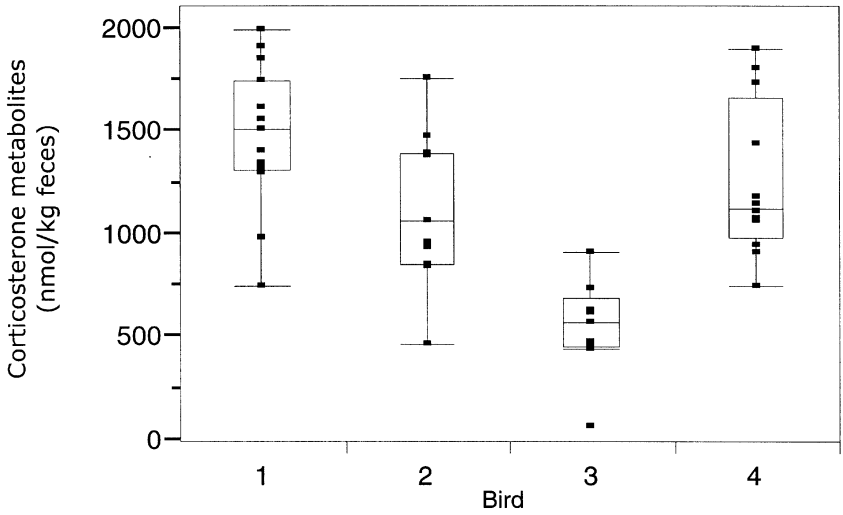

FIGURE 5. Boxplots of 3,11-dioxo-CM concentrations (nmol/kg feces) from samples from the snow burrows of four free-ranging black grouse males in February 2003.

mained close to that level after 48 h and 72 h (average decrease by 21% and 17%, respectively, from the control sample).

Concentrations of ^{3}H-Corticosterone Metabolites from Feces of Free-Ranging Birds

Concentrations of CM in free-ranging black grouse assayed by the cortisone-EIA ranged from 62 nmol/kg to 1993 nmol/kg feces (FIG. 5). There was a significant interindividual variation in metabolite concentrations (ANOVA, $F_{[3,43]} = 14.852$, $I < .0001$), but within the burrow variances did not differ significantly (Levene's test, $F_{[3,43]} = 1.048$, $P = .381$). This range of concentrations was similar to, or somewhat higher than that of captive birds on the control day prior to the ACTH challenge experiment, but much lower than concentrations induced by ACTH (FIG. 3) assayed with the same EIA.

DISCUSSION

In this study, we successfully tested and physiologically validated a noninvasive method for evaluating adrenocortical activity in the black grouse. This method offers the novel possibility of quantifying the level of stress, for instance, induced by human disturbance, in free-ranging populations of this endangered species, since feces can easily be collected from snow burrows in winter. In line with previous investigations, we could demonstrate that assaying CM by group-specific EIAs gives an accurate picture of the adrenocortical activity.[28] This is the first time that a noninvasive

technique for evaluating disturbances has been described via corticosterone metabolites measured by EIA in fecal material of a species of Phasianidae.

Birds' excreta consist of an inhomogeneous cloacal mixture of urine and feces. Steroid metabolites are excreted in urine and feces at different time intervals, first in urine, and in feces some hours later.[30,31,42] In the black grouse, as in chicken,[30] the first peak of radioactivity appeared within the first two hours after injecting 3H-corticosterone (FIG. 1). This corresponded to an initial period when most excreta in all four birds were rather liquid, containing a large number of urine components. Yet, contrary to what was found in other studies,[11,30,44] a second peak, reflecting excreted fecal metabolites, roughly corresponding to the timing of gut passage, was not detected in black grouse (except in one male), where the concentration of metabolites decreased almost continuously. The reason for this may be a more expressed urinary excretion, thus concealing the smaller amounts of fecal metabolites or some mixing of excreta in the cloaca.

Our HPLC analysis of CM from the feces of two captive males and females demonstrated that corticosterone is heavily metabolized mainly to polar metabolites, whereas corticosterone itself was almost absent. This corresponds to the findings of Goymann *et al.*[11] in European stonechats, Carere *et al.*[32] in great titmice, and Rettenbacher *et al.*[30] in chicken. Altogether, three to four prominent peaks, probably representing conjugated CM, were present in all four black grouse individuals. There were also some apparent sex differences in the pattern of metabolites formed, similar to those shown for other vertebrate species.[30,31]

Only three out of seven group-specific EIAs tested cross-reacted significantly with the ^{3}H-CM present in the HPLC fractions (FIG. 2). In order to choose the one best suited to assess adrenocortical activity, all three of those EIAs were physiologically validated. This was achieved by the ACTH challenge test. Blood samples were not taken, as corticosterone concentrations in plasma are known to correlate well with the concentration of metabolites in the feces.[45,46] Because we were interested in the pattern of CM in the droppings, it was important to avoid confounding effects of the stress experienced by the blood sampling procedure itself. In addition, we did not want to apply an invasive, frequent blood-sampling regime to the endangered birds.

Injection of ACTH resulted in a distinct increase in measured 3,11-dioxo-CM concentrations in all four birds. After the initial peak, which took place during the first 2 to 3 h after ACTH administration, there was a rapid decrease in CM values within 4 h. Peak concentrations were approximately 13 times higher than baseline values (FIG. 3). This increase was more pronounced than in chicken,[30] and makes it more probable that some less stressful events also can be monitored by fecal analysis.

Although sex differences were observed in the pattern of formed ^{3}H-CM, there were no statistically significant differences in measured levels of 3,11-dioxo-CM. This is another advantage of the cortisone-EIA, because the gender of an animal can be neglected in comparative analyses of stress levels faced by birds under various environmental conditions. The other two assays (11-oxoetiocholanolone and 11β-dihydrocorticosterone-EIA) were found to be unsuitable for evaluating stress properly; as the measured concentrations were much lower, no distinctive peaks could be recognized after ACTH injection, and pronounced individual differences were observed.

As Washburn and Millspaugh[33] and Morrow *et al.*[47] showed for "even-toed" ungulate feces, environmental conditions, particularly moisture in combination with higher temperatures, can significantly affect CM degradation in the feces, as they would favor the activity of bacteria. This could be a serious source of bias in the quantification and interpretation of stress levels. One advantageous trait of black grouse is that in winter they roost in snow burrows, in which they defecate. This enables the collection of fecal samples, which are, so to say, naturally stored at optimal temperature conditions (<0°C), which is lower than in our incubation experiment(6–7°C, where degradation is only slight).

Another question is whether there is variation in CM between the droppings of the same individual within a short time. This variation could, for example, be due to a diurnal rhythm. In our preliminary field experiment, the concentrations of CM taken from samples of free-ranging birds varied considerably within the individual. However, the mean of the individuals still differed significantly. It is therefore advisable to take all droppings from a snow burrow and homogenize them before analysis. We think that this is the best way to characterize the level of CM over the time the feces were excreted.

We conclude that measuring corticosterone metabolites from feces of black grouse with the cortisone-EIA provides a suitable, novel tool for quantifying noninvasively adrenocortical activity, and thus stress, in free-ranging black grouse populations. This enables one to investigate properly the levels of stress, acclimation, and facilitation actually faced by this endangered bird species, especially in mountain habitats, where the increasing intrusion of human leisure activities might potentially represent a serious additional source of threat to the fauna in general. In the future, we can envision modeling tolerance thresholds toward human disturbance within black grouse populations. This might be an essential step for proposing sound, targeted conservation measures to mitigate the impact of man on that emblematic species.

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