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Non-invasive assessment of adrenocortical function in the male African elephant (*Loxodonta africana*) and its relation to musth

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Abstract

Adult male elephants periodically show the phenomenon of musth, a condition associated with increased aggressiveness, restlessness, significant weight reduction and markedly elevated androgen levels. It has been suggested that musth-related behaviours are costly and that therefore musth may represent a form of physiological stress. In order to provide data on this largely unanswered question, the first aim of this study was to evaluate different assays for non-invasive assessment of adrenocortical function in the male African elephant by (i) characterizing the metabolism and excretion of [^{3}H]cortisol (^{3}H-C) and [^{14}C]testosterone (^{14}C-T) and (ii) using this information to evaluate the specificity of four antibodies for determination of excreted cortisol metabolites, particularly with respect to possible cross-reactions with androgen metabolites, and to assess their biological validity using an ACTH challenge test. Based on the methodology established, the second objective was to provide data on fecal cortisol metabolite concentrations in bulls during the musth and non-musth condition. ^{3}H-C (1 mCi) and ^{14}C-T (100 μCi) were injected simultaneously into a 16 year old male and all urine and feces collected for 30 and 86 h, respectively. The majority (82%) of cortisol metabolites was excreted into the urine, whereas testosterone metabolites were mainly (57%) excreted into the feces. Almost all radioactive metabolites recovered from urine were conjugated (86% ^{3}H-C and 97% ^{14}C-T). In contrast, 86% and >99% of the ^{3}H-C and ^{14}C-T metabolites recovered from feces consisted of unconjugated forms. HPLC separations indicated the presence of various metabolites of cortisol in both urine and feces, with cortisol being abundant in hydrolysed urine, but virtually absent in feces. Although all antibodies measured substantial amounts of immunoreactivity after HPLC separation of peak radioactive samples and detected an increase in glucocorticoid output following the ACTH challenge, only two (in feces against 3α,11-oxo-cortisol metabolites, measured by an 11-oxo-etiocholanolone-EIA and in urine against cortisol, measured by a cortisol-EIA) did not show substantial cross-reactivity with excreted ^{14}C-T metabolites and could provide an acceptable degree of specificity for reliable assessment of glucocorticoid output from urine and feces. Based on these findings, concentrations of immunoreactive 3α,11-oxo-cortisol metabolites were determined in weekly fecal samples collected from four adult bulls over periods of 11–20 months to examine whether musth is associated with increased adrenal activity. Results showed that in each male levels of these cortisol metabolites were not elevated during periods of musth, suggesting that in the African elephant musth is generally not associated with marked elevations in glucocorticoid output. Given the complex nature of musth and the variety of factors that are likely to influence its manifestation, it is clear, however, that further studies, particularly on free-ranging animals, are needed before a possible relationship between musth and adrenal function can be resolved. This study also clearly illustrates the potential problems associated with cross-reacting metabolites of gonadal steroids in EIAs measuring glucocorticoid metabolites. This has to be taken into account when selecting assays and interpreting results of glucocorticoid metabolite analysis, not only for studies in the elephant but also in other species.

Keywords: African elephant; Musth; Cortisol and testosterone metabolism; Feces; Urine; Non-invasive methodologies; Assay specificity; Crossreacting steroids

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1. Introduction

Adult male elephants live in a highly dynamic world of changing sexual state, rank, associations and behaviour. Most of the time they live alone or in small groups with other males except for brief, distinct, sexually active periods which they begin to show after the age of 20–25 years (African: Hall-Martin, 1987; Poole, 1987, 1994; Asian: Eisenberg et al., 1971; Jainudeen et al., 1972a). These sexually active periods in bulls are often, but not always, associated with the state of musth, a condition referring to a set of physical, physiological and behavioural characteristics, originally described for Asian elephants (Eggeling, 1901), but now also known to occur in the African species (Poole and Moss, 1981). The most obvious visible manifestations of musth are a sharp rise in aggressive behaviour, a continuous discharge of urine, and copious secretions from the swollen temporal glands (Poole, 1987). Physiologically, musth is known to be associated with elevated androgen levels (Hall-Martin, 1987; Jainudeen et al., 1972b; Poole et al., 1984; Rasmussen et al., 1984), although the temporal relationship between changes in androgens and musth-related behavioural and physical parameters is not clear. Furthermore, details of the physiological mechanisms underlying musth and of the factors regulating its onset and duration have yet to be determined.

Musth is also known to be associated with increased restlessness and reduced feeding activities (Poole, 1982, 1989). Since this often leads to significant weight reduction and progressive loss of condition (Poole, 1989), and is also associated with increased inter-male aggression (Hall-Martin, 1987; Poole, 1989), musth may represent a form of physiological stress. To date, however, there is no direct evidence for this and the question, whether musth is associated with increased adrenal activity is largely unanswered. Recently published preliminary data (Wingate and Lasley, 2002) report modest musth-associated elevations in serum cortisol levels in individual captive Asian and African bulls. Additional data in the form of longitudinal studies of animals under natural conditions are needed to examine this question more fully and to determine the extent to which increased adrenal activity is involved, either as a cause or consequence, of the musth condition. Clearly, such studies depend upon non-invasive sampling procedures, applicable to group-living or free-ranging animals, which in turn require appropriate endocrine techniques based on urine or, preferably, fecal hormone analysis.

Measurement of urinary cortisol has been used to assess adrenal function in elephants, but the data are limited to two studies in females (Brown et al., 1995; Schmid et al., 2001). Furthermore, collection of urine can present difficulties in extensively managed elephant groups in captivity (i.e., hands-off situations) and in general, regular urine sampling from animals in the wild, particularly over extended periods of time, is impractical. Thus, attention has focused more recently on establishing methods for determining glucocorticoid metabolites in feces as an index of adrenal function in elephants (Foley et al., 2001; Stead et al., 2000; Wasser et al., 2000), although again studies are limited to females. In order to assess the suitability of cortisol metabolite assays for application to studies in males, the potential measurement of other cross-reacting steroids, in particular androgen metabolites, needs to be carefully assessed (cf. Möstl et al., 2002). Due to a common androstane structure of the majority of androgen and even some glucocorticoid metabolites, which differ only in the functional group at C_{11} (see Fig. 1), antibodies against C_{19} cortisol metabolites are likely to substantially cross-react with androgen metabolites. This has been shown to be an important factor when these methods are applied to studies of adrenocortical function in males, as recently demonstrated for dogs (Schatz and Palme, 2001), and becomes especially important when, as is often the case, group-specific antibodies are used for such assays (Möstl et al., 1999; Teskey-Gerstl et al., 2000). Given the magnitude of the increase in androgen levels during musth in male elephants (Jainudeen et al., 1972b; Poole et al., 1984; Rasmussen et al., 1996), a potential measurement of cross-reacting testosterone metabolites could represent a significant problem when applying urinary and fecal glucocorticoid assays to monitor adrenal activity. Non-specific measurements could lead to false interpretations concerning the relationship between adrenal endocrine function and musth.

In order to establish glucocorticoid assays for non-invasive assessment of adrenocortical function in male

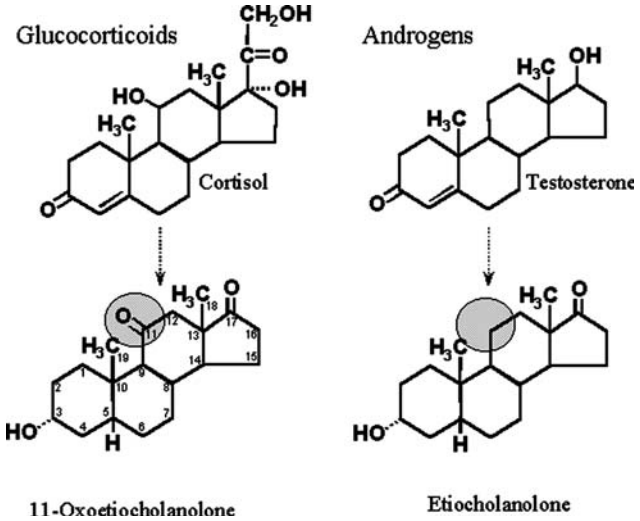

Fig. 1. Structural formula of 11-oxo-etiocholanolone (C-atoms are numbered) and etiocholanolone (both androstanes; 19 C-atoms) representing typical metabolites of cortisol and testosterone, respectively. The grey area indicates the position C_{11}, which in case of glucocorticoid, but not androgen metabolites, bears an oxygen.

elephants, not only information on the nature and relative abundance of excreted glucocorticoid metabolites, but also information on the degree of antibody cross-reactivity with prevailing androgen metabolites of testicular origin is required. Since such information is not available for either elephant species, the first aim of our study was to provide basic data on cortisol metabolism and excretion using a radiometabolism study and to assess the characteristics of urinary and fecal cortisol metabolites using HPLC analysis. Based on this information, our second objective was to evaluate the specificity of different antibodies for determination of fecal and urinary cortisol metabolites and to assess the biological validity of the different antibodies for monitoring adrenal function using an ACTH challenge test. Using the validated assays, our final aim was to provide data on concentrations of fecal cortisol metabolite concentrations in bulls during musth and non-musth conditions.

2. Materials and methods

2.1. Animals and housing conditions

A total of five male African elephants living in four mixed social groups were involved in this study (Table 1). The radiometabolism study and the ACTH challenge was performed on the 16 year old male *Rambo*, whereas the long-term fecal sample collection for the comparison of hormone concentrations in elephant feces during musth and non-musth condition was carried out in the four bulls *Chisco*, *Calimero*, *Kibo*, and *Mooti*. All elephants were housed in indoor/outdoor enclosures and were fed a daily diet of fresh grass, hay, fruits and vegetables with water available at all times. During the radiometabolism and ACTH study the bull was kept singly.

2.2. Radiolabel infusion study

An infusion mixture containing ~1 mCi [^{3}H]cortisol (64.0 Ci/mmol; TRK407 Batch 119; Amersham Pharmacia Biotech UK) and ~100 μCi [^{14}C]testosterone (57.0 mCi/mmol; CFA.129 Batch 71; Amersham Pharmacia Biotech UK) was prepared in 1 ml absolute ethanol. Sterile 0.9% NaCl solution (9 ml) was added to the radiolabel solution and the total volume injected into an ear vein. Prior to injection, the animal was sedated by an i.m. injection of 6 ml *Large Animal Immobilon* (Product Licence number 12809/4062). Following the procedure, 6 ml antidote (*Large Animal Revivon* 12809/4065) was given i.v.

After isotope administration, the syringe and tube containing the radiolabel solution were each rinsed four times with 5 ml scintillation fluid (Lumasafe, Groningen, The Netherlands), the residual radioactivity counted and subtracted from the pre-injection total to give the amount of radioactivity administered. All radioactive counting was conducted in a liquid scintillation counter (1414 WinSpectral, Wallac, Finland) using a dual ^{3}H/^{14}C quench compensation program.

Following radiolabel injection, all excreted urine and feces were collected separately for up to 4 days. Specifically, urine was collected for 30 h in 50 l plastic buckets by aspirating each voided volume from the concrete floor of the enclosure or external outlet of the drain. The total volume of urine was recorded, and two well-mixed portions (50 ml and 0.5 l) of each sample were stored at −20 °C for analysis. Feces was collected for 86 h in 50 l plastic bags from the concrete floor of the enclosure. To prevent cross-contamination with urine, the material was removed directly after defecation. The total amount of feces was recorded, and three well-mixed portions (2×50–100 g and 1×0.5 kg) of each sample were stored at −20 °C for analysis. All samples were frozen within 30 min of collection.

2.3. ACTH challenge

A total of 6 mg (2.5 μg/kg) of long-acting ACTH preparation (Synacthen, Novartis, Switzerland) was injected intramuscularly (Stead et al., 2000). Following ACTH administration, an aliquot of each voided urine and fecal sample was taken over the next five days. The urine samples were collected in 10 ml plastic tubes from the concrete floor of the elephant's enclosure or the external outlet of the enclosure drain. About 50–100 g of feces (from the middle of a bolus) were collected directly after defecation. All samples were frozen within 30 min of collection and stored at −20 °C until analysis.

Table 1
Elephant bulls involved in this study

Animal	Age[a]	Location	Number of months observed	Number of musth-periods
Chisco	24	Parque de la Naturaleza de Cabarceno, Spain	20	4
Calimero	26	Zoologischer Garten Basel A.G., Switzerland	15	2
Kibo	25	Boras Djurpark, Sweden	15	2
Mooti	18	Zoologická zahrada Dvur Králové, Czech Republic	11	3
Rambo	16	Parque de la Naturaleza de Cabarceno, Spain	—	—

[a] At the beginning of the study.

2.4. Long-term sample collection and signs of musth

To examine the relationship between musth and glucocorticoid (and androgen) metabolite concentrations, fecal samples were taken weekly over periods of up to 20 months from four bulls (see Table 1). For one male (*Chisco*), urine samples were also taken for the same time period. Feces (~40 g) was removed directly after defecation in order to avoid cross-contamination with urine or contamination with other fecal samples in the area. Urine samples were taken either mid-stream or from the concrete floor of the bull's enclosure. All material was frozen within 1 h of collection and stored at −20 °C until analysis.

The appearance of characteristic behavioral and physical changes in the four elephants was used to distinguish between the two conditions "musth" and "non-musth". The male was classified as being in musth, when one or a combination of the following behaviour and/or physical changes were continuously (for more than 3 days a week) observed: increased aggressive behavior, temporal gland swelling, temporal gland secretion, and urine dribbling (Poole, 1987). The changes were recorded *ad libitum* by the keeper staff using a standardized check sheet. Since musth is associated with elevated androgen levels (Poole et al., 1984), all fecal samples were also analysed for epiandrosterone (EA, see below) to provide a physiological marker of the male's reproductive condition (Ganswindt et al., 2002).

2.5. Sample analysis

2.5.1. Determination of radioactivity

From each urine sample, 0.5 ml was mixed with 20 ml scintillation fluid and counted for 10 min as described above. Urine samples were indexed by creatinine (Cr) (Bahr et al., 2000) and values of urinary radioactivity expressed as dpm/mg Cr.

For determination of radioactivity in fecal samples, about 3.5 g homogenised wet feces were lyophilized, pulverized and sieved through a mesh (Fieß et al., 1999) before the fecal powder was extracted with 60% methanol in water (3 ml). After vortexing for 30 min and centrifugation (2500*g*; 15 min), the supernatant was recovered and the fecal pellet re-extracted with 80% methanol (3 ml) as described above. The supernatants of both extracts were combined and an aliquot of 0.5 ml was counted for determination of radioactivity as described above. The radioactivity per fecal sample was expressed as dpm/g dry weight (DW). Total recovered radioactivity (%) was the ratio of the combined sums of the recovered radioactivity in urine and feces divided by the total amount of administered radioactivity. Urine and fecal samples containing peak radiolabel concentration were used for all subsequent analyses.

2.5.2. Separation of conjugated and unconjugated steroids

The proportion of steroid metabolites excreted in urine as free or conjugated forms was assessed by ether-water extraction. Briefly, 3×1 ml aliquots of the radioactive peak sample were extracted with 10 volumes of diethylether by vortexing for 10 min. The aqueous phases were frozen, the ether phases decanted, evaporated to dryness and the extracts reconstituted in 150 µl absolute ethanol. Radioactivity was determined in 50 µl aliquots (unconjugated fraction). The residual aqueous phases were subjected to enzyme hydrolyses by incubation with 1250 U β-glucuronidase/sulfatase (No. G 1512; Sigma Chemie) in 0.5 M sodium acetate buffer (pH 4.7) overnight at 37 °C (Bahr et al., 2000). Following diethylether extraction as described above, the radioactivity was counted in 50 µl aliquots of the reconstituted extracts (enzyme-hydrolyzable conjugated fraction). The remaining aqueous phases containing the non-hydrolyzable conjugated fraction were not further examined.

Three portions of 0.5 g wet feces of the well-mixed radioactive peak sample were each mixed with 80% methanol in water (5 ml) and centrifuged (3500*g*) for 10 min. From 3 ml of the supernatants, the methanol portion was evaporated and 5 ml diethylether and 0.2 ml $NaHCO_3$ (5%) were added to the remaining aqueous phases (Schatz and Palme, 2001). After vortexing (1 min) and centrifugation (3500*g*; 10 min), the ether fractions, containing unconjugated steroids, were recovered and the aqueous phases (containing conjugated steroids) were re-extracted with 5 ml diethlyether and 0.2 ml $NaHCO_3$ (5%). The supernatants of both diethlyether extracts were combined and evaporated to dryness in scintillation tubes (as well as aqueous phases). Each tube was mixed with scintillation fluid (Quicksafe A, no. 100800, Zinsser Analytic, Maidenhead, UK) and measured in a liquid scintillation counter (Packard Tri-Carb 2100TR, Meriden, USA) for 10 min (Schatz and Palme, 2001).

2.5.3. HPLC

Reverse-phase high performance liquid chromatography (RP-HPLC) for separation of cortisol metabolites (performed at the Institute of Biochemistry, Vienna, Austria) and androgen metabolites (performed at the German Primate Centre, Göttingen, Germany) were carried out using a NovaPak C-18 column (3.9×150 mm, Millipore, Milford, MA, USA).

For separation of glucocorticoid metabolites, the flow rate was 1 ml/min and a mixture of methanol:water (MeOH:H_2O) was utilised as mobile phase. The initial concentration of methanol was 50%, followed by a linear gradient up to 75% within 40 min and up to 100% until 45 min (Teskey-Gerstl et al., 2000). The androgen metabolites were separated using an isocratic solvent system of acetonitrile:water (ACN:H_2O; 40/60, v/v) at a flow rate of 0.3 ml/min (Ganswindt et al., 2002). In the

two HPLC systems used, fractions (3 per min for glucocorticoid metabolite separation) and (1 per min for androgen metabolite separation) were collected and in both cases an aliquot counted to determine the profiles of radioactivity. The remaining volume of each fraction was evaporated, reconstituted in assay buffer and an aliquot measured in different EIAs (see below). In this paper, however, only the glucocorticoid data are shown.

Prior to each HPLC, urine (6 ml) was hydrolysed with 1250 U β-glucucronidase/sulfatase and ether-extracted as described above. The extract was reconstituted in MeOH:H_2O (50/50) or ACN:H_2O (40/60) for glucocorticoid and androgen metabolite separation, respectively, 100 µl subjected to HPLC and fractions ($n = 90$ for glucocorticoid metabolites and $n = 110$ for androgen metabolites) collected. For separation of fecal steroids, 16 portions of 2 g well-mixed wet material were mixed with 80% methanol in water (5 ml), vortexed (2 min), and centrifuged (3500*g*) for 10 min. Extracts were pooled and cleaned up using a SepPak C-18 column (Waters, Milford, MA, USA) according to the method described by Palme et al. (1996). In brief, 3 volumes of sodium acetate buffer (0.2 M, pH 4.2; 20 % MeOH) were added to the extract and the total volume passed through the SepPak column. Steroids were eluted with 80% MeOH (10 ml), the MeOH evaporated to dryness as described above, the extract reconstituted in MeOH:H_2O (50/50) or ACN:H_2O (40/60), and 100 µl were separated by HPLC as described above.

In order to determine the specificity of the different cortisol metabolite measurements used (see below), HPLC chromatography was also carried out on urine (50 µl) and fecal (50 mg) samples ($n = 2$, each) collected during musth from the bull *Chisco*. The methanolic extraction of lyophilized fecal powder (and diethylether extraction of hydrolysed urine), as well as the separations on the different HPLC systems were achieved according to the methods described above and by Ganswindt et al. (2002).

2.5.4. Hormone assays

Four different enzymeimmunoassays (EIA; Table 2) using antibodies developed to measure a range of potential cortisol metabolites were used to determine immunoreactivity in (i) HPLC fractions, (ii) samples collected during the ACTH challenge test, and (iii) the long-term collected samples from the bull *Chisco*. Sensitivity of the assays as well as intra- and interassay coefficients of variation, determined by repeated measurements of quality controls, are shown in Table 2. Serial dilutions of extracted fecal and urine samples gave displacement curves, which were parallel to the respective standard curves in all assays. The cross-reactivities of the different antibodies are described by Palme and Möstl (1997); Schatz and Palme (2001) (for cortisol and 11-oxo-etiocholanolone I) and Möstl et al. (2002); Huber et al. (2003) (for 11-oxo-etiocholanolone II). Cross-reactivities of the 5β-androstane-3α,11β-diol-17-CMO antibody (100%) were 3.4% for 5β-androstane-3α-

Table 2
Characteristics of the four group specific EIAs, which were used to examine urinary and fecal glucocorticoid metabolites

EIA	Cortisol[a]	11-Oxo-etiocholanolone I[a]	11-Oxo-etiocholanolone II[b]	11β-Hydroxy-etiocholanolone
Antibody	Cortisol-3-CMO[c]	5β-Androstane-11,17-dione-3-HS[c]	5β-Androstane-3α-ol-11-one-17-CMO[c]	5β-Androstane-3α,11β-diol-17-CMO[d]
Label	Cortisol-3-CMO[e]	5β-Androstane-11,17-dione-3-glucoronide[e]	5β-Androstane-3α-ol-11-one-17-CMO[f]	5β-Androstane-3α, 11β-diol-17-CMO[e]
Standard	Cortisol	5β-Androstane-3α-ol-11,17-dione	5β-Androstane-3α-ol-11,17-dione	5β-Androstane-3α-11β-diol-17-one
Specificity[g]	Cortisol (ring A reduced)	11,17-DOA[h]	3α,11-Oxo-CM[i]	3α,11β-Dihydroxy-CM[i]
Sensitivity[j]	1.5	3	3	2
Intraassay CV[k]	4.8, 8.8 ($n = 16$)	2.1, 10.5 ($n = 17$)	2.6, 2.9 ($n = 18$)	6.3, 12.4 ($n = 14$)
Interassay CV[k]	11.2, 15.9 ($n = 24$)	11.1, 13.4 ($n = 65$)	9.7, 12.5 ($n = 96$)	5.6, 9.8 ($n = 50$)

[a] First described by Palme and Möstl (1997).
[b] First described by Möstl et al. (2002).
[c] Coupled with BSA and raised in rabbit.
[d] Coupled with BSA and raised in sheep.
[e] Coupled with *N*-biotinyl-1,8-diamino-3,6-dioxaoctane (DADOO-biotin).
[f] Coupled with biotinyl-3,6,9-trioxaundecanediamine (LC-biotin).
[g] Group of metabolites measured.
[h] 11,17-Dioxoandrostanes.
[i] 3α,11-Oxo-cortisol metabolites, 3α,11β-dihydroxy-cortisol metabolites.
[j] Given in pg/well.
[k] Values represent percentage variance for high and low quality controls.

ol-17-one, 1.8% for 11-oxo-etiocholanolone and <0.1% for corticosterone, cortisol, 5α-androstane-3,11,17-trione, 5β-androstane-3,11,17-trione, testosterone, 5α-andro- stane-3,17-dione, 5β-androstane-3,17-dione, androstendione, 5β-androstane-3β-ol-17-one, 5β-androstane-17-one, dehydroepiandrosterone, and androsterone.

In addition, in all fecal (and urine) samples collected from the four adult bulls, epiandrosterone (EA) concentrations were measured. Sensitivity of this assay at 90% binding was 3 pg/well, and the intra- and interassay coefficients of variation ranged between 2.4 and 11.9%.

All assays were performed on microtiter plates with a double antibody technique according to the methods described by Palme and Möstl (1997) and Ganswindt et al. (2002).

To adjust for variations in water content, urinary steroid concentrations were indexed against creatinine (Cr) and expressed as mass/mg Cr (Bahr et al., 2000), while fecal hormone levels were expressed as mass/g dry weight.

3. Results

3.1. Radiometabolism study

3.1.1. Time course and route of excretion

The total recovery of administered radioactivity was 59% for [^{3}H]cortisol and 78% for [^{14}C]testosterone. Eighty-two percent of the [^{3}H]cortisol recovered was excreted in urine compared to 18% in feces, whereas 43% of the [^{14}C]testosterone recovered was excreted in urine compared to 57% in feces (Fig. 2). In urine, highest levels of radioactivity for both steroids were measured in the initial sample, 4.5 h after radiolabel infusion and >95% of urinary radioactivity was excreted within the first day. Elimination of radioactivity was much slower in feces. Peak levels were recorded between 30 and 38 h postinjection and about 10–15% of [^{3}H]cortisol and [^{14}C]testosterone radioactivity were not excreted after 3 days.

Almost all radioactive metabolites recovered from urine were conjugated (86% [^{3}H]cortisol and 97% [^{14}C]testosterone) and the majority of these were enzyme-hydrolysable (53 and 78%, respectively). In contrast, 86 and >99% of the [^{3}H]cortisol and [^{14}C]testosterone metabolites recovered from feces were unconjugated steroids.

3.1.2. HPLC analysis

HPLC profiles of radiolabelled cortisol metabolites in feces and urine in combination with data on elution positions of immunoreactivity determined in the different EIAs (see Section 2) are shown in Fig. 3.

For feces there were three main peaks of radioactivity eluting in fractions 4–9, 33–38, and 40–46. The second peak co-eluted with 11β-hydroxy-etiocholanolone. No radioactive peak was detected at the position of authentic cortisol (fraction 20/21).

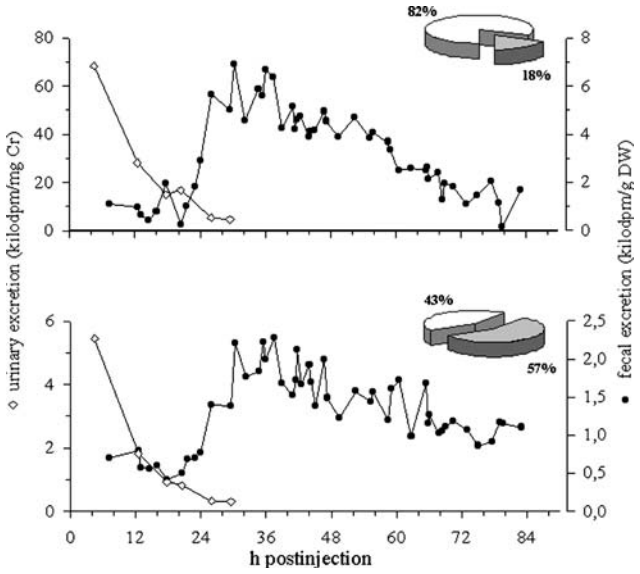

Fig. 2. Time course and route of excretion (pie charts) of [^{3}H]cortisol (A) and [^{14}C]testosterone (B) in urine (◇) and feces (●) in a male African elephant (Rambo). Pie charts show the proportion of [^{3}H]cortisol and [^{14}C]testosterone radioactivity recovered in urine (□) and feces (■). The values are presented as percentages and are based on the total radioactivity recovered during the first 30 h (for urine) and 86 h (for feces) post-injection.

Determination of immunoreactivity in HPLC fractions using three different glucocorticoid assays showed that the first (and smallest) peak of radioactivity was not detected by any of them. The second peak of radioactivity was associated with a single large peak of immunoreactivity in both the 11β-hydroxy-etiocholanolone (1) and 11-oxo-etiocholanolone I (2) assays, whereas the third peak of radioactivity was associated with the single major peak of immunoreactivity obtained with the 11-oxo-etiocholanolone II (3) assay.

For urine, HPLC indicated at least five peaks of radioactivity with those at fractions 18–21 and 31–35 clearly predominating. The more polar of these two peaks co-eluted at approximately the elution position of authentic cortisol, whilst the major peak (fractions 31–35) was found between the elution positions of corticosterone and 11β-hydroxy-etiocholanolone. The majority of immunoreactivity measured by the cortisol (4) assay was detected in fractions 18–24, with peak values (fractions 20–21) corresponding to the elution position of one of the two major peaks of radioactivity. Determination of immunoreactivity in HPLC fractions using the three different cortisol metabolite assays (1–3) showed peaks at the same positions as the respective peaks of immunoreactivity in feces. These peaks of immunoreactivity were for all three assays associated with only minor peaks of radioactivity. None of the four

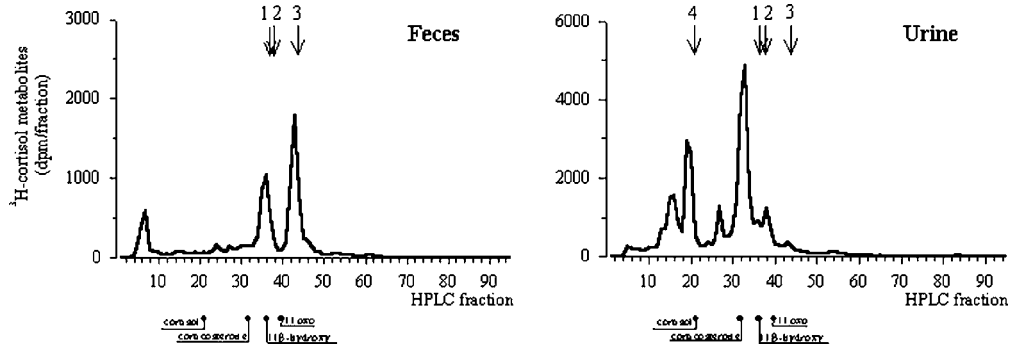

Fig. 3. HPLC profiles of [^{3}H]cortisol metabolites in feces and urine in a male African elephant (Rambo). Immunoreactivity of each fraction was determined with a (1) 11β-hydroxy-etiocholanolone; (2) 11-oxo-etiocholanolone I; (3) 11-oxo-etiocholanolone II; and (4) cortisol (only for urine) EIA. The arrows represent the elution position of the major peak of immunoreactivity obtained in each assay. Elution positions of standards were: fraction 20/21 for cortisol, fraction 31 for corticosterone, fraction 36 for 11β-hydroxy-etiocholanolone (11β-hydroxy), and fraction 39 for 11-oxo-etiocholanolone (11 oxo).

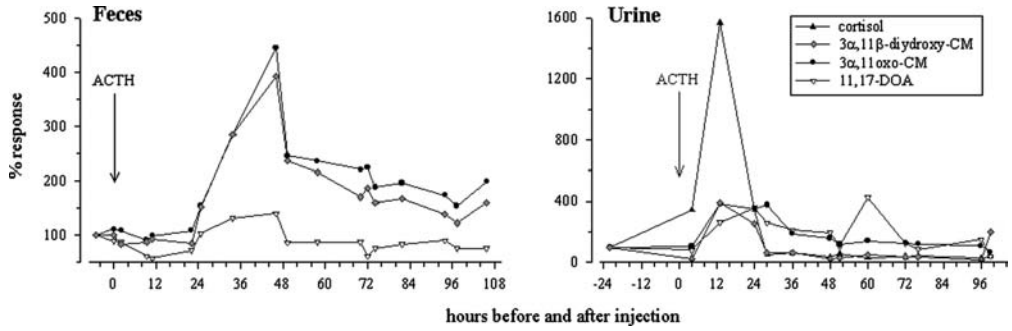

Fig. 4. Proportional responses of immunoreactive glucocorticoid concentrations in feces and urine following ACTH injection into a male African elephant (Rambo). Fecal extracts were assayed in the 11-oxo-etiocholanolone I; 11-oxo-etiocholanolone II and 11β-hydroxy-etiocholanolone EIA, and urinary extracts additionally in the cortisol EIA.

assays showed substantial immunoreactivity associated with the major peak of radioactivity.

3.1.3. ACTH challenge test

Application of ACTH resulted in a measurable increase in glucocorticoid metabolite concentrations in all assays used (Fig. 4), although the degree of response differed considerably. In feces, both the concentrations of 3α,11-oxo-CM and 3α,11β-dihydroxy-CM reached a peak 4- to 5-fold above baseline at about 46 h following ACTH administration, while 11,17-DOA concentrations showed a less pronounced increase (about 1.5-fold) and no clear peak.

In urine, the most pronounced increase (16-fold) was detected with the cortisol assay, with peak values occurring 13 h post-injection. In contrast, the rise in concentrations of the three immunoreactive cortisol metabolites was less pronounced (3- to 4-fold) and, compared to the cortisol measurement, showed a less well defined peak.

3.1.4. HPLC analysis of musth-phase samples

Fig. 5 shows the profiles of immunoreactivity for the different glucocorticoid metabolite measurements in a fecal and urine extract from a musth phase sample in comparison to the profiles of [^{3}H]cortisol and [^{14}C]testosterone radioactivity from the peak radioactive sample of the radiometabolism study. Since the purpose of this procedure was to assess the specificity of the different antibodies, in particular with respect of a potential measurement of cross-reacting androgen metabolites, steroids were separated by the androgen HPLC system (see above).

Several peaks of immunoreactivity were found in feces using all three assays. For the 11-oxo-etiocholanolone II assay, the two major peaks eluted between fractions 18–23 and 26–32, i.e., at positions where radiolabelled cortisol metabolites also eluted (see insert on the top). An additional minor peak of immunoreactivity was found around fraction 82, at the position of 5β-androstane-3α-ol-17-one and co-eluting with one of the two major radiolabelled testosterone metabolites (see insert). Of the three major peaks of immunoreactivity measured in the 11β-hydroxy-etiocholanolone EIA, one eluted at the position of authentic 11β-hydroxy-etiocholanolone (fractions 23–26), while the two others (fractions 52 and 82), co-eluted at the positions of the two major radiolabelled testosterone metabolites (see insert). The majority of immunoreactivity measured in

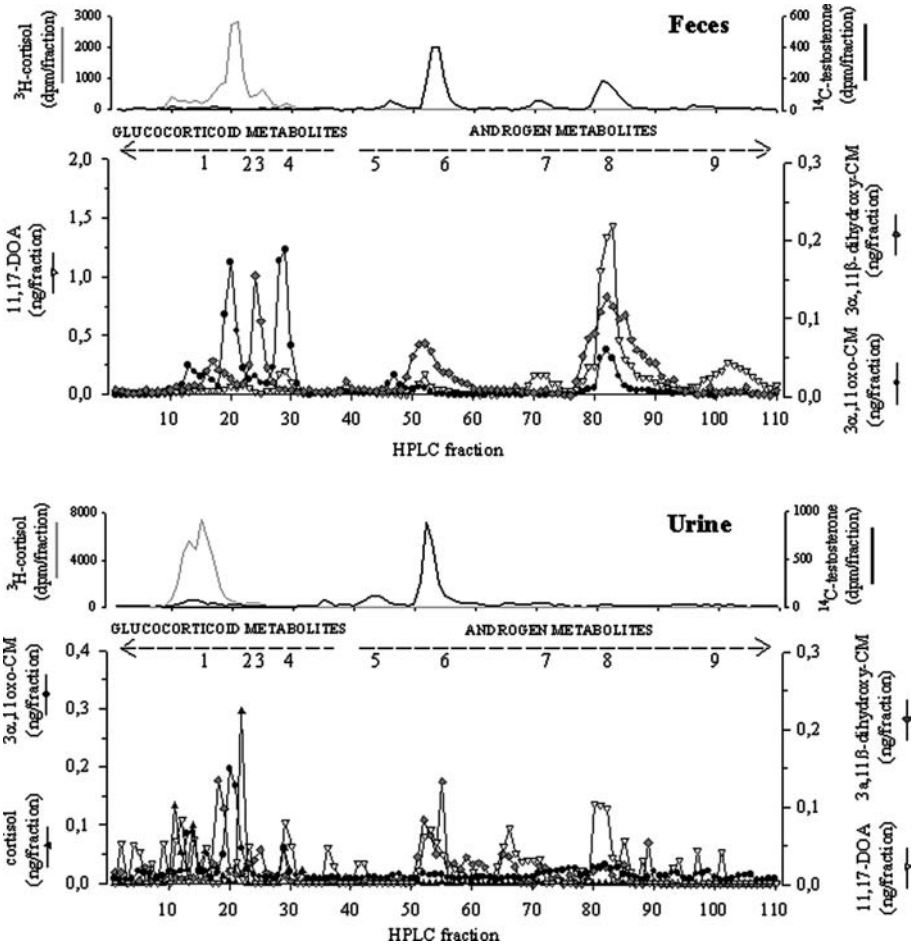

Fig. 5. HPLC profiles of cortisol (only urine); 11,17-DOA; 3α,11-oxo-CM and 3α, 11β-dihydroxy-CM immunoreactivity in a fecal and urine sample collected from an adult male African elephant (Chisco) during musth. Consecutive numbers represent elution positions of respective standards: fractions 14–15 for cortisol (1), fraction 22 for corticosterone (2), fraction 24 for 11β-hydroxy-etiocholanolone (3), fraction 29 for 11-oxo-etiocholanolone (4), fractions 43–44 for testosterone (5), fractions 55–56 for androstendione (6), fraction 72 for epiandrosterone (7), fraction 82 for 5β-androstane-3α-ol-17-one (8), and fraction 100 for androsterone (9). Inserts show the HPLC profiles of radioactivity in feces and urine following i.v. injection of [^{3}H]cortisol and [^{14}C]testosterone in a male African elephant (Rambo).

the 11-oxo-etiocholanolone I assay was located around position 82, again co-eluting with one of the major radiolabelled testosterone metabolites.

In urine, the measurement of 3α,11β-dihydroxy-CM and 11,17-DOA immunoreactivity revealed a number of relatively small peaks, many of which were apolar, eluting between fractions 40–100, beyond the elution positions of radiolabelled cortisol metabolites (see insert on the top). In contrast, the majority of immunoreactivity measured by the 11-oxo-etiocholanolone II assay was associated with two main peaks of high polarity (fractions 12–17 and 19–24). These peaks eluted in a region where also the radiolabelled cortisol metabolites eluted (see insert). Substantial levels of immunoreactivity, however, were also seen between fraction 70 and 100. Similarly, the two major peaks of immunoreactivity measured in the cortisol assay (fractions 10–13 and 21–23) were also of high polarity, eluting in the same area as the radiolabelled metabolites. With the cortisol assay, however, no immunoreactivity was detected beyond fraction 40.

3.1.5. Androgen and glucocorticoid excretion in relation to musth

Fig. 6 shows the longitudinal profiles of immunoreactive glucocorticoid metabolites in comparison to those of epiandrosterone in feces and urine over a period of 20 months in the adult male Chisco. In addition, the figure demonstrates the pattern of occurrence of musth shown by the bull over the whole study period. Although samples were initially measured in all glucocorticoid metabolite assays, based on the results of the ACTH challenge test and specificity of the antibodies reported in the previous sentences, only the profiles of 3α,11-oxo-CM (feces) and cortisol (urine) are presented.

For fecal measurements each of the four periods of musth was associated with clear elevations in EA. Although levels of 3α,11-oxo-CM varied also considerably

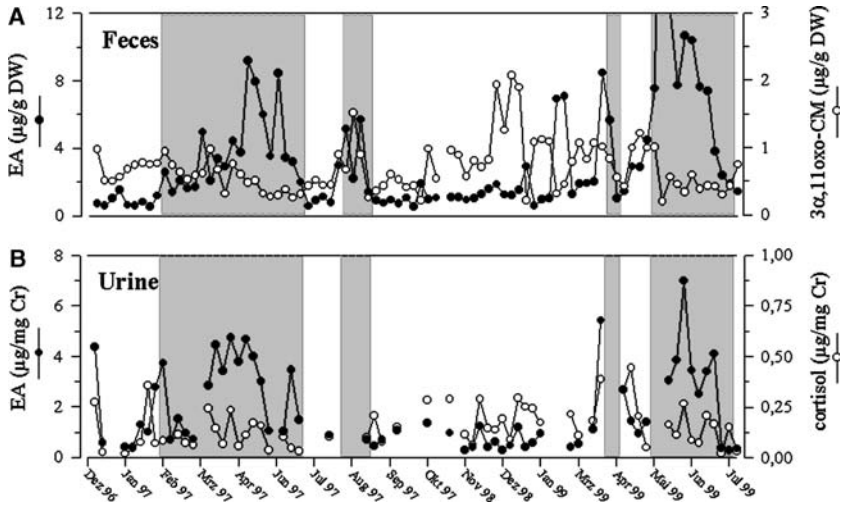

Fig. 6. Profiles of 3α,11-oxo-CM (feces), cortisol (urine) and EA (feces and urine) immunoreactivity throughout a 20-month period in an adult male African elephant (Chisco). Gray bars indicate the periods during which signs of musth were recorded.

over the sampling period, there was no consistent elevation during periods of musth and levels of both hormones were not correlated ($r = -0.2$, $p > 0.05$, $n = 85$). In contrast, levels of the less specific 11,17-DOA and 3α, 11β-dihydroxy-CM measurements (not shown) demonstrated a pattern similar to that of EA and concentrations of both groups of immunoreactive glucocorticoid metabolites were highly significantly correlated to those of EA (11,17-DOA: $r = 0.86$, $p < 0.0001$; 3α, 11β-dihydroxy-CM: $r = 0.49$, $p < 0.0001$).

Due to a number of missing samples, the hormone profiles measured from urine were less clear compared to those determined from feces, although in general the results were similar in that, (i) periods of musth were associated with elevations in EA; (ii) less specific glucocorticoid metabolite measurements obtained by the 11-oxo-etiocholanolone I, 11β-hydroxy-etiocholanolone, and 11-oxo-etiocholanolone II assays were significantly correlated to those of EA ($r = 0.26 - 0.86$, $p < 0.04 - 0.0001$); and (iii) only the specific glucocorticoid metabolite measurement obtained by the cortisol assay showed no elevation during periods of musth and no correlation with levels of EA ($r = 0.22$, $p > 0.05$, $n = 60$).

Fig. 7 shows the fecal 3α,11-oxo-CM (and EA) levels during musth and non-musth phases for four adult bulls. In each of the four males, median concentrations of 3α,11-oxo-CM during periods of musth were slightly lower compared to those recorded when the bulls did not show signs of musth. In contrast, and as expected, all four animals showed marked elevations in their fecal androgen concentrations during periods of musth.

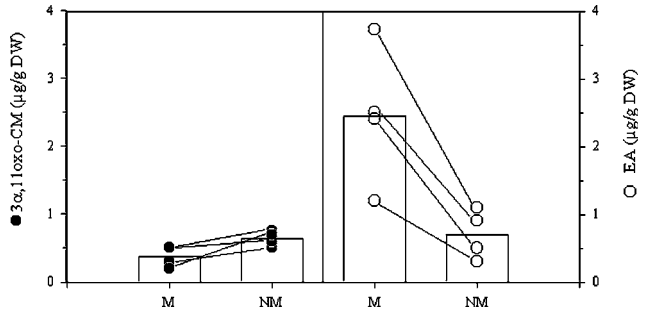

Fig. 7. Dot-bar plots of grouped concentrations of immunoreactive 3α,11-oxo-CM and EA in the feces from the four adult animals (Chisco, Calimero, Kibo, and Mooti). Each dot represents the median hormone value of each individual in musth (M) or non-musth (NM) condition. Values from the same individual are connected by a line. Each bar shows the resultant median value of all assay/condition combinations of the four individuals.

4. Discussion

This study provides basic information on the metabolism and excretion of administered radiolabelled cortisol and testosterone in a male African elephant. Following separation of fecal and urinary cortisol metabolites by HPLC, four different EIAs were evaluated for their ability to specifically detect these compounds. Based on the data obtained, EIAs suitable for non-invasive assessment of adrenocortical function in male African elephants were validated. Profiles of excreted glucocorticoid metabolites showed no clear indication of a musth-related increase in adrenal activity. Further studies are required to clarify the relationship between musth and adrenal endocrine function in the African elephant.

The radiometabolism experiment showed that [^{3}H]cortisol metabolites were predominantly excreted into the urine, whereas the majority of [^{14}C]testosterone metabolites were found in the feces. Differential routes of steroid excretion have also been reported in the fe-

male African elephant (Wasser et al., 1996), and numerous other species including domestic livestock (Palme et al., 1996), cats and dogs (Schatz and Palme, 2001), and some primates (Bahr et al., 2000). The time courses of cortisol and testosterone metabolite excretion are similar to those reported for ovarian steroids in the female elephant (Wasser et al., 1996) and, in general, are in agreement with data from a range of other mammals (e.g., Brown et al., 1996; Teskey-Gerstl et al., 2000; Möhle et al., 2002), showing that peak urinary steroid excretion occurs in one of the first two samples voided (2–6 h), whereas fecal excretion, dependent on gut passage time, generally peaks after 12–56 h (see Schwarzenberger et al., 1996 for review). The slow rate of elimination following peak excretion in feces for both steroids, but particularly for testosterone, is similar to the pattern reported in some domestic species (Lindner, 1972) and primates (Möhle et al., 2002) and, as previously proposed in these studies, is indicative of enterohepatic recirculation.

HPLC indicated the presence of various cortisol metabolites both in urine and feces. While the nature of these substances could not be confirmed with the analytical methods used, comparison of unknown peaks with elution position of standards suggested that one of the major glucocorticoid metabolites excreted into feces is 5β-androstane-3α,11β-diol-17-one, whereas cortisol appears to represent one of the more abundant metabolites in urine after hydrolysis. Virtually no radioactivity was found at the position of native cortisol in feces, indicating that, in the African elephant, as also shown in other taxonomic groups such as ungulates (Palme and Möstl, 1997), primates (Bahr et al., 2000), and carnivores (Graham and Brown, 1996; Schatz and Palme, 2001), cortisol is not excreted in significant amounts in the feces.

All tested EIAs measured substantial amounts of metabolites in the HPLC fractions of samples of both urine and feces. They also all detected an increase in glucocorticoid output following the ACTH challenge. However, only the 11-oxo-etiocholanolone II-EIA measuring 3α,11-oxo-CM detected the most abundant cortisol metabolite in feces, and only the cortisol assay recognised one of the two major metabolites in urine. More importantly though, only these two assays did not show substantial cross-reactivity with testosterone metabolites. Thus, whereas all antibodies detect glucocorticoid metabolites in excreta and are capable of measuring adrenal glucocorticoid output, only two (11-oxo-etiocholanolone II assay for feces and cortisol assay for urine) can be expected to provide measurements with an acceptable degree of specificity.

The importance of this distinction can be seen when applying the various assays to evaluate adrenocortical activity in relation to musth. Elevated levels of corticol metabolites during musth and a high degree of correlation with androgen metabolites were obtained with assays yielding non-specific measurements, whereas use of assays with reduced cross-reactivity with androgen metabolites resulted in profiles in which no clear elevation of cortisol metabolites during musth was observed. In the only other study of this nature, Wingate and Lasley (2002) reported a modest elevation of serum cortisol levels in a captive African and Asian bull during the time when physical and behavioural signs of musth were detected. Whether the discrepancy between these findings and our own reflects different assay specificities is difficult to determine, since no characterisation of antibody specificity in the radioimmunoassay used by Wingate and Lasley was given. In addition to assay specificity, age, housing conditions and nutritional status can all affect glucocorticoid output and differences in the definition of musth may also have a bearing on the divergent results. It may also be that the 2- to 3-fold elevations in cortisol levels in plasma are simply too small to be detected after excretion in urine or feces, but this is probably unlikely considering that we are dealing with chronic, rather than acute changes in hormone levels and that non-invasive hormone measurements generally reflect well changes in circulating levels of other steroids in the elephant (e.g., progestogens, Fieß et al., 1999; androgens, Ganswindt et al., 2002). Although our own data are not comprehensive, similar findings among all four males studied over extensive periods, together with the correspondence of results for urine and feces for one animal, are consistent with the interpretation that musth in African elephants is generally not associated with marked elevations in glucocorticoid output. Given the complex nature of musth, our limited knowledge of any aspect of its physiology and the variety of factors that are likely to influence its manifestation, it is clear that further studies are needed before a possible relationship between musth and adrenal function can be resolved.

As has been clearly illustrated in the present study, there can be certain limitations with the use of non-invasive methodologies for the measurement of cortisol metabolites in male elephants, as recently demonstrated for male dogs (Schatz and Palme, 2001). This emphasizes the need for caution when interpreting findings on adrenal endocrine function in males, especially in individuals with elevated androgen levels. Here we describe further procedures that enable a specific measurement of cortisol metabolites in male African elephants, allowing adrenocortical activity to be reliably assessed from both urinary and fecal analysis. The use of these methods should help to facilitate further studies of adrenocortical function in this species, particularly under conditions of extensive management or free-ranging conditions, and to clarify to what extent musth can be considered to represent a physiological stress.

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