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ORIGINAL PAPER

A non-invasive method for measuring glucocorticoid metabolites (GCM) in Mountain hares (*Lepus timidus*)

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Abstract In this study, we evaluated a non-invasive method for measuring glucocorticoid metabolites (GCM) in the Mountain hare (*Lepus timidus*). An adrenocorticotrophic hormone challenge test was performed in order to select an appropriate enzyme immunoassay (EIA) to measure faecal GCM. Finally, an 11-oxoaetiocholanolone EIA and a 5α-pregnane-3β,11β,21-triol-20-one EIA were chosen. Both assays showed small fluctuations in baseline values and a clear response after stimulation of the adrenocortical activity. To test the stability of faecal metabolites under field conditions, the effects of different storage conditions and periods on GCM concentrations were examined. The assays revealed low fluctuations in metabolite concentrations within the storage period of 12, 24, 48 and 72 h, both at ambient temperatures of 10°C and 25°C, respectively. A washing-out effect of water was found for both assays, which must be taken into account in field studies. The results indicate that this non-invasive method can be used to evaluate glucocorticoid levels of free-ranging Mountain hares.

Keywords Mountain hare · Faeces · Non-invasive · Cortisol · ACTH

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Introduction

Wild animals have to cope with predictable environmental conditions, such as seasonal changes in climate and resource availability, and with a variety of unpredictable events including social stress (e.g. Hackländer et al. 2003) or human disturbance (Wingfield and Romero 1999; Taylor and Knight 2003; Ingold 2005), which is increasing in most ecosystems worldwide. To evaluate the amount of stress experienced, concentrations of faecal glucocorticoid metabolites (GCM) have been measured in numerous studies showing that human disturbance was correlated with higher glucocorticoid secretion in these species (Dehnhard et al. 2001; Arlettaz et al. 2007; Thiel et al. 2008). Especially winter tourism leads to higher stress in alpine species living at high altitudes (Arlettaz et al. 2007; Thiel et al. 2008). In line with this, alpine Mountain hares (*Lepus timidus varronis*), which dwell in areas with intensive human outdoor activities like ski trails and ski runs (Meile 1984; Gamboni 1997), should also be affected. Investigations about the influences of seasonal changes on the physiological stress reaction of animals have also shown that these can be important to gain information about an animal's relationship to environment and its condition (e.g. Lund-Larsen et al. 1978; Bubenik and Brown 1989; Monfort et al. 1993; Reyes et al. 1997; Huber et al. 2003; Dalmau et al. 2007). However, the physiological reaction to seasonal changes and unpredictable events in Mountain hare is still unknown. In order to propose an appropriate method to quantify their impacts, evaluation of the assay is a necessary first step.

To gain important information about an animal's endocrine status, non-invasive methods for measuring steroid hormone metabolites in the faeces have become a widely accepted tool (Palme 2005). The advantage of such

methods is that samples can be collected easily without any need to handle the animal. Additionally, the process is almost free of feedback reactions, such as disturbance and stress. Therefore, this method is appropriate to evaluate the chronic stress faced by free-living wild animals. Metabolism and excretion of steroids differ significantly between species, and sometimes even between sexes and individuals within a given species (Rettenbacher et al. 2004; Touma et al. 2003; Palme 2005; Palme et al. 2005). Hence, non-invasive methods must be validated for each species (Touma and Palme 2005) by a challenge test with adrenocorticotrophic hormone (ACTH). Moreover, it is decisive to know the effect of various environmental conditions (e.g., ambient temperature or precipitation) and effects of bacterial activities in faeces between defecation and sampling, which may lead to altered GCM concentrations. Hence, we tested the effect of storage conditions and sampling design on GCM levels for selected enzyme immunoassays (EIAs).

Methods

ACTH challenge test

Five captive Mountain hares (two males and three females) in the Alpenzoo Innsbruck (Austria) and the Tierpark Goldau (Switzerland) were used in this study. The whole experiment was designed to last for a period of 7 days. All faeces were collected 3 days before (pre-treatment) and 4 days after an injection of 1 ml Synacthen® (0.25 mg, Novartis Pharma Schweiz SA, Bern, Switzerland), which stimulates adrenocortical activity. In a similar study with European hares (*Lepus europaeus*) the peaks of metabolites were observed in faeces after 24 (±7)h following injection (Teskey-Gerstl et al. 2000). Therefore, time intervals in this study were chosen in order to investigate the course of concentration in the smallest time spans possible by local conditions in zoos. In Goldau, samples were collected in the morning (faeces were a maximum of 16 h old then) and in the afternoon (8 h after the collection in the morning). In Innsbruck, faeces were collected only in the morning. To avoid the influence of possible diurnal variations (Palme 2005; Touma and Palme 2005), sampling was conducted at the same time in the morning and in the afternoon. Until the extraction in the laboratory, all samples were stored at −22°C to prevent changes in the steroid concentrations after collection.

Storage experiment

In order to optimise the collection of faecal samples of free-living Mountain hares, it is important to know whether the concentrations of metabolites changes with time after defecation, especially when ambient temperature increases above zero and allows activation of faecal bacteria. Bacterial enzymes are known to be the main source for observed changes in GCM (Palme 2005; Touma and Palme 2005). To study such effects presentable, four samples were selected. They were collected from one male and one female individual, on the first and third days after the ACTH injection (within 8 h after voidance). Each of the samples was pooled, homogenised and divided into nine equal subsamples. A subsample was frozen immediately at −22°C (control), while the eight other subsamples were incubated at 10°C and at 25°C for 12, 24, 48 and 72 h, respectively, frozen thereafter and stored at −22°C until analysis.

Washing-out experiment

To investigate the possible influence of rainfall on GCM concentrations in field samples, six other samples were selected. Each sample was pooled and homogenised, and three equal subsamples (0.5 g each) were soaked and shaken on a hand vortex with 2.0 ml water. In order to test the effect of washing out, 1 ml of the water of each subsample was removed after centrifugation. To the remaining, 4 ml of methanol was added for extraction. The amounts of GCM were determined in the supernatant of both the water and the methanolic extracts (diluted 1:10 with assay buffer) by both EIAs (see below).

Determination of faecal cortisol metabolites

The general procedure of the EIAs is described in detail by Palme and Möstl (1997). In this study, two 11-oxoaetiocholanolone EIAs and one 5α-pregnane-3β, 11β, 21-triol-20-one EIA were tested. At the start, faecal GCM in Mountain hares were measured with the same 11-oxoaetiocholanolone EIA used by Teskey-Gerstl et al. (2000) and Sheriff et al. (2009). As only very low amounts of immunoreactive GCM were measured with this EIA, another 11-oxoaetiocholanolone EIA (further referred to as EIA1) and a 5α-pregnane-3β,11β,21-triol-20-one EIA (further referred to as EIA2) were evaluated. EIA1 (developed for ruminants) determines corticoid metabolites with a 5β-3α-ol-11-one structure (Möstl et al. 2002), and EIA2 (first developed for laboratory mice) recognises GCM with a 5α-3β,11β-diol structure (Touma et al. 2003; 2004). Both tests detected high amounts of GCM and were used in the present study. Samples were homogenised after collection (Palme 2005) and 0.15 g (dry weight) were extracted with 5.0 ml of methanol (80%). After shaking on a hand vortex (1 min) and centrifugation (2,500 g, 15 min) the amounts of GCM were determined in the supernatant of the extracts (diluted 1:10 with assay buffer) by both EIAs.

Data analysis

All statistical tests were conducted using R 2.5.0. In the descriptive part of the ACTH challenge test, GCM baseline for faeces was defined for each individual as the mean of the morning concentrations in the pre-treatment stage. For determination the time of peaks at an individual level, samples were collected after treatment. To demonstrate the changes of concentrations in this study, values of all individuals were allocated into time frames of 24 h, and mean concentration (±95% CI) was calculated for each interval. In order to get a normal distribution (Shapiro–Wilk normality test), the data were logarithmised before analysis. We tested the effect of time on concentrations measured with EIA1 and EIA2 using analysis of variance (ANOVA), followed by Tukey's post hoc test to conduct multiple comparisons of the mean concentrations between days.

To determine the effects of time and temperature on GCM concentrations measured with EIA1 and EIA2 in the storage experiment, values of all samples were allocated into mean concentration for each interval. Logarithmisation of the data was unnecessary in this approach as they showed a normal distribution. Influences on the GCM levels were tested by ANOVA with levels measured with EIA1 and EIA2 as the dependent variable and treatment (frozen vs. not frozen), time, temperature and the interaction of the latter as independent variables. Tukey tests were used to determine differences between time intervals.

In the washing-out experiment, we calculated GCM loss in nanograms and as a percentage of the total amount present in the faeces in the control faecal pellets (mean ± standard deviation). The effect of elution was analysed by ANOVA with percentage of total pre- (100%) and post-treatment concentrations measured by EIA1 and EIA2 as the dependent variables and treatment (yes or no) as the independent variable. To analyse difference in GCM loss between assays, we used a *t* test.

Results

ACTH challenge test

There was considerable inter-individual variability across the means of all samples' baselines during the pre-treatment phase as well as in the peak values. In addition, there was inter-individual variability also in the time after which peak levels were reached and returned to the basal levels (Table 1). After the injection on the third day, GCM concentrations peaked after 8 to 48 h and returned to baseline levels between 48 and 72 h after treatment. In EIA1, maximum concentration of GCM ranged from 68 to 128 ng/g faeces (mean 88 ng/g) which reflects a 1.1- to 2.2-fold increase over the individual basal levels. In EIA2, maximum concentration of GCM ranged from 54 to 302 ng/g faeces (mean 137 ng/g) which reflects a 1.2- to 3.2-fold increase over the individual basal levels. The decrease of GCM measured by the second EIA was protracted when compared to EIA1 (Fig. 1).

Table 1 ACTH challenge: individuals' baseline and peak values (ng/g faeces; percent increase) of faecal GCM concentrations analysed by EIA1 and EIA2 and time after which faecal peak levels were reached

Animal	Baseline (mean±SD)		ACTH challenge				
			Maximum (ng/g)		% Increase		Hours after treatment
	EIA1	EIA2	EIA1	EIA2	EIA1	EIA2	
1	31±5	24±3	68	54	218	220	8
2	46±6	13±2	80	25	172	187	8
3	45±2	71±14	96	152	215	215	24
4[a]	61±13	95±13	69	302	113	318	48
5[a]	90±8	130±13	128	151	143	116	24

[a] Sampling in 24-h intervals

Statistical effects of time were found for both EIAs (EIA1: $F_{6,28}$=4.91, P=0.002; EIA2: $F_{6,28}$=3.17, P=0.02). Tukey's post hoc test showed significant differences between day 6 (t=−3.178, P=0.049) and day 7 (t=−3.014, P=0.07) to day 4 for EIA1, but for EIA2, no significant differences between days were found. The mean values of all individuals and both EIAs are shown in Fig. 1.

Storage experiment

At both temperatures (10°C and 25°C), GCM concentrations of both EIAs were almost on a constant level (Fig. 2). There were no statistical effects of temperature (EIA1: $F_{1,38}$=0.01, P=0.90; EIA2: $F_{1,38}$=0.03, P=0.87), storage time (EIA1: $F_{4,35}$=0.01, P=0.91; EIA2: $F_{4,35}$=0.72, P=0.40) or their interaction (EIA1: $F_{10,30}$=0.26, P=0.72; EIA2: $F_{10,30}$=3.17, P=0.61).

Washing-out experiment

In all used samples (n=6, with three subsamples each), a negative effect of the water treatment on GCM levels was found for both EIAs ($F_{1,70}$=649.3, P<0.001). Amounts of GCM transferred from pellets to water ranged from 1.4 to 12.5 ng (EIA 1) and 9.4 to 14.6 ng (EIA2) per 1 ml of water. The total proportion of GCM loss due to water treatment ranged between 16% and 40% (mean 32±10%) for EIA 1 and 27 to 33% (mean 29±3%) for EIA 2, respectively. There was no statistical difference in GCM loss between both EIAs (t=1.171, P=0.26).

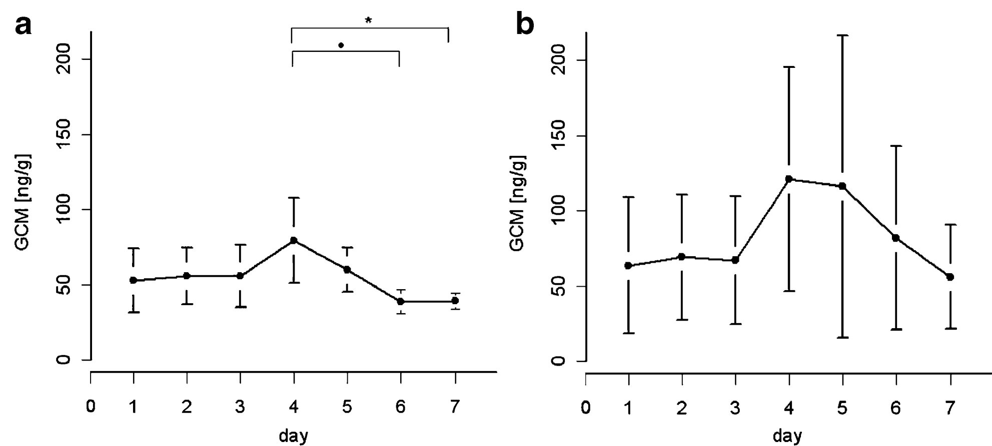

Fig. 1 GCM concentrations of adult Mountain hares (mean±95 CI; n=5) measured by EIA1 (**a**) and EIA2 (**b**) after an ACTH challenge on the third day

Discussion

In this study, we validated a non-invasive method for measuring stress in Mountain hares. It demonstrates the necessity to physiologically validate GCM measurements even in related species (Touma and Palme 2005), as the EIA used in European and Snowshoe hares (Teskey-Gerstl et al. 2000; Sheriff et al. 2009) detected only very small amounts of immunoreactive GCM in Mountain hares. However, the selected 11-oxoaetiocholanolone EIA (Möstl et al. 2002) and the 5α-pregnane-3β,11β,21-triol-20-one EIA (Touma et al. 2003), which were used for the first time in the genus *Lepus*, showed the required suitability for measuring GCM concentrations in faeces of Mountain hares. Results of both EIAs did not differ significantly, and none seemed more qualified. As groups of faecal GCM detected by the used EIAs are not identical, the observed differences in assessed concentrations are neither much surprising nor indicative of the quality of the assays. The protracted decrease of GCM measured by EIA2 indicates that this EIA may pick up metabolites (probably more polar ones) formed after an enterohepatic recirculation (Palme et al. 1996; Lexen et al. 2008).

Interestingly, we found contrasting results in the ACTH challenge test within the available documentations in the genus *Lepus*. In our study, GCM levels of Mountain hares increased after stimulating adrenocortical activity up to 218% (EIA 1) and 318% (EIA 2), respectively. Also, in European hares (*L. europaeus*), pre-treatment levels were exceeded up to five times (Teskey-Gerstl et al. 2000). However, following ACTH injection in Snowshoe hares (*Lepus americanus*), the levels of faecal cortisol metabolites increased up to 10-fold (Sheriff et al. 2009). Peak concentrations in Mountain hares were reached between 8 and 48 h after treatment. Similar delay times (about 24 h) were also found in European hares (Teskey-Gerstl et al. 2000), whereas in Snowshoe hares, highest concentrations of faecal cortisol metabolites were reached about 10 h post stimulation (Sheriff et al. 2009). Potential explanations of this variation can be found in the species-specific differences in GC metabolism and/or excretion, in diet and/or the sampling schedule: The faeces of European hares (Teskey-Gerstl et al. 2000) were collected once a day in the morning, and the sampling of faeces of Snowshoe hares began after 2 h of treatment in 4-h intervals (Sheriff et al. 2009). In general, sample collections in smaller time intervals provide more detailed information about changes in levels of GCM over time and thus result in more pronounced increases in peak concentrations. The time of treatment should also be considered. Tests performed at the beginning of the active phase of an animal can lead to a shorter time delay as compared to those carried out at the beginning of the inactive phase (Touma et al. 2003; Touma and Palme 2005).

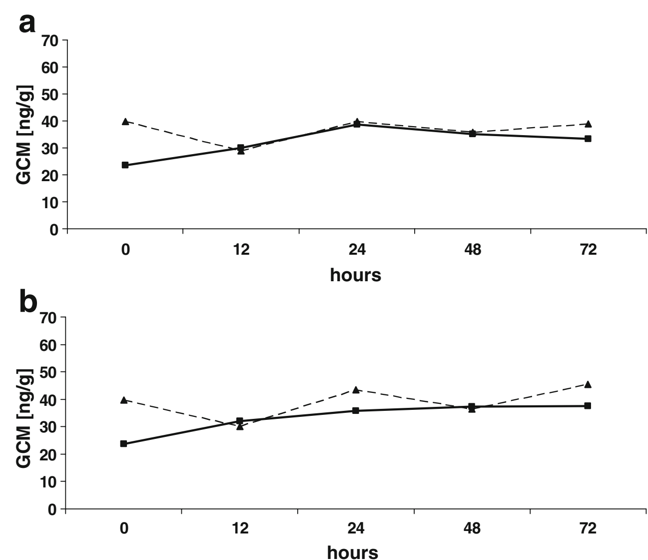

Fig. 2 Concentrations (mean; n=4) of faecal GCM concentrations (ng/g) measured by EIA1 (*triangles*) and EIA2 (*squares*) after incubation at 10°C (**a**) and at 25°C (**b**) for 12, 24, 48 and 72 h, respectively

In the performed storage experiments, neither different storage conditions nor time intervals resulted in a statistically significant variation of GCM concentrations. In other species investigated in a similar way, concentrations remained unchanged if faeces were incubated at 8°C (Thiel et al. 2005) or 6–7°C (Baltic et al. 2005). Results indicate that this temperature is not optimal for activity of bacterial enzymes. In Mountain hares, incubation of faeces at 25°C showed no statistical significant changes of GCM, either. However, in capercailles (*Tetrao urogallus*) and domestic livestock, GCM concentrations were significantly higher in samples which were exposed to a higher temperature for a longer time period (Thiel et al. 2005; Möstl et al. 1999). Interestingly, a negative influence of water treatment on GCM levels could be found for both assays. The high amounts of GCM present in the water portion might be explained by the prior homogenisation of the faecal pellets. Here, the original form of faeces was destroyed, and the effect of the water could have been more expressed compared to dried intact pellets. However, still rainfalls are important for any field application of this non-invasive method, and any precipitation during field sampling must be documented before analysis.

This study demonstrates that GCM are reliably measured in faeces collected from Mountain hares under field conditions in various seasons. Faecal GCMs are successfully determined with the selected 11-oxoaetiocholanolone EIA and 5α-pregnane-3β, 11β, 21-triol-20-one EIA. In the field, faeces can be collected within 3 days after defecation. Only rainfalls must be monitored during periods of field sampling because of the potential washing-out effects.

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