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Measurement of Glucocorticoid Metabolites in feces of Capricorns (Alpine Ibex)

Diploma thesis

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Abbreviations

ACTH	Adrenocorticotropin hormone
EIA	Enzyme immunoassay
GCM	Glucocorticoid metabolites
GC	Glucocorticoids
11,17-DOA	immunoreactive substances with an 5 β-androstane-11,17-dione structure
3α, 11 oxo-A	immunoreactive substances with a 5 β-androstane-3α-hydroxy, 11-one structure
Assay A	11-oxoetiocholanolone EIA measuring substances with a 5 β-androstane - 3α-hydroxy, 11-one structure
Assay B	11-oxoetiocholanolone EIA measuring substances with a 5 β-androstane – 11,17-dione structure
T0	samples collected shortly after defecation
T3	samples collected three hours after defecation
T6	samples collected six hours after defecation
T24	samples collected 24 hours after defecation

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Abstract

The aim of this study was to validate an appropriate enzyme immunoassay (EIA) to analyze fecal glucocorticoid metabolites (GCM) in Alpine ibex Therefore an ACTH stimulation test was performed at the Wildlife Park Langenberg, Switzerland. Two animals, an adult male and an adult female ibex, were narcotized and ACTH was injected intramuscularly. Fecal samples were collected prior to and several days after the ACTH stimulation test. After collection, parts of the samples collected prior to the ACTH test, were divided into four equal subsamples. One fraction was taken to a local freezer and stored immediately at -18°C, the other parts were retained in the enclosure to perform a storage test. The test was performed to ascertain the stability of GCM over a specific time (3, 6, 24 hours) in the fecal samples. Samples were analyzed by two different 11-oxoetiocholanolone EIAs which were already used successfully for the determination of GCM in other ungulate species. Both were compared for their performance in Alpine Ibex. Assay A detected the highest quantities of immunoreactive metabolites prior and after the ACTH injection in fresh fecal samples. The percentage increase, after ACTH injection, showed that assay B reacted a little more expressed than assay A. The result of the storage test showed, for both assays, that there was no significant difference between samples collected shortly after defecation and samples collected three, six and 24 hours after defecation. In addition to the GCMs, fecal estrogen levels were measured too, to see if the ACTH injection has an influence on the estrogen level in the feces. Estrogens displayed the same pattern after ACTH injection as the GCMs. Thus, my study demonstrates that fecal GCMs can be used as a reliable and non-invasive parameter to determine stress in Alpine ibex.

Zusammenfassung

Die Studie beschäftigt sich mit der Bestimmung von Glukokortikoidmetaboliten im Kot von Steinböcken. Es wurde ein ACTH Stimulationstest im Wildpark Langenberg (CH) durchgeführt. Zwei Tiere, der führende Bock und die führende Geiss, wurden narkotisiert und ACTH wurde intramuskulär verabreicht. Die Probensammlung erfolgte vor und nach dem ACTH Test. Einen Teil der Proben, die vor dem ACTH Test gesammelt wurden, teilte ich in vier gleiche Teile. Der erste Teil dieser Proben wurde sofort in den lokalen Gefrierschrank gebracht und bei einer Temperatur von -18°C gelagert. Mit den restlichen Teilen der Proben führte ich einen Lagerungstest durch, um den Abbau von Glukokortikoidmetaboliten über eine gewisse Zeit (3, 6, 24 Stunden) im Kot zu bestimmen. Die Proben wurden mit der Hilfe zweier verschiedener 11-Oxoätiocholanolon Enzymimmunoassays analysiert. Assay A wies die höchste Menge von immunreaktiven Metaboliten vor und nach der ACTH Injektion im frischen Kot auf. Der prozentuelle Anstieg nach der ACTH Injektion zeigte das Assay B ein wenig sensitiver reagierte als Assay A. Bei dem Lagerungstest stellte sich heraus, dass für beide Assays, kein signifikanter Unterschied besteht zwischen der Sammlung von Proben zum Zeitpunkt der Defäkation und der Sammlung von Proben nach drei, sechs und 24 Stunden nach der Defäkation. Zusätzlich zu den Glukokortikoid-Metaboliten wurde in meiner Studie noch der Östrogen Spiegel im Kot gemessen, um festzustellen ob die ACTH Injektion den Estrogen Spiegel im Kot beeinflusst. Die Östrogen Konzentration verhielt sich, nach ACTH Injektion, gleich wie die Glukokortikoid-Metaboliten Konzentration. Diese Studie zeigt, dass Glukokortikoidmetaboliten im Kot ein verlässlicher nicht invasiver Parameter sind um Stressreaktionen Steinbock zu bestimmen.

1. Introduction

Wild animals, such as Alpine Ibex (*Capra ibex*), inhabiting alpine regions have to cope with pronounced seasonal changes in environmental conditions and resource availability. These changes may cause a stress response in animals which leads to the activation of the hypothalamic-pituitary-adrenocortical axis. This hormonal cascade results in an increase of glucocorticoid production (ARLETTAZ et al., 2007).

Glucocorticoids (GC), like cortisol, are steroid hormones produced predominantly by the adrenal glands. Their endocrine production is regulated by the hypothalamus and the pituitary gland. The hypothalamus produces the corticotrophin releasing hormone, which stimulates the pituitary gland. This stimulation leads to the increased production of the adrenocorticotropic hormone (ACTH). Thereafter, ACTH causes an increased production of GCs by the adrenal glands. GCs have effects on the glucose metabolism, act in a catabolic manner, induce lipolysis and cause an involution of the lymphatic tissue (THUN and SCHWARTZ-PORSCHE, 1994). GCs are metabolized in the liver and excreted via urine and feces (PALME et al., 1996).

There are different methods to measure GCs in animals (MÖSTL and PALME, 2002). While measuring plasma glucocorticoid levels is the best indicator to quantify the activity of the hypothalamic-pituitary-adrenal system, blood collection is an invasive procedure that requires skilled technical capabilities, sample collection, and storage capabilities (MÖSTL and PALME, 2002). Other methods to determine GCs or GCMs are collecting salvia, urine and milk (MÖSTL and PALME, 2002) but this is rather difficult under field conditions and therefore impracticable.

In the past years different methods and parameters have been used to assess stress reactions and welfare in various ruminants. From the above mentioned matrices all need, except the collection of feces, handling of the animals. Therefore the measurement of fecal GCMs has become an important tool for estimating stress (MÖSTL and PALME, 2002). Fecal samples are easily obtained, can be stored in the freezer for a long time and are feedback free, meaning that the collection of the samples does not stress the animal.

The integrated measure of hormone metabolites over an extended period of time and its non-invasiveness make the quantification of steroid hormone metabolites in feces a tool which is particularly suitable for field applications. This method has already been used to determine stress in domestic livestock including cattle, sheep and goats (DEHNHARD et al., 2001; KLEINSASSER et al., 2010; LEXEN et al., 2008; MÖSTL and PALME, 2002; PALME et al., 1999). This led to the development of group specific enzyme immunoassays (EIA) for the determination of GCMs (MÖSTL et al., 2005). Studies showed that there are also differences in GCMs excreted between species (PALME et al., 2005), and therefore various EIAs had to be developed, since each EIA reacts with another group of metabolites excreted in feces (MÖSTL et al., 1999). Physiological validation of an EIA is possible by an ACTH stimulation test and continuous collection of fecal samples. In addition to the GCMs, estrogen concentration in the fecal samples were also measured, to discover if there is a correlation between ACTH injection and the estrogen level in feces, because a small amount of estrogens is also secreted by the adrenal cortex.

The aim of this study was to select an appropriate EIA to determine GCM levels in the feces of Alpine Ibex. As fecal GCM levels can be used as a parameter of stress, such a method is important for studies on wildlife management, conservation biology, and behavioral ecology of this species.

2. Material and Methods

2.1. ACTH Stimulation Test

An ACTH stimulation test was performed with a male (15 years old) and a female (10 years old) Alpine Ibex at the Wildlife Park Langenberg, Switzerland. The animals were kept in an enclosure together with 20 animals of both sexes and different age classes. It was obviously from the age and the attitudes of the other animals that these two animals held the leading position in the hierarchy among the same-sex individuals of the group.

Fecal samples were collected over approximately 12 hours every day on six consecutive days. The collection started in the early morning between six and seven. At lunch time, always one of the observers stayed in the enclosure. The assemblage ended between six and seven in the evening. The two animals were observed at every minute during the day time. No observation of the animals and no collection of fecal samples were done over night. After defecation the samples were collected immediately by hand and stored in a plastic bag. When the samples were brought to the local freezer always one observer stayed in the enclosure and watched the animals. In the morning of the third day, the ACTH-stimulation was performed. The animals were narcotized with the Hellabrunner mix (Ketamin (Ketasol®, Graeub, Dr. E., AG, CH-Bern; 10 mg/kg BW) and Xylazin (Rompun®, Bayer Pharma; 0,02 – 0,05 mg/kg BW i.m.); the male with 2.5 ml, the female with 1.8 ml. The dose was calculated referring to prior experience in anesthesia of ruminants. Once narcotized, they were weighed, blood was taken and 0.5 mg ACTH (Synacthen®, Novartis Pharma, Basel, Switzerland) was injected intramuscular to stimulate the glucocorticoid production from the adrenal glands. While recovering from anesthesia, both animals were separated from the herd, taken to a shelter and received 0.025-0.05 mg/kg Atipamezol (Antisedan®, Orion Corporation, Turku, Sf-Turku) as reversal. Treatment of the animals was approved by the district veterinary office of the Canton of Zurich (No. 3993).

2.2. Storage Test

Seven samples, four from the male and three from the female, were used for the storage test. All samples utilized for the storage test were collected prior to the ACTH stimulation test. Each sample was divided into four equal parts. One part was taken immediately (within less than 30 min after defecation) to a local freezer and stored at -18°C. The other parts were retained in the enclosure to perform a storage test. They were stored in contact with the soil in a corner of the enclosure. The samples were exposed to all weather changes during day and night. At the beginning of the study (Monday and Tuesday) it was raining and the temperatures were about 7 °C. In the lapse of the week the weather changed from rain into snow (Tuesday evening) and the temperatures decreased reaching temperatures between 3 °C over the day and -7 °C over the night. The subsamples collected on Monday and Tuesday, were wet and cold. On Wednesday the subsamples were partly frozen and partly wet. After three, six and 24 hours one fraction of the samples was brought to the local freezer. The aim of the storage test was to investigate the depletion pattern of GCMs in the feces, since it is not always possible to collect the fecal samples directly after defecation under field conditions.

2.3. Laboratory analysis

Laboratory analyses of fecal samples were performed at the Institute of Chemistry and Biochemistry, University of Veterinary Medicine Vienna, Austria. The samples were defrosted and 0.5 g of every sample was put into a test tube. Four milliliters of 100 % Methanol and 0.5 ml of water were added to each sample. The test tubes were vortexed for 30 min on a multivortex (Rapid Vap, company Labconco) or 1-2 min on a handvortex (Vortex mixer, company Velp). Thereafter, samples were centrifuged for 15 min at 2,500 *g* (Allegra X-12R, company Beckmann / GS-6KR). After centrifugation, an aliquot of the supernatant was diluted (1:10) with assay buffer (pH 7.5) and stored at -20°C. Then the EIA was performed (MÖSTL et al., 2002; PALME and MÖSTL, 1997).

I used two different EIAs to determine the GCM levels in the samples. The standard for assay A used was 11-oxoetiocholanolone (5β-androstane-3α-ol-11,17-dione). The standard curve ranged from 2-500 pg/well. The antibody was raised against 11-oxoetiocholanolone-17-

CMO:BSA in rabbits (working dilution 1:60,000; Ak 3199/6/96). The label was 11-oxoetiocholanolone-17-CMO-biotinyl-3,6,9-trioxaundecanediamin (working dilution; 1:2x10^6; lab-code EL 71; MÖSTL et al, 2002). The standard for Assay B used was the same as in Assay A. The range of the standard curve was 2-500 pg/well. The antibody was raised against11-oxoetiocholanolone -3-HS: BSA (working dilution 1:20,000; lab-code Ak 7/42/95), as label 11-oxoetiocholanolone-3-glucosiduronate-DADOO-biotin (1:25,000; lab-code EL 54) was used (PALME and MÖSTL, 1997).

For the measurement of estrogens I used the biotin-streptavidin enzyme immunoassay described by PALME and MÖSTL, 1994.

2.4 Statistics

The correlation for both assays was calculated with SigmaStat (Version 3.1). For the calculation of the significance, for the storage test, we used the t-test function and the Mann-Whitney-Rank Sum Test.

3. Results

3.1. ACTH Stimulation Test

3.1.1. Assay A vs. assay B in the male ibex

Using the assay A (lab code EIA 72T), GCM levels showed a first moderate increase (approximately 490 %) approximately 23 hours after the injection of ACTH. In the male ibex, the level of excreted GCM peaked after 25 hours (approximately 1780 %) at ~6000 ng/g, and thereafter declined over the following 24 hours.

With assay B (lab code EIA 72a), the levels of excreted GCM in the male ibex increased over the first 23 hours (approximately 1160 %), and reached its peak (~1.100 ng/g) after 25 hours

(circa 1920 %) followed by a constant phase over the next hours until it started decreasing (Fig. 1). The correlation between both assays was R = 0.949 (P = <0.001).

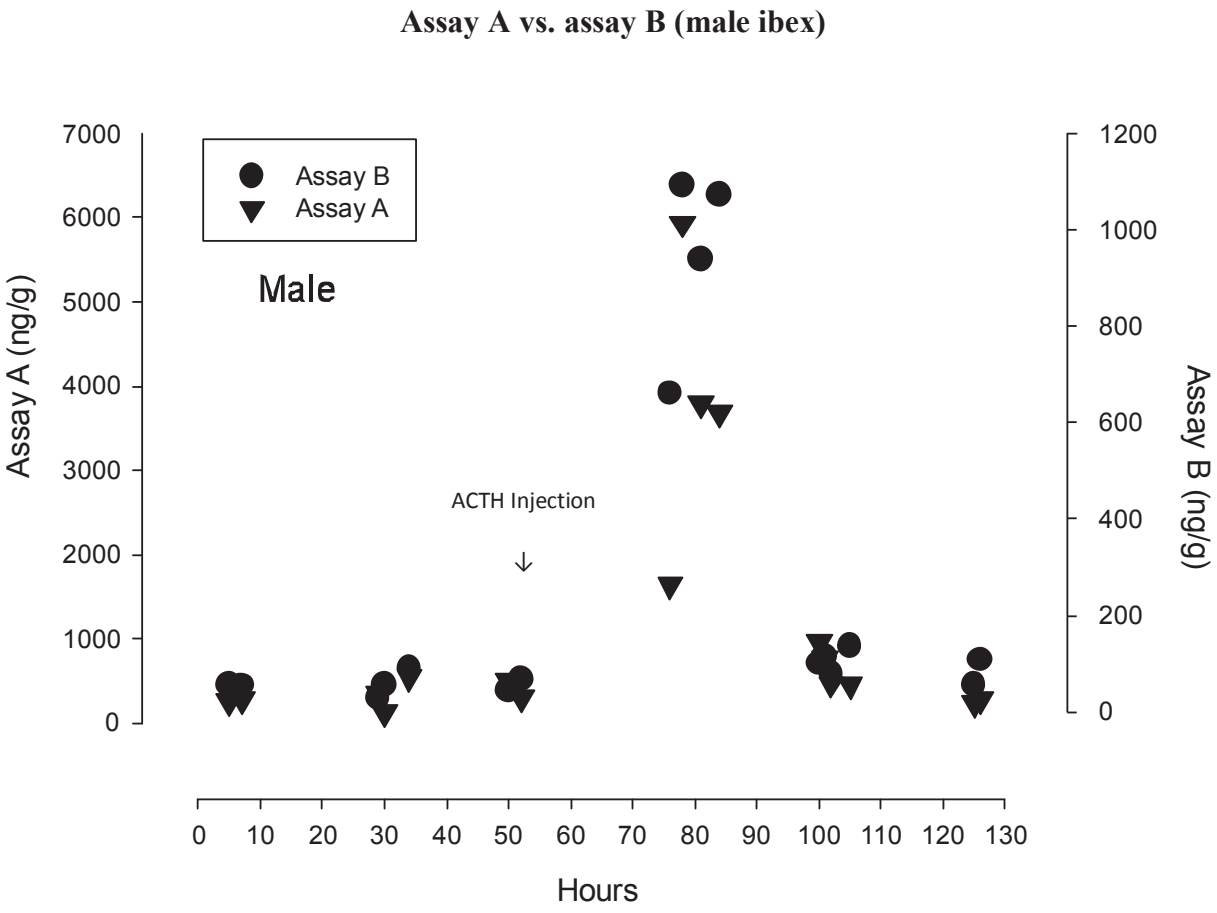

Fig. 1: Concentration of GCMs over the whole observation period in the feces of the male ibex

3.1.2. Assay A vs. assay B in the female ibex

In the female ibex, using assay A, GCM levels reached its peak (~12.000 ng/g) approximately 48 hours (approximately 2310 %) after injection of ACTH, when the animal was observed defecating for the first time. After reaching the peak, excreted GCM levels drastically decreased within the subsequent 24 hours.

As with assay B (Fig. 2) the GCM level of the female showed a comparable pattern as when using assay A, and reached its peak after 48 hours (~6.100 ng/g, approximately 3300 %)

The correlation between both assays was R = 0.968 (P = 0.029)

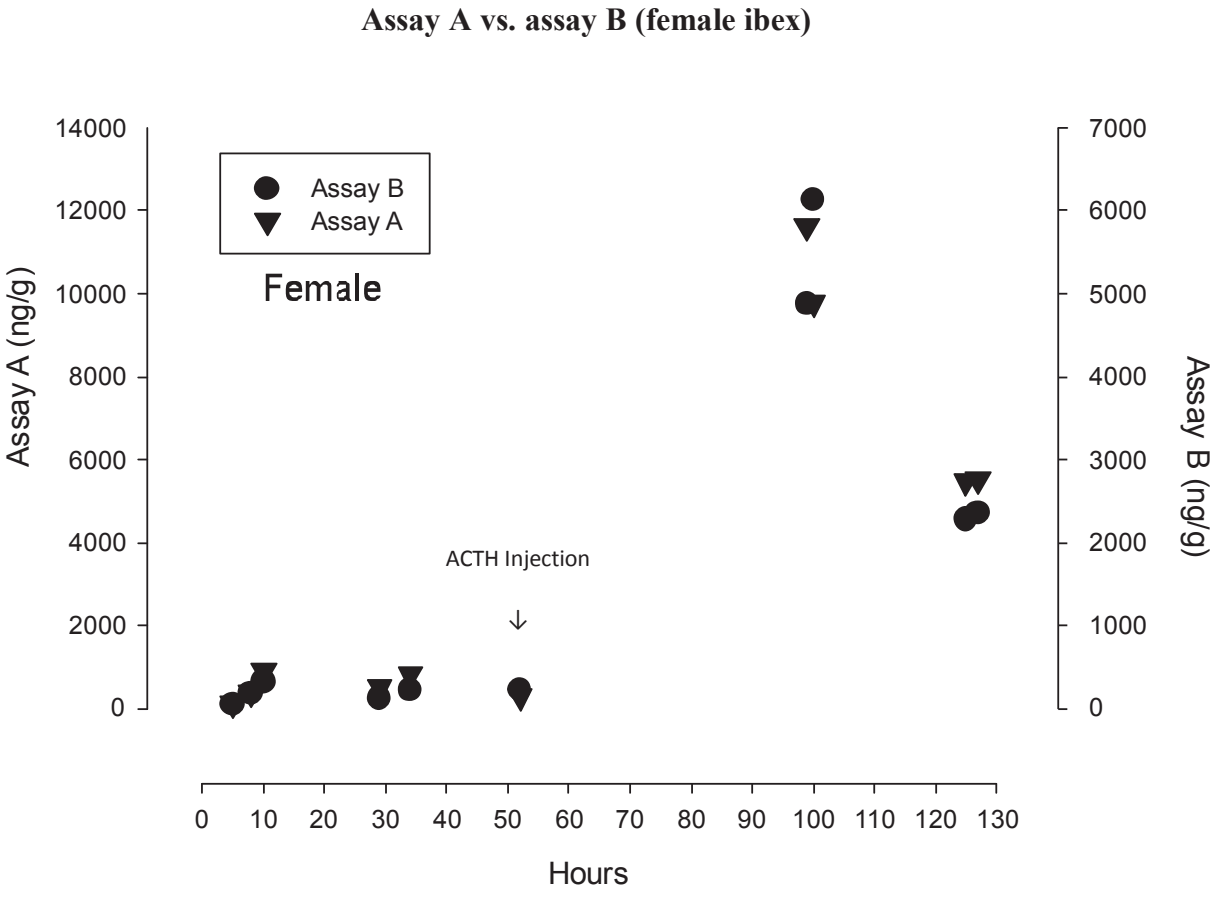

Fig. 2: Concentration of GCMs over the whole observation period in the feces of the female ibex

In summary, assay A detected the highest quantities of immunoreactive metabolites prior (male 333 (± 142) ng/g, female 503 (± 294) ng/g) and after the ACTH injection (male 1822 (± 1963) ng/g, female 8100 (± 3094) ng/g) in fresh fecal samples. As with assay B the metabolites prior (median values: male 57 (± 19) ng/g, female 185 (± 92) ng/g) and after injection (median values: male 436 (± 450) ng/g, female 3909 (± 1908) ng/g) were lower.

Fig. 3 shows the percentage increase in the male ibex. Assay B reacted a little more sensitive to the ACTH injection than assay A.

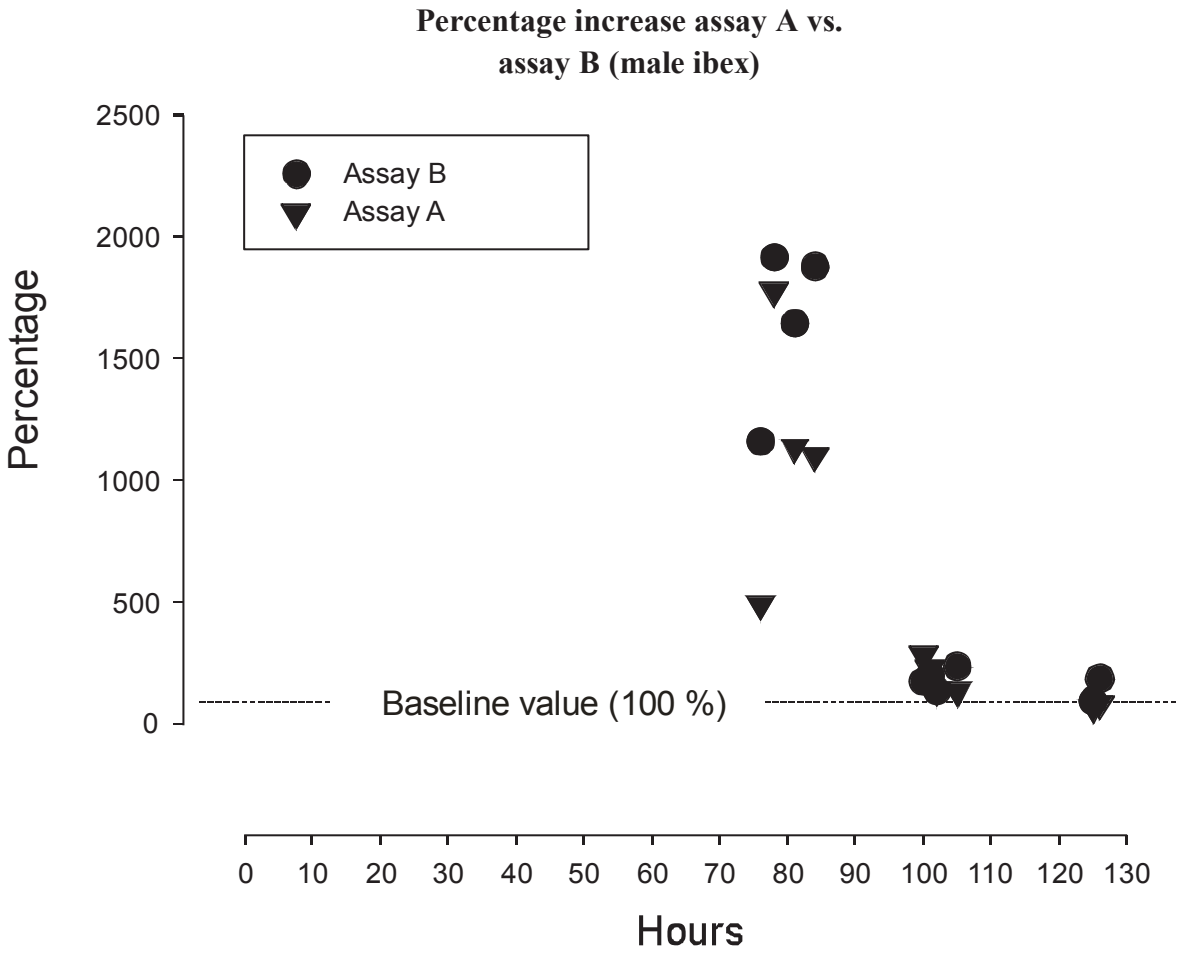

Fig. 3: Percentage increase of assay A and assay B in the male ibex

3.1.3 Measurement of Estrogens

The estrogen level in the male and the female showed the same pattern as the GCM level. As for the male the estrogen level increased 23 hours (approximately 367 %) after injection (Fig. 4). It peaked after 25 hours (34 ng/g, circa 1220 %) and declined over the next 24 hours.

As for the female the estrogen level reached its peak after 48 hours (1033 ng/g, approximately 870%) when it was first seen defecating (Fig. 4). Afterwards the estrogen level dropped drastically. The correlation between assay A and estrogens is R = 0.975 (P = 0.250) and the correlation between assay B and estrogens is R = 0.970 (P = 0.145) for the male.

As for the female the correlation between assay A and estrogens is R = 0.578; (P = <0.001) and the correlation between assay B and estrogens is R = 0.522 (P = 0.081).

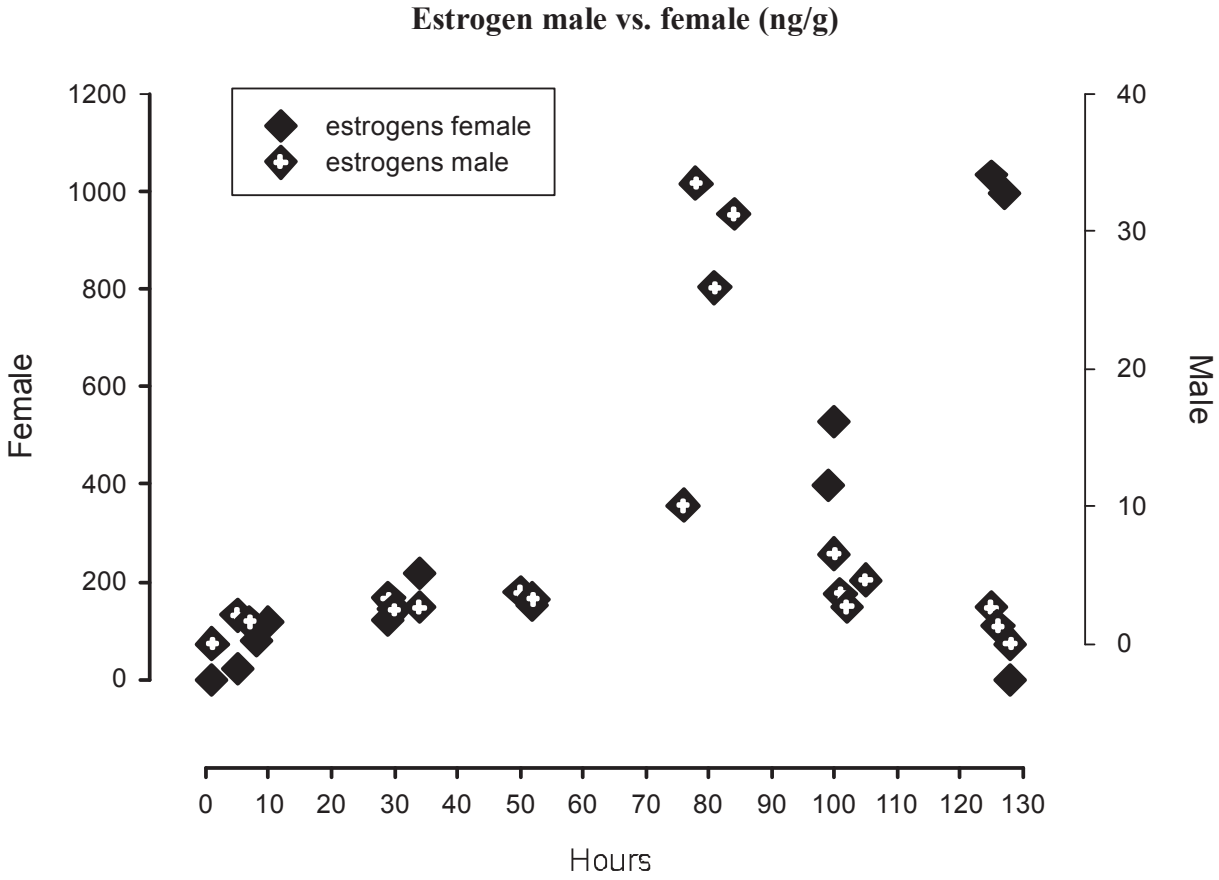

Fig. 4: Measurement of estrogens in the male and the female ibex over the whole observation period

3.2. Storage Test

Fig. 5 shows a decrease in the concentrations measured using assay A compared to using assay B, which shows an increase of GCM in the feces of Alpine Ibex during storage. For assay A there is no statistically significant difference between fecal samples collected shortly after defecation (T0) and samples collected three hours after defecation (T3) ($P = 0.300$). There is also no statistically significant difference between T0 and samples collected six hours after defecation (T6) ($P = 0.975$). No statistical significant difference can be found between T0 and samples collected 24 hours after defecation (T24) later ($P = 0.185$). For Assay B there is no statistically significant difference between T0 and T3 ($P = 0.165$).There is no significant

difference between T0 and T6 (P = 0.165) and also no significant difference can be found between T0 and T24 (P = 0.097).

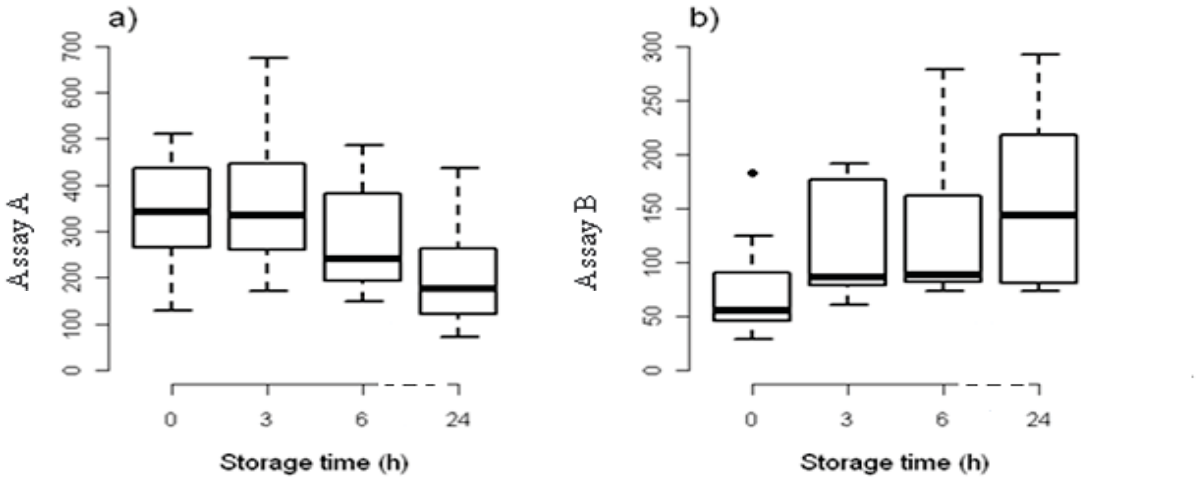

Fig. 5: Storage test. Depletion (assay A) and increase (assay B) of GCMs in fecal samples collected shortly after defecation, three hours, six hours and 24 hours

4. Discussion

The aim of my study was to find and validate an appropriate EIA to determine GCM levels in the feces of Alpine Ibex. This method offers a non-invasive approach to measure stress in free-ranging animals and is therefore important for wildlife management, conservation biology and behavioral ecology (MÖSTL and PALME, 2002).

GCMs are measured in feces with the help of EIAs. As there are species-specific differences in the metabolism of glucocorticoids in the gut, a physiological validation of an EIA for measuring GCM is obligatory for each species (PALME et al., 2005). This can be achieved with an ACTH stimulation test.

To determine the concentration of cortisol metabolites in feces, infusion studies in sheep and characterization of the fecal metabolites had been done (PALME and MÖSTL, 1997). These studies resulted in the establishment of group-specific EIAs, for example 11,17-dioxoandrostanes (11,17-DOA; PALME and MÖSTL, 1997) and 3α, 11 oxo-A (MÖSTL et al., 2002).

In my study, I first of all compared the results of the ACTH stimulation test. I used two different assays. There were individual differences in the baseline levels of the GCMs found in both assays. The baseline level for assay A was, over the whole observation time, higher than for assay B which is in accordance with the findings of KLEINSASSER et al. (2010). Assay A detected the highest quantities of immunoreactive metabolites prior and after the ACTH injection in fresh fecal samples. As with assay B the metabolites prior and after injection were lower. The reason for the difference in the results, although both EIAs utilize the same standard (11-oxoeticocholanolone) is, that the antibodies used are directed against different groups of metabolites (3α, 11 oxo-A versus 11,17 DOA; KLEINSASSER et al., 2010). This explains the higher values measured by assay A, because it mirrors the higher amount of cross reacting GCMs in feces of Alpine Ibex. Similar researches have shown the same results in different species, such as cattle (MÖSTL et al., 2002), sheep (LEXEN et al., 2008) and goats (KLEINSASSER et al., 2010). Both assays showed an increase in the GCM excretion 24-48 hours after injection of ACTH. However, the elevation measured using assay B, after ACTH injection, was somewhat more pronounced compared to assay A, especially in the female ibex. The percentage of the increase in assay B was approximately 1920 % for the male and 3300 % for the female ibex compared to assay A where the percentage of the increase was about 1780% for the male and 2310 % for the female ibex. Summing up, although assay A showed higher values prior and after ACTH injection, the percentage increase was a little more pronounced with assay B. Therefore assay B should be favoured for the determination of GCMs in alpine ibex.

Peak concentrations of both assays appeared earlier in the male than in the female ibex. This may be due to post anesthesia problems of the female. The day after the ACTH stimulation test, the female turned out to be apathetic and sample collection was not possible due to lacking defecation. This problem may have been caused by over-dosage of the anesthetic drugs, as Xylazin is known to affect the gastrointestinal system while reducing rumen activity (LÖSCHER et al., 2006). Over-dosage of anesthetic drugs therefore may explain the delayed defecation, the apathetic behavior, and the drastically increased GCM levels compared to the male.

Compared to other studies, the time between the ACTH injection and peak GCM found here was different. In my study the interval between injection and peak was about 24 hours for the

male and 48 hours for the female. KLEINSASSER et al. (2010) found that the interval between ACTH injection and the peak were about 12 – 15 hours in goats and PALME et al. (1999) revealed the same in cattle and sheep. In some species like roe deer (DEHNHARD et al., 2001) and red deer (HUBER et al., 2003) the interval was much shorter (6 – 9 hours) or longer (18 hours).

The prolonged interval until peak excretion found in ibex could be due to the prior mentioned post anesthesia problems. These may have caused delayed defecation in both study animals. The second problem which we also have to keep in mind is that the sample collection was only done during the day. There was no observation or collection over the night. Therefore, we cannot be sure that there was no defecation in the night and the true interval may well be much shorter.
And thirdly for example in the study of KLEINSASSER et al. (2010), ACTH was injected intramuscular without anesthesia and the interval was much shorter, which again brings us to the point that the anesthesia was the problem. Therefore further studies have to be made with a change in the anesthesia protocol or with the injection of ACTH via blow pipe to avoid anesthesia and its problems.

In my study, in addition to the GCMs, the estrogen level was measured as well. Interestingly the estrogen levels showed the same pattern as the GCM levels, meaning that they also increased and peaked at the same time as the GCMs in both animals. A correlation was calculated which showed that the correlation between the GCMs and the estrogens were higher for the male than for the female.
However, a small amount of estrogens is also secreted by the adrenal cortex. Therefore it can be expected that an increase in ACTH also leads to an increase of estrogens. To prove this finding a chromatography should be done. BAUER et al. (2008) showed a correlation in female guinea pigs between plasma and fecal gonadal steroids. In this study the gonadal steroids also increased in the fecal samples due to an ACTH injection. BAUER et al. (2008) revealed that fecal samples can be used for the non-invasive assessment of the female reproductive status in guinea pigs. It also has to be kept in mind, as PALME et al. (1996) described, that in some species estrogens are mainly excreted via the feces. This could explain the high estrogen level in the feces in my study, meaning that estrogens are mainly excreted via feces in alpine ibex. To prove this finding also urine samples of alpine ibex have to be

measured for the estrogen level. Another factor which could explain the increase of estrogens is, because my study was done at the end of the mating season that sexual behavior plays an important role. But there is no explanation why GCMs and estrogens showed the same characteristics in their course. As for the female we can assume that an ovulation would also lead to such an increase, but as mentioned above the question is why the male ibex showed the same pattern. Thinking of the anesthesia problems of both animals we should consider the drug combination as a possible trigger too. To prove this further animals have to be tested and more investigations are needed.

The storage test clearly demonstrated a difference between both assays. The storage test was done under field conditions. The fecal samples were exposed to all weather conditions (rain on the first two days, followed by snow). GCM concentrations measured with different EIAs increased or decreased, similar to results found in other ruminants (MÖSTL et al., 2002). In my study, with assay A the GCM level continuously decreased over the first 24 hours (Fig. 5a). This result is in agreement with LEXEN et al. (2008). In this study a bacterial conversion (e.g. oxidation of the hydroxyl group at position 3 of the steroid molecule) was assumed (LEXEN et al., 2008). Due to this conversion the antibody is not able to detect cortisol metabolites (MÖSTL et. al., 2005) which are specific for the position 3 and out of that reason the values decrease (LEXEN et al., 2008). MÖSTL et al. (2002) also showed in their study that the above mentioned facts may be explained by the effect of bacterial side chain cleavage, forming increased amounts of $C_{19}O_3$ steroids. In their study the authors explained that assay A crossreacts with some $C_{21}O_4$ steroids, whereas assay B does not.
The GCM level in assay B increased over the same period of time (Fig. 5b). This finding is in agreement with the result of a study in cattle (MÖSTL et al., 1999), where a significant increase in the concentration of immunoreactive metabolites using the same assay was revealed. Assay B measures the $C_{19}O_3$ steroids and that is the explanation why assay B showed an increase and assay A not. In my study, the collection of the fecal samples took place directly after defecation. This is, under field conditions, not always possible. My study showed that there is, for both assays, no significant difference between samples collected directly after defecation and samples collected three, six and 24 hours after defecation. To generally avoid changes in the GCM concentration, if fresh fecal samples are collected, the samples have to be frozen, heated or dried immediately to inactivate bacterial enzymes.

Summing up my study shows that measuring the concentration of cortisol metabolites in feces of Alpine Ibex is possible. When it comes to the question which assay should be preferred, I recommend assay B due to the higher percentage increase after the ACTH injection. When the age of the fecal samples is not known both assays can be used for the determination of GCMs. Further studies have to be made for the correlation between ACTH injection and estrogen in fecal samples. The non-invasiveness of this method will be helpful in further investigations about stress in animals and can be used in every research field.

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References:

ARLETTAZ, R., PATTHEY, P., BALTIC, M., LEU, T., SCHAUB, M., PALME, R., JENNI-EIERMANN, S. (2007): Spreading free-riding snow sports represent a novel serious threat for wildlife. Proc Roy Soc B 274, 1219-1224

BAUER, B., PALME, R., MACHATSCHKE, I. H., DITTAMI, J., HUBER, S. (2008): Non-invasive measurement of adrenocortical and gonadal activity in male and female guinea pigs (*Cavia aperea f. porcellus*). Gen Comp Endocrinol 156, 482-489

DEHNHARD, M., CLAUSS, M., LECHNER-DOLL, M., MEYER, H.H.D., PALME, R. (2001): Noninvasive monitoring of adrenocortical activity in Roe Deer (*Capreolus capreolus*) by measurement of fecal cortisol metabolites. Gen Comp Endocrinol 123, 111-120

EDMONDS, J.S, MORITA, M., TURNER, P., SKELTON, B.W., WHITE, A.H. (2006): Aerial oxidation of the glucocorticoid sidechain under pH control. Steroids 71, 34-41

HUBER, S., PALME, R., ZENKER, W., MÖSTL, E. (2003): Non-invasive monitoring of the adrenocortical response in red deer. J Anim Sci 77, 258-266

KLEINSASSER, C., GRAML, C., KLOBETZ-RASSAM, E., BARTH, K., WAIBLINGER, S., PALME, R. (2010): Physiological validation of a non-invasive method for measuring adrenocortical activity in goats. Vet Med Austria 97, 259-262

LEXEN, E., EL-BAHR, S.M., PALME, R., MÖSTL, E. (2008): Monitoring disturbances in sheep by measuring glucocorticoid metabolites in faeces. Vet Med Austria 95, 64-71

LÖSCHER, W. UNGEMACH, F.R., KROKER, R. (editor 2006): Pharmakotherapie bei Haus- und Nutztieren. 100-103

MÖSTL, E., MESSMANN, S., BAGU, E., ROBIA, C., PALME, R. (1999): Measurement of Glucocorticoid Metabolite Concentrations in Faeces of Domestic Livestock. J Vet Med A 46, 621-631

MÖSTL, E., PALME, R. (2002): Hormones as indicators of stress. Domest Anim Endocrinol 23 (2002), 67-74

MÖSTL, E., MAGGS, JL, SCHRÖTTER, G., BESENFELDER, U., PALME, R. (2002): Measurement of cortisol metabolites in faeces of ruminants. Vet Res Commu 26, 127-139

MÖSTL, E., RETTENBACHER, S., PALME, R. (2005): Measurement of corticosterone metabolites in birds‘ droppings: an analytical approach. Ann NY Acad Sci 1046, 75-80

PALME, R., FISCHER, P., SCHILDORFER, H., ISMAIL, M.N. (1996): Excretion of infused ^{14}C-steroid hormones via faeces and urine in domestic livestock. Anim Reprod Sci 43, 43-63

PALME, R., MÖSTL, E. (1994): Biotin-Streptavidin enzyme immunoassay for the determination of oestrogenes and androgens in boar faeces. Szombathely: Proc 5th Symp Analys Steroids, 111-117

PALME, R., MÖSTL, E., BREM, G., SCHELLANDER, K., BAMBERG, E. (1997): Faecal metabolites of infused 14C-Progesteron in Domestic Livestock. Reprod Dom Anim 32, 199-206

PALME, R., MÖSTL, E. (1997): Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. Int J Mam Biol 62 (Suppl 2), 192-197

PALME, R. ROBIA, C., MESSMANN, S., HOFER, J., MÖSTL, E. (1999): Measurement of faecal cortisol metabolites in ruminants: a non-invasive parameter of adrenocortical function. Vet Med Austria 86, 288-294

PALME, R., ROBIA, C., BAUMGARTNER, W., MÖSTL, E. (2000): Transport stress in cattle as reflected by an increase in faecal cortisol metabolites. Vet Rec 146, 108-109

PALME, R. (2005): Measuring fecal steroids: guidelines for practical application. Ann NY Acad Sci 1046, 75-80

THUN, R., SCHWARTZ-PORSCHE, D. (1994): Nebennierenrinde. In: Döcke, F. (ed), Veterinärmedizinische Endokrinologie, 309-356. Gustav Fischer-Verlag, Jena Stuttgart.

TOUMA, C., PALME, R. (2005): Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. Ann NY Acad Sci 1046, 54-74