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Characterisation and validation of an enzyme-immunoassay for the non-invasive assessment of faecal glucocorticoid metabolites in cheetahs (*Acinonyx jubatus*)

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ABSTRACT

The non-invasive measurement of adrenocortical function in cheetahs is an important tool to assess stress in captive and free-ranging individuals, because stress has been suggested to be one of the causes of poor reproductive performance of captive cheetahs. We tested four enzyme immunoassays (EIA) in two captive cheetahs in Germany using adrenocorticotropic hormone (ACTH) challenges and identified the corticosterone-3-CMO EIA to be most sensitive to the increase in faecal glucocorticoid metabolite (fGCM) concentrations after the ACTH challenge. This EIA performed also well in five captive cheetahs in South Africa. The fGCM concentrations across all seven cheetahs increased within 24 h by 681% compared to the baseline levels prior to ACTH. Storage of faecal samples at 0–4 °C did not strongly affect fGCM concentrations within 24 h, simplifying sample collection when immediate storage at −20 °C is not feasible. The two cheetahs in Germany also received an injection of [^{3}H]cortisol to characterise fGCMs in faecal extracts using high-performance liquid chromatography (HPLC) immunograms. HPLC fractions were measured for their radioactivity and immunoreactive fGCM concentrations with the corticosterone-3-CMO EIA, respectively. The results revealed a polar peak of radiolabelled cortisol metabolites co-eluting with the major peak of immunoreactive fGCMs. Thus, our EIA measured substantial amounts of fGCMs corresponding to the radioactive peaks. The peaks were of higher polarity than native cortisol and corticosterone, suggesting that the metabolites were conjugated, which was confirmed by solvolysis of the HPLC fractions. Our results show that the corticosterone-3-CMO EIA is a reliable tool to assess fGCMs in cheetahs.

1. Introduction

The number of cheetahs (*Acinonyx jubatus*) in the wild are diminishing rapidly which is mainly a consequence of habitat loss and a declining prey base [17]. Thus, captive breeding of cheetahs might become increasingly important as an insurance measure, facilitating re-introductions in the wild once conditions for cheetahs have improved. However, whereas reproductive performance of free-ranging cheetahs is high [16,29], cheetahs in captivity reproduce rather poorly [18,32].

Numerous studies in zoos and in free-ranging populations investigated possible reasons for this phenomenon [5,14,19,20,27]. Recently, Wachter et al. [29] tested the predictions of four hypotheses and demonstrated that the "asymmetric reproductive aging" hypothesis was the most likely explanation. This hypothesis predicts rapid aging of the inner reproductive organs associated with the early development of pathologies in older, nulliparous captive females compared to younger nulliparous captive females or young and old multiparous free-ranging females [11,12]. In captivity, females often start to reproduce later than in the wild which diminishes their reproductive performance, as also found in other species [11,12].

Previously published findings on the possible impact of stressors that may impair reproductive performance in female cheetahs are inconsistent. Wachter et al. [29] used ultrasonography to assess the potential extent of chronic stress in free-ranging and captive cheetahs in Namibia and demonstrated similar sizes of the adrenal glands in both study groups, with little indication that they were enlarged, suggesting that – if anything – levels of stress were at best moderate and similar for both conditions. They described substantial pathologies in the inner reproductive organs in the

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captive individuals but found little evidence of such anomalies in free-ranging animals. On the other hand, female cheetahs in North American zoos had significantly higher stress levels than free-ranging cheetahs in Namibia or cheetahs kept in large enclosures in their natural Namibian environment, as measured by the size of their adrenal cortices and faecal corticoid metabolite concentrations [26,27]. Without reliable measurements of stress levels in captive cheetah females on a regular basis and a large scale the conclusion that in zoos stress might complement asymmetric reproductive aging in depressing reproductive performance of cheetah females should remain tentative.

The development of immunoassays to measure faecal steroid metabolites in domestic and non-domestic felids provides a useful approach to non-invasively assess an individual's endocrine status. Steroid metabolism studies in domestic cats demonstrated that the majority of steroid excretion occurs mainly via faeces rather than urine [4,9,23]. Steroid metabolites in faeces represent a pooled value of steroids excreted by the adrenal glands during the previous 12–24 h (depending on defaecation rates), thus are less affected by episodic fluctuations or the pulsatility of hormone secretion and are therefore useful to evaluate the adrenal activity in felids in an integrated manner.

The development of immunoassays to measure faecal steroid metabolites in domestic and non-domestic felids provides a useful approach to non-invasively assess an individual's endocrine status but need to be validated for each species and each hormone, respectively, because they are often based on antibodies generated against the native steroid but with unknown cross-reactivities with the excreted steroid metabolites. Steroid metabolism by the liver as well as microbial impact during the intestinal passage, and re-absorption into the enterohepatic circulation generate a vast number of faecal steroid metabolites, different in even closely related species [10,21]. In addition, metabolism of stress hormones such as cortisol, and gonadal hormones such as testosterone may lead to faecal metabolites with similar structures because the native hormones have similar structures. Thus, immunoassays designed to assess stress by measuring the concentration of faecal glucocorticoid metabolites (fGCM) may cross-react with other steroid metabolites, distorting the results. To ensure that a proposed immunoassay reliably measures an animal's endocrine status, it should be experimentally demonstrated that the concentrations of fGCMs increase with defined stressors experienced by the animal. The most widely used approach is to stimulate the adrenocortical activity with an adrenocorticotropic hormone (ACTH) challenge test. If the assay works well, there should be a sharp increase of fGCM concentrations with a lag time proportional to the defaecation rate after ACTH application. Furthermore, to characterise the species-specific predominance of fGCMs and to identify the affinity of the antibody to these fGCMs, an injection of ^{3}H labelled cortisol and high-performance liquid chromatography (HPLC) immunograms are a useful approach (e.g. [2]). Comparing the pattern of excreted radiolabelled metabolites with the metabolites produced after an ACTH challenge reveals the major fGCMs for a particular species and the fit of the respective immunoassay.

For cheetahs several RIAs and one EIA validated by ACTH challenges are available [14,25,30,33], but no comprehensive characterisation of the fGCMs and the exact affinity of the immunoassays were conducted, or the performances of the different EIAs compared. Our first aim was therefore to test four available EIAs and to identify the most reliable antibody for accurately assessing adrenal status with a non-radioactive immunoassay in the cheetah by conducting ACTH challenges. Our second aim was to characterise the fGCMs and measure their relative abundance in the best assay by performing a radiometabolism study and subsequent HPLC analyses. Owing to logistical reasons, especially in the field, faecal samples can be often only kept cooled in an ice box rather than be deep frozen immediately after defaecation. Our third aim was therefore to examine the stability of fGCM concentrations when faeces are stored for variable amounts periods of time at temperatures of 0–4 °C.

2. Materials and methods

2.1. Study animals and housing conditions

Four adult male cheetahs and three adult female cheetahs were involved in this study. One male and one female were kept in German zoos in Münster and Wuppertal, respectively, whereas the other cheetahs were kept at the Ann van Dyk cheetah centre, North West Province, South Africa. The cheetahs in Germany were fed daily with whole rabbit or beef meat, the cheetahs in South Africa received daily a mixture of cat food (Iams®), horse-, and chicken mince. Water was available ad libitum. As two males were kept together, their food was mixed with rice and split peas, respectively, to permit the allocation of faeces to the correct individual.

All procedures were performed in accordance with the requirements of the Ann van Dyk cheetah centre as well as the Leibniz Institute for Zoo and Wildlife Research Ethics Committee on Animal Welfare.

2.2. Radiometabolism study

The two cheetahs in Germany were immobilised with a mixture of ketamine 10% (CP-Pharma, Burgdorf, Germany, 4.5 mg/kg) and medetomidine (Janssen Animal Health, Neuss, Germany, 0.04 mg/kg). A radiolabelled solution of 0.25 ml containing ~250 μCi 1,2,6,7-[3H] cortisol (TRK407, Amersham Bioscience, UK) in ethanol was mixed with 2.25 ml sterile 0.9% NaCl solution and injected into the cephalic vein. The cheetahs were observed 24 h during six days (two days before and four days after the injection) and all faeces were collected. Samples were collected within one hour after defaecation, homogenised and stored at −20 °C until they were brought to the Leibniz Institute for Zoo and Wildlife Research (IZW) in a portable freezer. Aliquots of each sample were extracted for cortisol metabolite determination and radioactivity counting. The samples with the highest amount of radioactivity (23.2 and 20.3 h following injection of the male and the female, respectively) were used for HPLC analysis.

2.3. ACTH challenge

Synthetic ACTH (Synacthen, 0.25 mg, Ciba-Geigy, Wehr, Germany) corresponding to 25 IU was injected i.m. with a blow pipe in the two cheetahs in Germany. Faeces were collected four days before and seven days after the injection and homogenised. The enclosures were checked twice a day, except for the two days following the ACTH administration when cheetahs were observed 24 h a day. Samples were therefore collected within 12 h or within one hour after defaecation, respectively, and frozen at −20 °C until transported to the IZW.

The five cheetahs in South Africa received i.m. 50 IU of long-acting ACTH preparation corresponding to 0.5 mg (Synacthen Depot©, Novartis South Africa [Pty] Ltd). Faeces were collected 10 days before and 7 days after ACTH administration and homogenised. Cheetahs were observed from 7:30 a.m. to 5 p.m. and samples frozen at −20 °C within 10 min after defaecation. All overnight samples were collected early the next morning.