The measurement of glucocorticoid concentrations in the serum and faeces of captive African elephants (*Loxodonta africana*) after ACTH stimulation

S K Stead[a], D G A Meltzer[a] and R Palme[b]

ABSTRACT

Conventionally, the assessment of adrenal responses to stress relies on blood sample collection. However, blood collection from animals is impossible without restraint or immobilisation that influences results. This study was undertaken to validate recently established enzyme immunoassays that measure faecal glucocorticoid metabolites in elephants, and to perform a preliminary investigation into the biological relevance of this non-invasive method for use in assessing the degree of stress in this species. Four juvenile African elephants were injected i.m. with 2.15 mg synthetic adrenocorticotrophic hormone (Synacthén, Novartis, Switzerland). Blood and faecal samples were collected over 4 h and 7 d respectively. Concentrations of serum cortisol and faecal cortisol metabolites were determined using immunoassay. Variability of basal and peak values in blood and faeces was observed among the elephants. After ACTH injection, serum cortisol concentrations increased by 400–700 %. An 11-oxoaetiocholanolone enzyme immunoassay (EIA) proved best suited to measure cortisol metabolites (11,17-dioxoandrostanes) when compared to a cortisol and corticosterone EIA in faecal samples. Concentrations of faecal 11,17-dioxoandrostanes increased by 570–1070 %, reaching peak levels after 20.0–25.5 h. Greater levels of glucocorticoid metabolites were measured in faecal samples from elephants kept in small enclosures compared to levels in the faeces of animals ranging over a larger area. The results of this preliminary study suggest that non-invasive faecal monitoring of glucocorticoid metabolites is useful in investigating adrenal activity in African elephants.

Key words: ACTH, animal welfare, cortisol, EIA, elephant, faeces, glucocorticoids, non-invasive.

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INTRODUCTION

In 1998, 30 juvenile elephants were captured in Botswana and transported to training facilities at African Game Services (AGS) (25°47′ S, 27°46′E) in South Africa. The move ignited a heated debate between animal rights and conservation organisations concerning the welfare of the so-called 'Tuli Elephants'. On a broader scale, the psychological well-being of elephants maintained in zoos and circuses was highlighted, and the need to optimise husbandry conditions for behaviour, health and well-being was reiterated. As Garaï[7] noted, it is reasonable to assume that juvenile elephants separated from their families, captured and translocated have experienced a certain degree of stress. Stress is a subjective experience, and thus the extent to which individuals are 'stressed' is difficult to quantify[5].

Although there is no universal scientific agreement on the definition of stress, stress responses cause an increase in glucocorticoids, primarily cortisol and corticosterone, in the blood[9]. Conventionally, the assessment of adrenal responses to stress relies on collection of blood samples and measurement of corticosteroids[10]. However, the process of blood collection is impossible without the use of capture drugs when studying free-ranging wild animals and will, in itself, elicit elevated cortisol levels[16].

Metabolism of glucocorticoids occurs primarily in the liver[3]. There are large inter-species differences with respect to the metabolites formed and their route of excretion[1,11–13,18], therefore the efficacy of measuring faecal cortisol metabolites should be evaluated for each species.

Palme and Möstl[13] have established an 11-oxoaetiocholanolone enzyme immunoassay (EIA) that measures 11,17-dioxoandrostanes (11,17-DOA), a group of faecal cortisol metabolites. The biological relevance of this method has been proven in ruminants following ACTH stimulation of cortisol release by the adrenal cortex[15] and used to monitor transport stress in cattle[14]. This non-invasive technique has been applied to a number of domestic, zoo and wildlife species[1,11,18,19].

In elephants, cortisol has been measured in the saliva from 2 Asian elephants[4] and in urine from 1 African and 1 Asian elephant[2] as a means of assessing adrenocortical activity in a non-invasive manner. However, the collection of faecal samples is more practical, especially when dealing with free-ranging animals, and provides measurements that are independent of short-term fluctuations[2,15]. The aim of our study was to validate a method for measuring glucocorticoid metabolites in elephant faeces, and to conduct a preliminary investigation into the method's biological relevance.

MATERIALS AND METHODS

Animals

Twenty elephants at AGS made up a large proportion of the animals studied. Of these, 14 animals, referred to as Group 1, were being trained by Indonesian mahouts and were kept in a relatively large enclosure of approximately 2500 m². The remaining 6 animals, Group 2, were kept some distance away and out of sight and sound from the main hub of activity centred around the training process. Two of these animals were in an enclosure of 150 m² and 4 in an enclosure 350 m² in extent. Both groups had been in their respective enclosures for 12–14 months when the study was undertaken. The elephants were of a similar age, estimated between 5 and 7 years.

Three further elephants, Group 3, which were kept on the farm of the Glen Afric Lodge, Broederstroom (25°49′S, 27°51E), approximately 10 km distant from the AGS premises, were included in

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the study. These animals had been reared from an early age on the farm, were habituated to humans and roamed about in a 750 ha enclosure during the day accompanied by a game guard. One animal, a male, was approximately 10 years old and the 2 females were 4 and 6 years of age.

None of the elephants studied were pregnant.

Management of the elephants

Husbandry of all the AGS elephants, Groups 1 and 2, was the responsibility of AGS under the supervision of the National Council of the Society for the Prevention of Cruelty to Animals. The Group 1 elephants were tethered by a front and a back leg in a barn overnight (between 17:00 and 10:00) and provided with fresh fruit, vegetables, *Eragrostis curvula,* tef or oat hay, lucerne and bedding. During the day they were released into their enclosure and had free access to a similar variety of feed.

The elephants in Group 2 remained free in their respective enclosures. They had little contact with humans, who only attended to them when providing feed and when the enclosures were cleaned.

Group 3 elephants spent the day wandering about in the 750 ha enclosure feeding as they pleased and were tethered by 1 leg in a barn overnight between 16:00 and 07:00. They were given 2–3 kg horse cubes, lucerne and bedding during the evening.

ACTH administration and sample collection

Only 4 elephants were available for the purposes of this experiment. As a result, an experimental design in which control animals would have been given an injection of saline solution instead of ACTH could not be used.

Each elephant was injected intramuscularly with 300 mg azaperone. After 15 min, an 18-gauge catheter was inserted into an ear vein and a blood sample collected using a 10 mℓ syringe. Two further blood samples were collected at 15 min intervals before the intramuscular administration of 2.15 mg ACTH (Synacthén, Novartis, Switzerland). Thereafter, a venous blood sample was collected every 30 min for 4 h.

Faecal samples from almost all defaecations were collected for 3 days before and 4 days after the ACTH injection.

Blood samples

Five-millilitre samples of whole blood were placed in plain vacutainer tubes (Becton Dickenson, USA). Blood was allowed to clot for 1 h and centrifuged at 1700 × g. Serum was placed in cryotubes
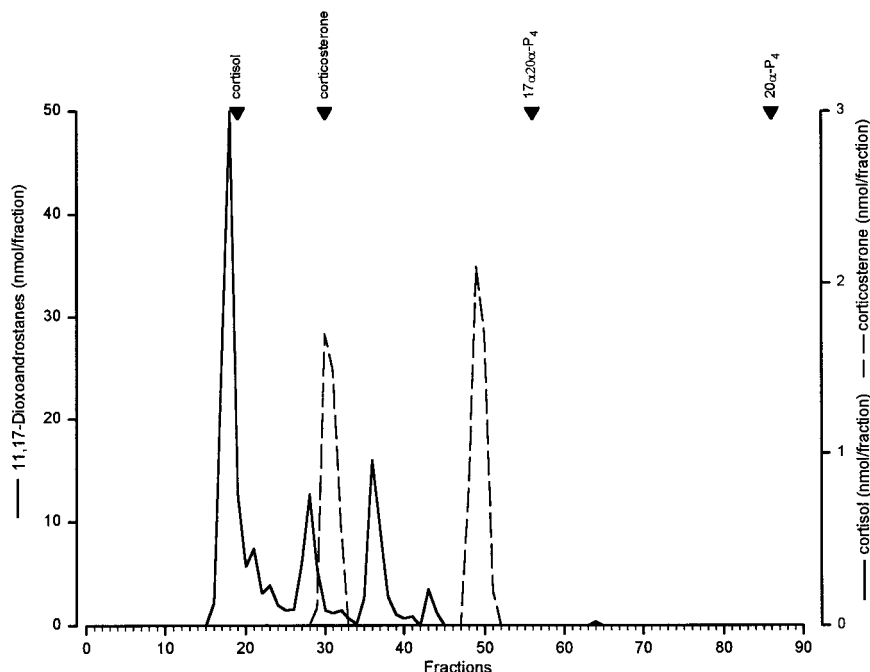
Fig 1: **HPLC separation of immunoreactive glucocorticoid metabolites in 1 faecal sample from an elephant as tested in a cortisol-, corticosterone- and 11-oxoaetiocholanolone-EIA. Fractions marked with ▼ represent the approximate elution time of respective standards** ($17^a,20^aP = 17^a,20^a$-dihydroxy-progesterone).

(Amersham, Johannesburg) and stored at –20 °C until analysis.

Measurement of serum cortisol

Serum concentrations of cortisol were determined using a Clinical Assays™ GammaCoat™ Cortisol ^{125}I Radio-immunoassay kit (DiaSorin; SA Scientific, Johannesburg).

Faecal samples

Samples were collected within 30 min of defaecation. A single faecal bolus was mixed by hand and then a handful of faeces was placed into a plastic freezer bag and stored at –20 °C until the preparation for extraction and EIA analysis.

Faecal samples were collected from each group of elephants.

Analysis of faecal cortisol

Frozen faecal samples were oven-dried at 100 °C. Each sample was powdered and mixed thoroughly. A 0.5 g subsample was mixed with 10 mℓ 80 % ethanol, shaken for 30 min and centrifuged at 1700 × g for 15 min. One millilitre of the supernatant was drawn off and stored at –20 °C until EIA analysis. Aliquots of the extract were analysed with 3 EIA systems (cortisol, corticosterone and 11-oxoaetiocholanolone) as described by Palme and Möstl[13].

High-performance liquid chromatography (HPLC)

HPLC of the faecal metabolites was performed at the Institute of Biochemistry, Vienna, as described by Teskey-Gerstl

et al.[18]. Faecal extracts containing peak 11,17-DOA concentrations were subjected to a clean-up procedure (Sep-Pak C18). Separation was performed on a reverse-phase Nova-Pak C18 column (3.9 × 150 mm, Millipore Corporation, Milford, Massachusetts, USA) using a linear gradient starting at 50 % methanol. Three fractions per minute were collected, dried under a stream of nitrogen, and reconstituted in assay buffer. Immunoreactive glucocorticoid metabolites were quantified with the cortisol, corticosterone and 11-oxoaetiocholanolone EIAs as previously described[13].

RESULTS

HPLC analysis

HPLC separations revealed a number of immunoreactive substances present in elephant faeces. They showed a chromatographic mobility between cortisol and 17,20-dihydroxyprogesterone (Fig. 1). The main metabolite determined with the 11-oxoaetiocholanolone-EIA eluted around cortisol. Lower amounts of immunoreactive substances were detected by testing the HPLC fractions with the corticosterone-EIA and negligible amounts with the cortisol-EIA (detection limit = 2 nmol/kg faeces).

ACTH challenge

Injection of ACTH resulted in an increase of serum cortisol (Fig. 2) and faecal cortisol metabolite concentrations (Fig. 3). Serum cortisol levels began to rise after
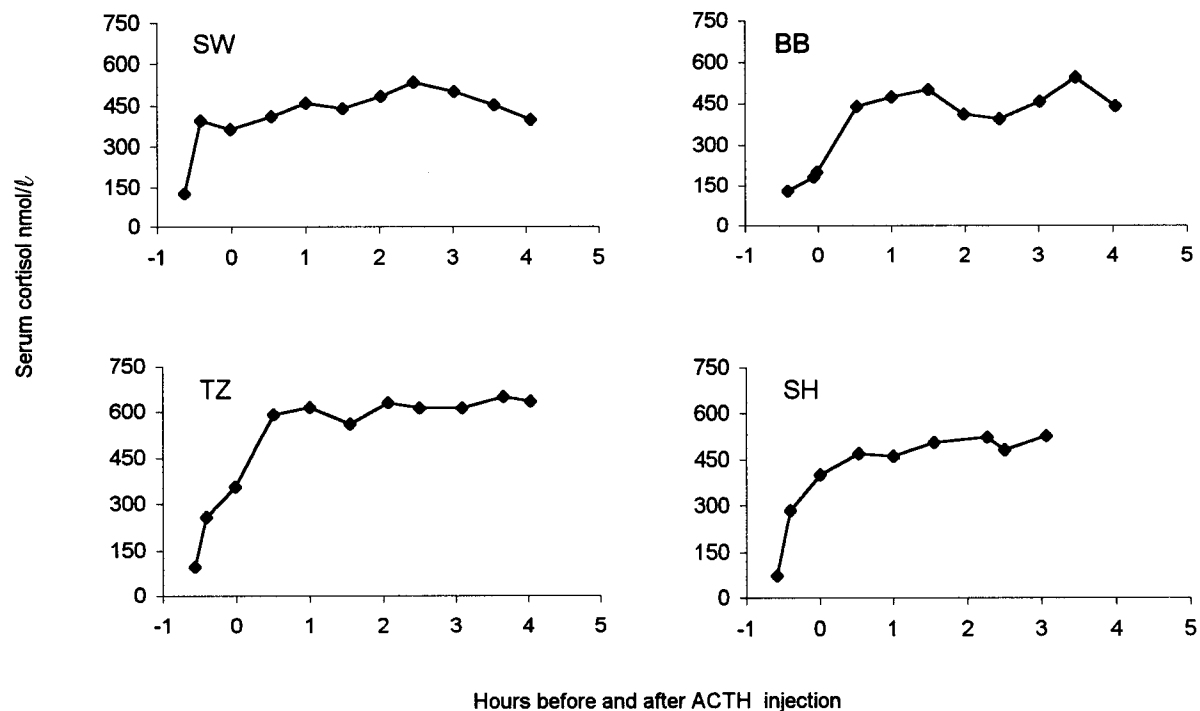
Fig. 2: **Time course of serum cortisol concentrations** (nmol/ℓ) **in 4 elephants before and after intramuscular injection of 2.15 mg ACTH at time zero. Individual elephants were identified as SW, BB, TZ and SH.**

injection with azaperone and insertion of catheters. Following ACTH administration serum cortisol increased between 4- and 7-fold, reaching highest recorded values (526–652 nmol/ℓ) after 2 h. No distinct peaks were observed.

Individual differences in basal and peak values of faecal cortisol metabolites were observed. Basal values of faecal 11,17-DOA and corticosterone equivalents ranged from 21 to 168 nmol/kg (median: 48 nmol/kg) and 33 to 133 nmol/kg

(median: 50 nmol/kg) respectively. ACTH-induced peaks were between 572–1104 % (11,17-DOA) and 160–353 % (corticosterone) higher than basal values. These peak concentrations occurred 20–25.5 h after the injection. Additional
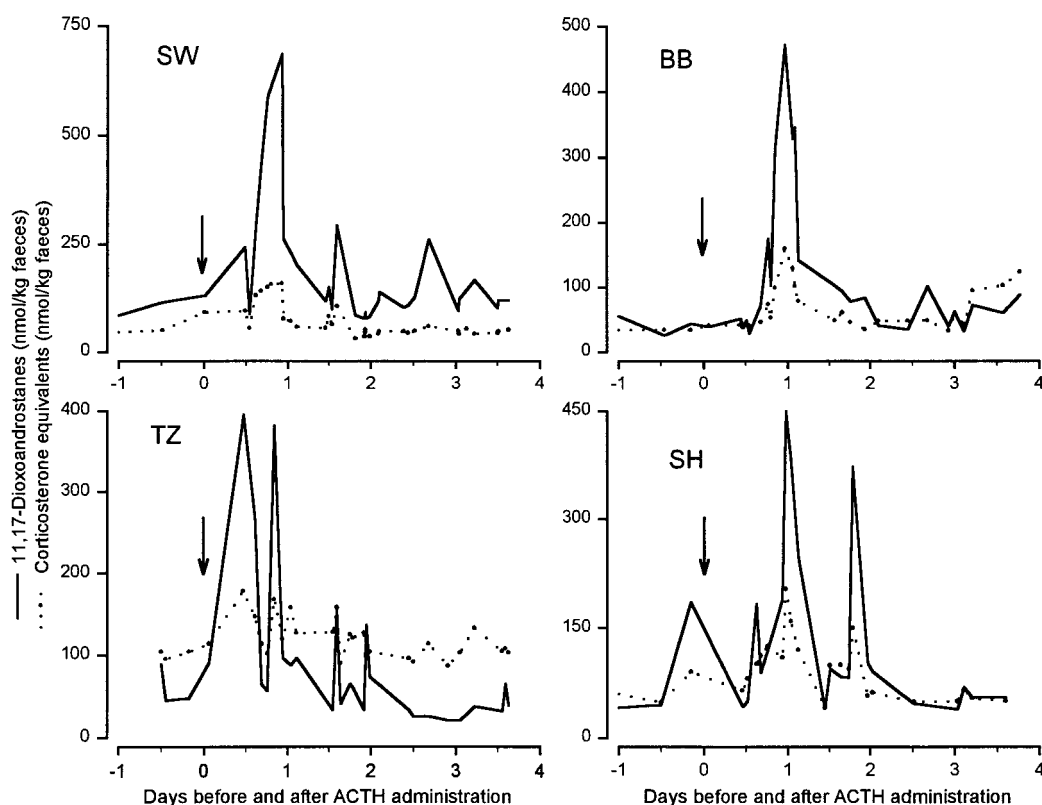

Fig. 3: **Time course of concentrations of faecal 11,17-dioxoandrostanes and corticosterone equivalents** (nmol/kg) **in 4 elephants before and after intramuscular injection of 2.15 mg ACTH at time zero. Individual elephants were identified as SW, BB, TZ and SH.**

Table 1: The effect of enclosure size on faecal 11,17-dioxoandrostane concentration.

Group	Number of elephants	Enclosure size (m²)	Range of 11,17-DOA (nmol/kg)	Median	Number of samples analysed
1	14	2 500	21–168	48	42
2	6	350	62–1000	176	6
3	3	7 500 000	15–47	39	6

peaks of varied height were observed for both groups of metabolites before and after the ACTH-induced peaks.

Faecal glucocorticoids in elephant groups

The results of faecal glucocorticoid analyses are summarised in Table 1.

DISCUSSION

There have been few studies to investigate the possibility of using non-invasive methods to assess adrenocortical activity in elephants[2,4]. Methods to identify and measure faecal glucocorticoid metabolites have been successfully used in a number of domestic livestock[11,13,15] and some wild species[1,7,18,19]. The aim of this study was to assess whether the measurement of faecal cortisol metabolites is more suitable than using blood cortisol values to monitor adrenocortical activity in elephants.

Serum cortisol levels began to rise before ACTH was injected, suggesting that handling or the injection of azaperone affected levels of serum cortisol within 30 min. These findings are in accordance with Sire *et al.*[17] and Fulkerson and Jamison,[6] who reported that physical restraint and blood-sampling can produce an increase of blood cortisol levels within 15 min of handling. The lowest values recorded before ACTH injection (73–131 nmol/ℓ) were similar to those found by Hattingh *et al.*[8] in plasma from 5 undisturbed adult female elephants that had been shot (mean = 111 nmol/ℓ). However, it must be noted that their results are associated with a high standard deviation (24.8). The results are not directly comparable to those in this study owing to the elephants' age, unknown reproductive status and undefined environmental stressors at the time of sample collection. Morton *et al.*[10] collected blood samples from 27 elephants immobilised with etorphine/xylazine. A mean plasma cortisol concentration of 347 ± 0.95 nmol/ℓ was estimated. It is likely that these values were a result of capture procedures and are not representative of baseline cortisol levels.

After ACTH injection, serum cortisol levels continued to rise, and in 3 of 4 cases reached a plateau. No further blood samples were taken, as it was regarded as unethical to collect blood for longer than 4 hours. The tranquillising effect of azaperone began to wear off and it became increasingly difficult to prevent the elephants from pulling the indwelling catheters out of the ear veins. Sampling from other sites, such as the tail, had been attempted previously and proved unsuccessful.

The highest recorded cortisol concentrations were within the range of those found in 5 elephants that had been herded for 6–20 minutes and darted with succinyldicholine before sample collection (mean: 688 nmol/ℓ, SD: 269.5)[8]. This gives an indication of the type of stressor that may produce such elevated levels.

A number of glucocorticoid metabolites were detected in elephant faeces using HPLC analysis. Although the exact identity of the metabolites was not determined due to the cross-reactions of the EIA, a group of them may be collectively described as 11,17-dioxoandrostanes (11,17-DOA)[13,15]. As reported in domestic livestock[11,13], negligible amounts of cortisol and low amounts of corticosterone were found in elephant faeces. These findings support the suggestion that the recently-developed 11-oxo-aetiocholanolone EIA, which measures 11,17-DOA, is the most suitable EIA to use for the non-invasive monitoring of adrenocortical activity in elephants.

As observed in other species, the time course of faecal cortisol metabolite concentrations reflected the ACTH-induced stimulation of glucocorticoid production[11,15]. Concentrations peaked 20–25.5 h after the ACTH injection. Similar times were found by Möstl *et al.*[11] in ponies. It has been suggested that the delay in faecal glucocorticoid excretion is correlated with the transit time of digesta from the duodenum to the rectum[12]. Our findings fit well with the total passage time from mouth to rectum of 33 h reported for Indian elephants[20]. Differences in diet and individual adaptations in hepatic or gastrointestinal function may explain differences in excretion rates[21].

The large increase of 11,17 DOA (572–1104 % above basal levels) after ACTH injection was higher than that observed in ponies (200–660 %) by Möstl *et al.*[11], but within the range of reported increases in cattle during transport (400–1100 %)[14]. Lower percentage increases in corticosterone were measured, supporting the earlier suggestion that 11,17-DOA is a more suitable group of metabolites to measure.

Additional peaks after the ACTH-induced peak could be due to an enterohepatic circulation of the metabolites[12]. Alternatively, additional peaks may have been caused by stressful events approximately 24 h before they were recorded. More prolonged periods of behavioural observations conducted before, during and after the trial may have made it possible to identify events that had caused these responses.

As reported in other species[11,13,18], individual variation in basal and peak values was observed. This may be due to differences in previous experiences, body mass, metabolism, age, diet or sex. Further investigations with greater numbers of animals are necessary to identify the influence of these confounding factors. Preliminary investigations into the application of the technique to assess welfare showed good correlation between behavioural observations, environmental stressors and faecal glucocorticoid metabolite concentrations. Concentrations of 11,17-DOA from elephants allowed to range in 750 ha fell within the lower end of the range of basal values measured in Group 1. These Glen Afric elephants were exposed to fewer stressors than those housed at AGS and had more opportunity to perform species-specific behavioural activities such as foraging in a large area. The elephants kept in the small enclosures at AGS, Group 2 animals, had 11,17-DOA concentrations 450 % higher than the elephants at Glen Afric.

The primary advantages of faecal sample collection are that the collector does not require special skills and there is no need to handle the animals. Secondly, concentrations of faecal glucocorticoid metabolites probably more closely reflect the amounts of cortisol produced and excreted than cortisol measured in blood, which only reflects a point in time during a dynamic process of absorption, metabolism and excretion[15]. We conclude that measuring faecal 11,17-DOA is a valuable

tool for non-invasive monitoring of adrenocortical activity in African elephants. This could help to optimise the capture, transport and husbandry of Afrian elephants and be useful in investigating stress in free-ranging situations.

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