Journal of Chromatography B 1215 (2023) 123576

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

Ultra-performance convergence chromatography tandem mass spectrometry analysis of adrenal and gonadal steroid hormones in southern white rhinoceros (*Ceratotherium simum simum*) faeces and serum

Rachelle Gent [a], Inge D. Barbier [a], Stephen L. Atkin [c], Annie E. Newell-Fugate [d], Amanda C. Swart [a,b,*]

[a] Department of Biochemistry, Stellenbosch University, Stellenbosch 7600, South Africa
[b] Department of Chemistry and Polymer Science, Stellenbosch University, Stellenbosch 7600, South Africa
[c] Royal College of Surgeons in Ireland Bahrain, Adliya, Bahrain
[d] Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, TX 77843, United States

ARTICLE INFO

Keywords:
Wildlife conservation
Steroid reproductive monitoring
Stress/distress
Cytochrome P450
Cortisol
Androgens
11-ketotestosterone/11KT
11-hydroxyandrostenedione/11OHA4
21-deoxycortisol

ABSTRACT

Steroid hormone analysis is routinely undertaken in the assessment of stress response and reproductive function in the management of both captive and free-ranging wildlife species. Faecal samples have become the preferred sample type for analysis as collection is non-invasive and easily assessable. These investigations are generally aimed at aiding successful translocations, enhanced survival outcomes in captivity and improvement of reproductive rate. Immunoassays are the most common approach in the analysis of hormones, particularly in the case of the southern white rhinoceros (*Ceratotherium simum simum*). Non-specificity, attributed to structural similarity of steroid metabolites impedes accurate evaluations which can be eliminated by chromatographic techniques which are more specific, selective and provide comprehensive analyses.

This study developed and validated three methods using ultra-performance convergence chromatography tandem mass spectrometry for the assessment of classical androgens, progestogens and adrenal steroids, as well as the C11-oxy androgens and C11-oxy progestogens in serum and faeces from white rhinoceros. The limit of detection and quantification were determined for each steroid, parameters such as accuracy (<19.8 % RSD) and precision (<20.2 % RSD) were established with recovery, matrix effect, and process efficiency within acceptable limits. Subsequent analysis of serum and faecal samples from five white rhinoceros identified novel steroids for the first time in this species. In addition to the classical adrenal steroids, the following C11-oxy steroids were detected in faecal samples: 11α-hydroxydihydroprogesterone (168 ng/g), 11α-hydroxyprogesterone (125.9 ng/g), 11β-hydroxyprogesterone (210.2 ng/g) and 11-ketoandrostenedione (3.3–19.6 ng/g) with 11-deoxycortisol being the major glucocorticoid (24.2–67.3 ng/g) together with 21-deoxycortisone (40.7 ng/g) and deoxycorticosterone (7.6–14.6 ng/g). In serum samples 11β-hydroxyandrostenedione (0.35–2.34 ng/mL) and 11β-hydroxytestosterone (0.18–1.62 ng/mL) were the predominant androgens with cortisol (5.8–20.5 ng/mL), the predominant glucocorticoid, while corticosterone, 18-hydroxycorticosterone and aldosterone were also detected.

These methods can be applied independently to assess either androgens, progestogens, or adrenal steroid panels or in combination to assess the cohort of gonadal and adrenal steroids in faeces and/or serum, in southern white rhinoceros as well as other wildlife species. Analysis would enable the accurate assessment of reproductive health and stress responses while also distinguishing between stress and distress thus contributing to the conservation of wildlife species.

Abbreviations: 11K5αdione, 11-keto-5α-androstanedione; 11KA4, 11-ketoandrostenedione; 11KAST, 11-ketoandrosterone; 11KDHT, 11-ketodihydrotestosterone; 11KT, 11-ketotestosterone; 11OH5αdione, 11β-hydroxy-5α-androstanedione; 11OHA4, 11β-hydroxyandrostenedione; 11OHDHT, 11β -hydroxydihydrotestosterone; 11OHT, 11β-hydroxytestosterone; 5αdione, androstanedione; A4, androstenedione; DHT, dihydrotestosterone; T, testosterone; 11KDHP4, 11-ketodihydroprogesterone; 11KP4, 11-ketoprogesterone; 11KPdiol, 11-ketopdiol; 11KPdione, 11-ketopdione, 11β-hydroxydihydroprogesterone, 11β-hydroxyprogesterone; 11αOHDHP4, 11α-hydroxydihydroprogesterone; 11αOHP4, 11α-hydroxyprogesterone; 3,11diOHDHP4, 3α,11β -dihydroxydihydroprogesterone; 16OHP4, 16α-hydroxyprogesterone; 17OHP4, 17α-hydroxyprogesterone; 17OHP5, 17α-hydroxypregnenolone; 18OHCORT, 18-hydroxycorticosterone; CORT, corticosterone; DHEA, dehydroepiandrosterone; DHP4, dihydroprogesterone; DOC, 11-deoxycorticosterone; E_1, estrone; P4, progesterone; P5, pregnenolone.

* Corresponding author at: Department of Biochemistry, Stellenbosch University, Stellenbosch 7600, South Africa.
E-mail address: acswart@sun.ac.za (A.C. Swart).

https://doi.org/10.1016/j.jchromb.2022.123576
Received 1 December 2021; Received in revised form 4 December 2022; Accepted 10 December 2022
Available online 13 December 2022

1. Introduction

Steroid analysis is widely used in assessing hormonal patterns in captive and free-ranging wildlife species, especially in animals at risk of extinction such as the southern white rhinoceros (*Ceratotherium simum simum*). Evaluation of hormonal patterns not only contributes to the basic biological understanding of a species but also augments wildlife conservation efforts monitoring reproductive health and response to stressors and relocation processes—both of which contribute to population management of threatened and endangered species [1,2].

Investigations into the role of steroid hormones in physiological and pathophysiological processes span many decades and have remained rather challenging due to the similarity in steroid structures while also presenting at low concentrations. Immunoassays (IA) are more commonly used to determine steroid hormones in serum, faeces, urine, hair and saliva to assess stress responses and reproductive health in wildlife species rather than liquid chromatography (LC) or gas chromatography (GC) separation techniques coupled to a detection system such as mass spectrometry (MS). Since blood, urine, hair and saliva from both captive and free-ranging wildlife are not readily accessible, faecal steroid analyses are frequently undertaken in the assessment of stress and reproductive health [1,3,4]. IA utilize antibodies raised against specific steroids but often cross-reactivity results in the antibody also detecting structurally similar steroids [4,5]. Therefore, IA analyses of steroid hormones extracted from faeces are typically reported as faecal glucocorticoid metabolites (fGMs), faecal progesterone metabolites (fPMs) or faecal androgen metabolites (fAMs) [6–10]. While some studies using IA consider matrix effects [7,11–13], the reliability of IA in the identification of specific steroids and their concentrations remains contentious as cross-reactivity impedes accuracy and precision. Unfortunately, cross-reactivity influences specificity and analyses of grouped steroids do not necessarily present accurate physiological interpretations. A study which utilized an enzyme immunoassay (EIA) to assess the effects of dehorning on fGMs in white rhinoceros found a significant increase in fGM levels post-procedure which indicated a significant stress response. However, the antibody aimed at detecting the downstream metabolites of cortisol and corticosterone (CORT), cross-reacted with the a C11-oxy C_{19} steroid, 5α-androstane-3β,11β-diol-17-one (230 %) [8], suggesting that the assay would not have accurately assessed changes in glucocorticoid levels while potentially also measuring other structurally related androgens. It is possible that the limited specificity of IA, which hinders the accurate analysis of structurally related steroid hormones, would impact biological interpretations of steroid hormone analyses [14–18].

Reports of studies which have followed the chromatographic route—GC–MS, LC-MS, and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) in the assessment of stress and reproductive health in white rhinoceros, and in other species from Perissodactyla families, are limited. Far fewer studies utilising LC-MS have been reported than those using IA to determine hormone levels. LC-MS has been used to analyse faecal and urinary sex steroids in southern white rhinoceros [19]; faecal sex steroids in Indian rhinoceros, *Rhinoceros unicornis* [20]; faecal cortisol in southern white rhinoceros and black rhinoceros (*Diceros bicornis*) [21]; and serum cortisol and CORT in Grevy's zebra, *Equus grevyi*, and in Przewalski's wild horse, *Equus caballus przewalskii* [22]. Two studies have clearly demonstrated that LC-MS/MS analyses generate more comprehensive steroid profiles with improved efficiency and specificity over IA while being similar to IA in cost due to the number of steroids being assessed. LC-MS/MS analyses provide steroid profiles which include glucocorticoids as well as sex steroids, reported for mammalian wildlife sera, avian plasma [23] and bottlenose dolphins (*Tursiops truncatus*) [24]. These analyses maximize data acquisition and therefore allow for greater insight into hormone homeostasis regarding stress and reproductive management.

The C11-oxy C_{19} steroids (Fig. 1) have only recently come to the forefront of steroid analysis in clinical conditions characterised by androgen excess and have not been considered in mammalian wildlife species. In addition, the C11-oxy progestogens are rarely considered, except in adrenal disorders such as cytochrome P450 21-hydroxylase deficiency. The C11-oxy progesterone metabolites (Fig. 2) are similar in structure to the C_{19} steroids but have a C17 side chain with either a hydroxy- or keto-group at C11. It has come to light that these steroid metabolites are present in circulation in a number of species [25,26] and play a role in normal physiology while also associated with pathophysiology [27,28]. These metabolites have not, to date, been identified in southern white rhinoceros.

Chromatography coupled to MS has long been considered to be superior to IA in steroid analysis, eliminating the negative factors associated with IA. Both GC- and LC-MS are techniques that have been successfully applied and are far more specific than IA in the accurate identification and quantification of steroids. Although LC-MS methods are superior, IA have nonetheless been the preferred method for analysing steroids by many research groups due to practicality and convenience at a relatively low cost [4,14,16,29,30]. Technologies such as ultra-high-performance liquid chromatography (UHPLC)-MS/MS and UltraPerformance Convergence Chromatography™ (UPC^2)-MS/MS have high sample throughput with run times often less than 10 min while allowing for the identification and quantification of a cohort of steroid metabolites with high sensitivity. UPC^2-MS/MS has superior sensitivity to UHPLC-MS/MS with an increase in signal of 5–50-fold, which is attributed to the use of supercritical fluid chromatography (SFC). SFC improves resolution while providing faster analysis compared to UHPLC. Supercritical fluids have densities and dissolving capacities similar to liquids as well as high diffusion and low viscosity comparable to gasses [31]. The UPC^2-MS/MS system utilised in this study therefore combines the benefits of both GC–MS and LC-MS without incorporation of their individual pitfalls. We have shown in previously established methods that comprehensive steroid panels within a single sample can be assessed with high specificity and accuracy [32,33].

The aim of this study was to develop and validate three UPC^2-MS/MS methods with optimised run-times, developed for the identification and accurate quantification of the following adrenal and gonadal steroids in faeces and serum from southern white rhinoceros: (i) the C_{19} and C11-oxy C_{19} steroids, (ii) the C11-oxy C_{21} steroids and (iii) the adrenal steroid panel. We refined the methods to target the analysis of glucocorticoids and mineralocorticoids, androgens and progestogens with the focus on steroid hormone analysis in the southern white rhinoceros. Application of these methods to faecal matter, the most accessible matrix in wildlife species, could improve the non-invasive assessment of steroids in both animals as well as humans.

2. Methods

2.1. Materials

Steroid reference standards, 11-keto-5α-androstanedione (11K5αdione), 11-ketoandrostenedione (11KA4), 11-ketoandrosterone (11KAST), 11-ketodihydrotestosterone (11KDHT), 11-ketotestosterone (11KT), 11β-hydroxy-5α-androstanedione (11OH5αdione), 11β-hydroxyandrostenedione (11OHA4), 11β-hydroxytestosterone (11OHT), 11-ketodihydroprogesterone (11KDHP4), 11-ketoprogesterone (11KP4), 11-ketopdione (11KPdione), 11β-hydroxyprogesterone (11βOHP4), 11α-hydroxydihydroprogesterone (11αOHDHP4), 11α-hydroxyprogesterone (11αOHP4), 21-deoxycortisone, 21-deoxycortisol and alfaxalone were purchased from Steraloids (Newport, USA). Steroids, 11β-hydroxydihydrotestosterone (11OHDHT), 11-ketopdiol (11KPdiol), 11β-hydroxypdiol (11OHPdiol), 11β-hydroxypdione (11OHPdione), 11β-hydroxydihydroprogesterone (11βOHDHP4) and 3,11-dihydroxydihydroprogesterone (3,11diOHDHP4) were also purchased from IsoScience (Pennsylvania, USA), while 5α-androstanedione (5αdione), androstenedione (A4), cortisone, dihydrotestosterone (DHT),

testosterone (T), 11-deoxycortisol, 16α-hydroxyprogesterone (16OHP4), 17α-hydroxyprogesterone (17OHP4), 16α-hydroxypregnenolone (17OHP5), 18-hydroxycorticosterone (18OHCORT), aldosterone, CORT, cortisol, dehydroepiandrosterone (DHEA), deoxycorticosterone (DOC), estrone (E_1), progesterone (*P4*) and pregnenolone (P5) were all purchased together with methyl *tert*-butyl ether (MTBE), absolute ethanol, analytical grade methanol and formic acid from Sigma-Aldrich (St. Louis, USA). Deuterated steroids were acquired from Cambridge isotopes (Andover, USA) and included 4-androsten-11β-ol-3,17-dione 2,2,4,6,6,16,16-D7 (D7-11OHA4; 98 %), progesterone 2,2,4,6,6,17,21,21,21-D9 (D9-*P4*; 98 %), testosterone 1,2-D2 (D2-T; 98 %) and 4-androsten-3,17-dione 2,2,4,6,6,16,16-D7 (D7-A4). FOOD-FRESH CO_2 was supplied by Afrox (Cape Town, South Africa). The ACQUITY UPC^2 ethylene-bridged hybrid (BEH) column (3 mm × 100 mm, 1.7 μm particle size) and VanGuard Pre-columns were purchased directly from Waters (Milford, USA).

2.2. Sample collection

Southern white rhinoceros included in the study were located at Amakhala Game Reserve in Eastern Cape, South Africa. Faecal samples were collected between October 2018 and October 2019 from two female and two male white rhinoceros ranging from four to 18 years of age. Faecal samples were collected within 10 min of defaecation upon visual confirmation of animal identity, transported on ice (~2 h), and stored at −20℃ for 2–4 weeks after which samples were kept at −80℃ until analysis. Matched serum samples from each animal and day of collection were supplied by Ikahala Veterinary Wildlife Services, South Africa and stored at −80℃.

Faecal matter from a southern white rhinoceros was collected from Buffelsfontein Game and Nature Reserve in Western Cape, South Africa for the determination of matrix effects. The faecal sample was stored at −20℃ until use. Steroids were extracted after which the faecal matter analysed ensuring it to be void of all steroids and appropriate for determining matrix effects.

2.3. Preparation of standard series and samples

2.3.1. Standard series

Reference steroid standards comprised stock solutions of the aforementioned listed steroids (excluding deuterated standards) which were prepared in absolute ethanol (1 mg/mL). Stocks were subsequently used to prepare two master mixes (1 ng/mL and 1000 ng/mL) in 100 % methanol in specific combinations of standards which would allow optimum chromatographic separation and elimination of cross-talk. Combined reference steroids were as follows: (A) androgens (C_{19} and C11-oxy C_{19} steroids) and cortisone; (B) C11-oxy progestogens (C11-oxy C_{21} steroids), 21-deoxycortisol and 21-deoxycortisone; and (C) adrenal steroid hormones and estrone (E_1) and used for methods A, B and C, respectively (Table 1). Using these master mixes, three standard series ranging from 0.01 ng/mL to 350 ng/mL were prepared in faecal matrix (preparation in section 2.3.2) for use with each method. Using grouped reference steroids, A, B and C, two additional standard series consisting of five concentrations (0.05, 0.25, 05, 50 and 100 or 150 ng/mL) were prepared for use with all methods. Two sets of steroid standards were prepared for validation: the first set was prepared in 50 % methanol comprising pure standards, no matrix; the second set required matrix to be extracted, dried and resuspended in 50 % methanol (75 μL) prior to the addition of the relative steroid master mixes and internal standards (post-extraction spiked standards).

2.3.2. Preparation of faecal matrix and faecal samples

Faecal samples were prepared and extracted as previously published [34,35] with minor changes. Briefly, in the preparation of a faecal matrix, a faecal sample was lyophilised, after which the dried sample was ground using an ULTRA-TURRAX® homogeniser to produce a fine powder. The ground powder, 0.2 g dry weight, was resuspended in autoclaved ddH_2O (1 mL) and the steroids and matrix extracted as described in section 2.4. The extracted matrix was subsequently incubated overnight with dextran-coated charcoal. Charcoal bound steroids were removed by centrifugation (500xg; 10 min) and two rounds of filtration (0.4 μm and 0.2 μm particle sizes). Extracted matrix was analysed using UPC^2-MS/MS and confirmed to have no detectable steroids.

Each faecal sample (±1 g wet weight) was lyophilised and once dried prepared as described above. Samples, ±0.1 g dry weight (exact weights noted for final data analyses), were resuspended in autoclaved ddH_2O (500 μL) and steroids extracted as described in section 2.4.

2.3.3. Preparation of serum matrix and serum samples

Endogenous steroids were removed from foetal bovine serum (FBS) by means of dextran-coated charcoal as described in section 2.3.2. Samples were extracted as outlined in section 2.4. Upon confirmation that steroids were successfully removed, this was used as pure matrix for

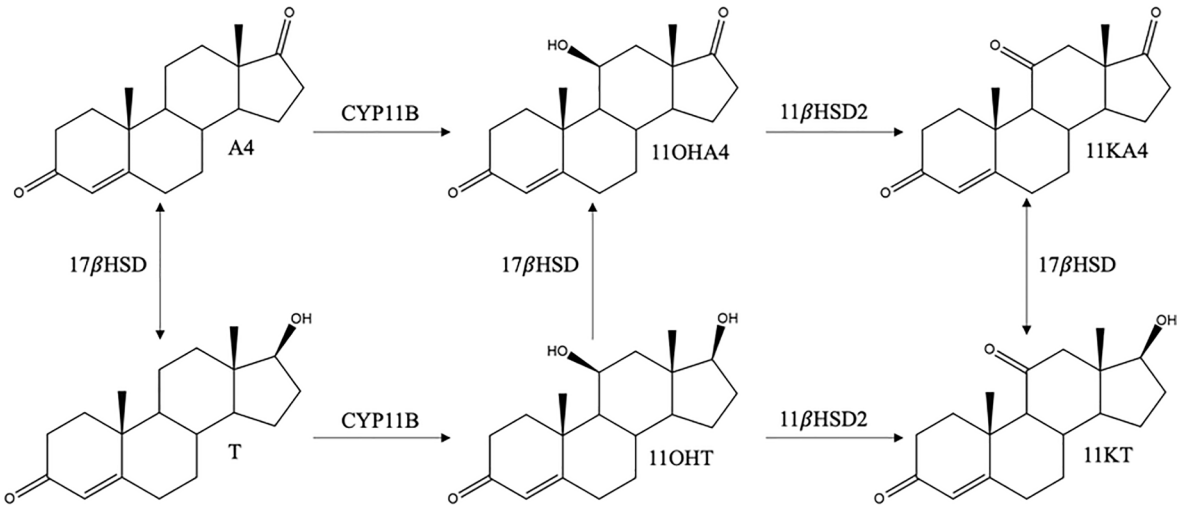

Fig. 1. Androgens, androstenedione (A4) and testosterone (T), are converted to C11-oxy steroids by cytochrome P450 11β-hydroxylase (CYP11B1) and 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2), yielding 11β-hydroxyandrostenedione (11OHA4) and 11β-hydroxytestosterone (11OHT) as well as 11-ketoandrostenedione (11KA4) and 11-ketotestosterone (11KT). 17β-hydroxysteroid dehydrogenase (17βHSD) isoforms catalyse the interconversion of the metabolites as indicated.

the validation of serum as a matrix.

2.4. Steroid extraction

Prior to extraction, deuterated steroids, 100 µL, containing 15 ng D9-*P*4, 15 ng D7-11OHA4 (or 15 ng D7-A4) and 1.5 ng D2-T were added to all standard series, pure standards as well as faecal and serum samples. A liquid–liquid extraction was carried out and MTBE chosen as the organic solvent. This solvent has been demonstrated to efficiently extract steroids over other commonly used solvents for extraction of steroids from various matrices including faeces [35,36]. MTBE was added in a 1:6 ratio (0.5 mL sample to 3 mL MTBE) followed by samples being vortexed at 800 RPM for 5 min and placed at −80 °C for 20 min to freeze the aqueous phase. The liquid organic phase containing steroids was transferred to a clean test tube which was dried under a constant stream of N_2 at 45 °C. Dried samples were resuspended in 50 % methanol (75 µL), transferred to MS vial inserts and stored at −20 °C prior to UPC^2-MS/MS analysis.

2.5. Instrumentation and analytical conditions

Steroids were separated using an ACQUITY UPC^2 system (Waters Corporation, Milford, USA) with an ACQUITY UPC^2 BEH column (3 mm × 100 mm, 1.7 µm particle size) coupled to a Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, USA) for quantitative mass spectrometric detection which was coupled to a make-up pump used to feed 1 % formic acid in methanol into the mixer preceding the MS line at a flow rate of 0.2 mL/min. The mobile phase consisted of liquid CO_2 (solvent A) modified with methanol (solvent B). Three methods were developed for the comprehensive steroid profiling of samples, for the separation of C_{19} steroids, C11-oxy C_{19} steroids and cortisone (4 min total run time); for the separation of C11-oxy C_{21} steroids, 21-deoxycortisol and 21-deoxycortisone (3.8 min total run time); and for the separation of adrenal steroid hormones and E_1 (3 min total run time). Analyses were performed in MRM ESI + mode with an injection volume of 2 µL. Instrumentation parameters were the same and listed as follows: source temperature of 150 °C, desolvation temperature of 500 °C, cone gas flow of 150 L/hour, desolvation gas flow of 900 L/hour and collision gas flow of 0.15 mL/min. Gradients used for the successful separation of steroids in methods as well as mass transitions, cone voltages, collision energies and retention times (R_T) are summarised in the Supplementary tables 1 – 4.

2.6. Method validation parameters

Standard curves were generated with matrix at the following concentrations: 0.01, 0.05, 0.1, 0.25, 0.5, 1, 5, 10, 25, 50, 100, 250 and 350 ng/mL and r^2 values were above 0.96 for each steroid following the exclusion of points below the limit of quantification (LOQ). The limit of detection (LOD) and LOQ were determined using the signal-to-noise (S/N) ratio. LOD is defined as the lowest concentration at which the S/N ratio is greater than 3 for the qualifier ion while LOQ is defined as the lowest concentration at which the S/N ratio for the quantifier ion is greater than 10 and greater than 3 for the qualifier ion. Accuracy and precision were determined for low (0.05, 2.5 and 5 ng/mL), medium (50 ng/mL) and high (100 or 150 ng/mL) concentrations. Accuracy was defined as the % relative standard deviation (RSD) from the average calculated concentration of a single sample repeatedly injected (n = 5). Precision was defined as %RSD of independent replicate samples (n = 5). Recovery, process efficiency and matrix effect were determined at the same five concentrations (0.05, 0.25, 5, 50 and 100/150 ng/mL; n = 3) and are represented as %. Recovery indicated the amount of steroid recovered following extraction and was therefore determined by the comparison of standards with matrix versus post-extraction spiked standards. Process efficiency was determined by the comparison of matrix standards to pure standards and represents the combination of matrix effects and the recovery of the compound from the matrix. Lastly, matrix effects indicated the effect of the matrix on the system, in which the ionisation of a compound was either suppressed (negative value) or enhanced (positive value) and was defined as the difference in concentration between post-extraction spiked standards and pure standards divided by pure standards [31,37,38]. All data were collected, analysed and quantified using the MassLynx 4.1 software package.

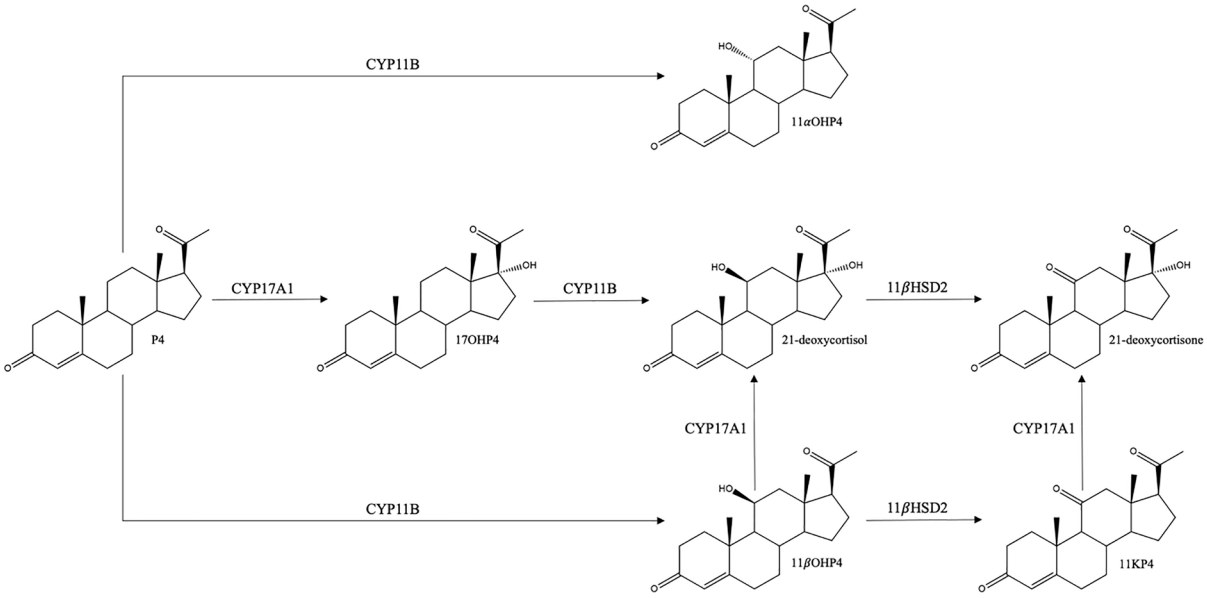

Fig. 2. Progesterone (*P4*) is converted to 17α-hydroxyprogesterone (17OHP4) by cytochrome P450 cytochrome 17α-hydroxylase/17,20-lyase (CYP17A1). 17OHP4 and P4 yield C11-oxy C_{21} steroids, 11β-hydroxyprogesterone (11βOHP4) and 21-deoxycortisol catalysed by cytochrome P450 11β-hydroxylase (CYP11B1). 11βOHP4 and 21-deoxycortisol are converted to 11-ketoprogesterone (11KP4) and 21-deoxycortisone by 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2). 11βOHP4 and 11KP4 are also converted by CYP17A1 to 21-deoxycortisol and 21-deoxycortisone, respectively. *P4* is additionally metabolised to 11α-hydroxyprogesterone (11αOHP4).

Table 1
Steroids detected in methods A, B and C used in the analysis of white rhinoceros faecal samples. Common steroid names and respective abbreviations are listed.

Method A	Method B	Method C
11-keto-5α-androstanedione (11K5αdione)	11-ketodihydroprogesterone (11KDHP4)	11-deoxycortisol
11-ketoandrostenedione (11KA4)	11-ketoprogesterone (11KP4)	16α-hydroxyprogesterone (16OHP4)
11-ketoandrosterone (11KAST)	11-ketopdiol (11KPdiol)	17α-hydroxyprogesterone (17OHP4)
11-ketodihydrotestosterone (11KDHT)	11-ketopdione (11KPdione)	17α-hydroxypregnenolone (17OHP5)
11-ketotestosterone (11KT)	11β-hydroxypdiol (11OHPdiol)	18-hydroxycorticosterone (18OHCORT)
11β-hydroxy-5α-androstanedione (11OH5αdione)	11β-hydroxypdione (11OHPdione)	aldosterone
11β-hydroxyandrostenedione (11OHA4)	11β-hydroxydihydroprogesterone (11βOHDHP4)	corticosterone (CORT)
11β-hydroxydihydrotestosterone (11OHDHT)	11β-hydroxyprogesterone (11βOHP4)	cortisol
11β-hydroxytestosterone (11OHT)	11α-hydroxydihydroprogesterone (11αOHDHP4)	11-deoxycorticosterone (DOC)
5α-androstanedione (5αdione)	11α-hydroxyprogesterone (11αOHP4)	estrone (E_1)
androstenedione (A4)	21-deoxycortisol	progesterone (P4)
cortisone	21-deoxycortisone	pregnenolone (P5)
dihydrotestosterone (DHT)	3α,11β-dihydroxydihydroprogesterone (3,11diOHDHP4)	dehydroepiandrosterone (DHEA)
testosterone (T)	alfaxalone	testosterone (T)
		androstenedione (A4)

3. Results

3.1. UPC^2 separation of C_{19}, C_{21}, C11-oxy steroids and adrenal steroids

Separation of metabolites was achieved by means of a single chromatographic step (Supplementary Fig. 1) using a flow rate of 1.5 mL/min and ABPR of 1800 psi for the three UPC^2-MS/MS methods. The SFC mobile phase allowed for the separation of stereoisomers due to differences in chirality and allowed for the separation of structurally similar steroids. Additionally, specific MRMs enabled the identification and accurate quantification of steroids. Quantifier and qualifier ions for each metabolite were optimised for best selectivity and reduced cross-talk within other channels.

3.2. Performance and validation of methods

3.2.1. Calibration range

LOD and LOQ values were determined for methods A, B and C (Table 2). LODs ranged from 0.01 to 25 ng/mL (0.07–333.3 pg on the column) and LOQs were 0.01–50 ng/mL (0.07–666.67 pg on column). The calibration range for individual metabolites is shown in Table 2. Each steroid ionises differently and thereby has different LOQs and upper limits of quantification (ULOQ). Metabolites which ionise well, such as T, have low ULOQs due to saturation of the detector at higher concentrations. Due to this, points in the calibration curve above the ULOQ (which ranged from 100 to 350 ng/mL) are excluded from analyses. Once calibration range parameters were determined for each steroid a quadratic fit with an acceptable r^2-value (greater than0.97) was obtained for each metabolite enabling quantification during further analysis.

3.2.2. Accuracy and precision

Acceptable %RSDs (<20 %) were achieved for accuracy and precision. These parameters were determined for each method in either faecal (Table 3) or serum (Table 4) matrix for five concentrations. Accuracy and precision could only be determined across all concentrations for steroids which were detected above LOQ. Moreover, parameters at concentrations below a metabolite's LOQ could not be determined in each respective calibration range. Metabolites in the faecal matrix at 0.05 ng/mL showed %RSD ranging from 0.5 to 19.8 % for accuracy and 3.9–13.9 % for precision while 6.5–19.8 % for accuracy and 3.9–19.3 % for precision was obtained for metabolites in the serum matrix. For 0.25 ng/mL, accuracy ranged from 4.4 to 18.4 % and precision ranged from 0.7 to 20.1 % in the faecal matrix while the serum matrix resulted in a % RSD for accuracy of 1.8–16.8 % and for precision of 2.8–20.2 %. Moreover, a range of 1.2–19.7 % for accuracy and 2.30–18.8 % for precision was obtained for 5 ng/mL in the faecal matrix and the serum matrix 2.80–18.4 % for accuracy and 2.90–15.7 % for precision. For accuracy and precision, %RSD, were further determined at the midpoint of the calibration ranges for each metabolite in faecal matrix and ranged from 3.0 to 19.0 % and 2.0–19.5 %, respectively. An accuracy of 1.5–18.1 % and precision of 4.1–19.5 % was achieved for each metabolite, mid-concentration, in serum matrix. Accuracy and precision were further determined at a high concentration and demonstrated to be 2.10–19.5 % and 0.70–17.7 % in the faecal matrix and, 2.20–16.0 % and 3.20–20.0 % in serum matrix.

3.2.3. Recovery, process efficiency and matrix effect

Additional parameters were determined and included recovery, process efficiency and matrix effect. Parameters are described for all methods in faecal matrix (Table 5) as well as in serum matrix (Table 6). Faecal matrix metabolite recovery ranged from 98.1 to 110 % at 0.05 ng/mL, 87.8–116 % at 0.25 ng/mL, 46.4–129 % for 5 ng/mL, 52.6–129 % for 50 ng/mL and 62.0–120 % for 100 ng/mL. Serum matrix steroid recovery ranged from 92.1 to 128 % for 0.05 ng/mL, 87.8–116 % for 0.25 ng/mL, 61.5–141 % for 5 ng/mL, 66.0–140 % for 50 ng/mL and 57.8–130 % for the highest concentration. The recovery of 170 % was only shown for 16OHP4 in the serum matrix and may have been due to cross-talk with 17OHP4 which we have shown to interfere due to similar product ion species. Process efficiency of faecal matrix fell within the

Table 2
Limit of detection (LOD) and limit of quantification (LOQ) are shown for all steroid metabolites analysed in methods A, B and C together with the calibration range and the associated r^2-value.

Method A

Steroid	LOD		LOQ		Range	r^2
	ng/ml	pg on column	ng/ml	pg on column		
5αdione	0.5	6.67	5	66.67	5–350	0.998
A4	0.05	0.67	0.1	1.33	0.1–250	0.992
11K5αdione	1	13.33	5	66.67	5–250	0.975
DHT	1	13.33	5	66.67	5–250	0.998
11KA4	0.25	3.33	1	13.33	1–250	0.990
11OH5αdione	1	13.33	10	133.33	10–350	0.992
T	0.01	0.13	0.05	0.67	0.05–100	0.996
11KAST	10	133.33	25	333.33	25–250	0.996
11OHA4	0.05	0.67	0.25	3.33	0.25–250	0.992
11KDHT	0.5	6.67	0.5	6.67	0.5–100	0.994
11KT	0.05	0.67	0.25	3.33	0.25–250	0.991
cortisone	0.5	6.67	0.5	6.67	0.5–250	0.991
11OHDHT	0.5	6.67	5	66.67	10–250	0.987
11OHT	0.25	3.33	0.5	6.67	0.5–250	0.995

Method B

Steroid	LOD		LOQ		Range	r^2
	ng/ml	pg on column	ng/ml	pg on column		
11KDHP4	0.5	6.67	5	66.67	5–350	0.999
11KP4	0.1	1.33	0.25	3.33	0.25–100	0.994
11βOHDHP4	5	66.67	10	133.33	10–250	0.997
11KPdione	5	66.67	5	66.67	5–350	0.975
21-deoxycortisone	0.5	6.67	0.5	6.67	0.5–350	0.998
alfaxalone	0.5	6.67	5	66.67	5–350	0.999
11βOHP4	0.5	6.67	0.5	6.67	0.5–100	0.997
11αOHDHP4	5	66.67	5	66.67	5–250	0.986
11OHPdione	10	133.33	50	666.67	50–350	0.993
11αOHP4	0.25	3.33	5	66.67	5–250	0.991
3,11diOHDP4	25	333.33	25	333.33	25–350	0.999
21-deoxycortisol	0.5	6.67	5	66.67	5–250	0.998
11KPdiol	10	133.33	25	333.33	25–350	0.999
11OHPdiol	5	66.67	25	333.33	25–350	0.993

Method C

Steroid	LOD		LOQ		Range	r^2
	ng/ml	pg on column	ng/ml	pg on column		
P4	0.01	0.07	0.01	0.07	0.01–150	0.991
A4	0.01	0.07	0.01	0.07	0.01–200	0.993
P5	0.5	3.33	1	6.67	1–300	0.995
E_1	1	6.67	10	66.67	10–300	0.995
DOC	0.01	0.07	0.01	0.07	0.01–150	0.998
DHEA	5	33.33	25	166.67	25–150	0.995
T	0.01	0.07	0.01	0.07	0.01–150	0.999
11OHA4	0.05	0.33	0.05	0.33	0.05–150	0.999
17OHP5	0.25	1.67	10	66.67	10–300	0.993
11-deoxycortisol	0.05	0.33	0.1	0.67	0.1–200	0.997
CORT	0.01	0.07	0.5	3.33	0.5–300	0.997
aldosterone	0.1	0.67	0.25	1.67	0.25–300	0.995
11OHT	0.05	0.33	0.1	0.67	0.1–150	0.999
18OHCORT	0.1	0.07	0.5	0.33	0.05–300	0.991
16OHP4	0.1	0.67	0.25	1.67	0.25–250	0.993
cortisol	0.01	0.07	0.05	0.33	0.05–300	0.991

range of 46.2–122 % at the low end concentration, 57.7–111 % at 2.5 ng/mL, 43.6–189 % at 5 ng/mL, 56.3–130 % at 50 ng/mL and 71.0–129 % at the 100 ng/mL. Process efficiency of serum matrix fell within the range of 46.8–121 % at 0.05 ng/mL, 68.8–151 % at 0.25 ng/mL, 42.3–139 % at 5 ng/mL, 48.8–157 % at 50 ng/mL, and 43.5–118 % at 150 ng/mL. In addition, matrix effect was established and determined to either enhance (positive value) or suppress (negative value) ionisation of the metabolites. Matrix effect ranged from − 89.6–36.5 % across all methods and concentrations in both the faecal and serum matrices.

3.3. Methods applied in the analysis of biological samples

The three methods were applied in the detection of steroid hormone metabolites in faeces and matched serum from four individual southern white rhinoceros (Table 7). Internal reference standards for each steroid were allocated as previously reported [33]. Each rhinoceros was provided with an identification code: PND, SIP, KON, and KTH.

In all rhinoceros, 11-deoxycortisol (27.1–67.3 ng/g) and 11KA4 (3.32–19.6 ng/g) were detected in faecal samples while 11OHA4 (0.35–2.34 ng/mL) was detected in serum samples. Of note is that T was only detected in the older male in both serum and faeces while the C11-

Table 3
Accuracy and precision parameters determined for the separation of C_{19}, C11-oxy C_{19} and cortisone metabolites (method A), C11-oxy C_{21}, 21-deoxycortisol and 21-deoxycortisone steroids (method B), as well as adrenal steroids and E_1 (method C) in faecal matrix, expressed as relative standard deviation (%RSD). * = <LOQ.

Method A

Steroid	Accuracy (%RSD)					Precision (%RSD)				
	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL
5αdione	*	*	9.2	12.9	11.1	*	*	12.5	16.2	14.5
A4	*	13.9	15.3	8.5	7.6	*	11.7	15.2	15.6	12.9
11K5αdione	*	*	17.3	11.9	11.8	*	*	13.4	5.7	6.6
DHT	*	*	5.9	14.2	6.5	*	*	14.2	10.8	6.5
11KA4	*	*	19.6	11.4	9.7	*	*	18.3	5.6	10.7
11OH5αdione	*	*	*	13.9	13.6	*	*	*	10.9	4.9
T	18.6	15.4	15.3	3.5	10.5	12.9	19.3	15.9	2.0	1.6
11KAST	*	*	*	19.0	7.2	*	*	*	19.5	11.0
11OHA4	*	14.5	12.9	16.5	7.5	*	20.1	5.1	7.7	10.4
11KDHT	*	*	9.6	12.5	6.8	*	*	10.0	6.4	13.5
11KT	*	10.6	7.7	17.5	4.2	*	16.0	11.7	6.6	6.9
cortisone	*	*	18.7	15.1	6.2	*	*	14.0	3.4	17.7
11OHDHT	*	*	*	18.0	13.9	*	*	*	13.8	5.3
11OHT	*	*	18.8	15.0	5.1	*	*	11.8	3.9	4.4

Method B

Steroid	Accuracy (%RSD)					Precision (%RSD)				
	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL
11KDHP4	*	*	9.6	5.1	7.4	*	*	9.2	9.8	5.1
11KP4	*	6.7	5.7	5.0	11.3	*	7.2	9.6	4.7	2.4
11βOHDHP4	*	*	*	12.4	13.7	*	*	*	15.5	12.1
11KPdione	*	*	9.0	14.3	14.3	*	*	14.0	7.3	15.7
21-deoxycortisone	*	*	4.4	4.5	5.4	*	*	4.6	6.6	6.3
Alfaxalone	*	*	17.9	4.0	6.1	*	*	8.7	7.7	9.9
11βOHP4	*	*	6.9	6.1	5.8	*	*	5.5	7.9	7.6
11αOHDHP4	*	*	19.7	4.5	5.1	*	*	8.3	18.9	7.0
11OHPdione	*	*	*	6.4	2.8	*	*	*	4.5	11.9
11αOHP4	*	*	10.2	3.8	4.9	*	*	9.4	11.2	9.5
3,11diOHDP4	*	*	*	8.7	7.0	*	*	*	3.4	10.8
21-deoxycortisol	*	*	14.6	4.4	5.8	*	*	10.9	5.3	13.7
11KPdiol	*	*	*	16.9	2.1	*	*	*	10.3	10.4
11OHPdiol	*	*	*	13.6	10.5	*	*	*	13.7	17.4

Method C

Steroid	Accuracy (%RSD)					Precision (%RSD)				
	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL
P4	2.5	9.3	10.3	5.4	10.2	6.0	12.8	18.8	12.4	7.0
A4	0.5	11.7	10.4	5.3	14.9	9.1	9.0	16.2	8.1	16.7
P5	*	*	*	5.8	19.5	*	*	*	13.6	9.0
E_1	*	*	*	5.1	17.1	*	*	*	5.0	14.3
DOC	5.6	18.4	9.4	10.4	2.8	7.6	19.6	13.7	10.1	8.8
DHEA	*	*	*	5.8	19.5	*	*	*	13.4	15.4
T	9.0	14.4	6.5	9.6	11.0	13.8	13.4	18.0	6.4	5.9
11OHA4	4.3	7.7	9.5	12.4	8.1	13.5	0.9	11.4	10.9	12.4
17OHP5	*	*	*	8.4	6.6	*	*	*	2.1	12.3
11-deoxycortisol	*	13.5	6.1	9.0	3.6	*	0.7	13.3	6.3	0.7
CORT	*	*	6.7	7.5	6.9	*	*	8.0	7.3	10.6
aldosterone	*	4.4	13.5	8.9	12.4	*	14.1	10.2	12.5	11.9
11OHT	*	8.4	12.2	6.1	3.5	*	7.6	14.8	9.9	8.9
18OHCORT	*	*	12.8	8.3	12.5	*	*	7.2	10.7	15.9
16OHP4	*	6.2	5.5	9.9	9.2	*	20.0	14.2	9.5	14.8
17OHP4	19.8	16.3	1.2	3.0	15.0	5.0	11.6	2.3	2.5	11.4
cortisol	11.3	9.4	15.2	9.6	9.2	13.9	11.5	9.1	9.2	8.2

oxy androgens were detected in all four rhinoceros—11OHA4 and 11OHT in serum only, and 11KA4 only in faeces. Although glucocorticoids were not detected in all samples, faecal 11-deoxycortisol was present as the major glucocorticoid followed by cortisol and DOC. *P*4, DOC and A4 were detected in faecal samples of both males while these metabolites were not detected in the faeces of female subjects.

In female southern white rhinoceros, cortisol and 11OHT were the most abundant steroids measured in serum, while A4, 18OHCORT and aldosterone concentrations were low. These same metabolites were present at higher concentrations in the older male together with cortisone and CORT. Differences in steroid profiles were also observed—the older male also had 11-deoxycortisol and T in serum and cortisol and T in faeces while 11KT was unique to the serum steroid profile of the younger male together with 16OHP4 in the faecal sample. 11αOHDHP4 was only detected in one female faecal sample while the younger female had no detectable steroids which differed from the other animals (Table 7).

A southern white rhinoceros faecal sample exposed to the environment for an unknown period of time following defaecation was collected from either a male (±16 years old) or a female (±12 years old) subject.

Table 4
Accuracy and precision parameters determined for the separation of C_{19}, C11-oxy C_{19} and cortisone metabolites (method A), C11-oxy C_{21}, 21-deoxycortisol and 21-deoxycortisone steroids (method B), as well as adrenal steroids and E_1 (method C) in serum matrix, expressed as relative standard deviation (%RSD). * = <LOQ.

Method A

Steroid	Accuracy (%RSD)					Precision (%RSD)				
	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL
5αdione	*	*	13.3	16.9	14.9	*	*	10.3	9.4	4.6
A4	*	10.9	7.5	17.1	7.1	*	15.8	5.4	12.8	10.5
11K5αdione	*	*	15.0	16.2	8.3	*	*	4.5	4.10	10.3
DHT	*	*	5.0	18.1	10.1	*	*	2.9	20.0	6.8
11KA4	*	*	15.3	5.5	10.5	*	*	14.6	9.8	19.3
11OH5αdione	*	*	*	14.4	12.2	*	*	*	8.3	11.9
T	6.5	16.8	3.2	8.4	5.2	6.9	9.4	10.7	9.6	3.2
11KAST	*	*	*	1.5	16.0	*	*	*	14.5	14.0
11OHA4	*	15.5	14.8	7.6	4.1	*	3.2	12.5	14.0	4.1
11KDHT	*	*	12.6	13.1	8.9	*	*	7.0	4.2	13.7
11KT	*	10.6	5.4	6.6	3.5	*	2.8	7.4	6.7	5.2
cortisone	*	*	12.5	2.1	10.0	*	*	5.9	14.6	7.4
11OHDHT	*	*	*	10.9	4.3	*	*	*	11.1	6.5
11OHT	*	*	5.0	5.9	2.9	*	*	4.8	6.2	9.4

Method B

Steroid	Accuracy (%RSD)					Precision (%RSD)				
	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL
11KDHP4	*	*	18.4	17.6	6.9	*	*	11.7	7.6	9.3
11KP4	*	7.8	7.2	5.7	14.3	*	20.0	9.8	4.6	5.9
11βOHDHP4	*	*	*	11.1	4.2	*	*	*	7.1	11.4
11KPdione	*	*	18.2	11.4	5.0	*	*	11.0	9.6	3.4
21-deoxycortisone	*	*	4.4	3.7	3.4	*	*	8.9	8.1	4.6
Alfaxalone	*	*	7.8	6.0	5.9	*	*	11.5	8.0	5.9
11βOHP4	*	*	9.8	6.0	5.9	*	*	9.3	10.8	5.7
11αOHDHP4	*	*	14.5	9.9	2.2	*	*	13.7	15.4	12.3
11OHPdione	*	*	*	14.1	4.2	*	*	*	16.4	13.6
11αOHP4	*	*	7.5	6.2	3.3	*	*	8.0	7.3	10.1
3,11diOHDP4	*	*	*	4.6	7.0	*	*	*	15.0	10.2
21-deoxycortisol	*	*	2.8	2.3	4.7	*	*	6.6	13.1	6.0
11KPdiol	*	*	*	6.3	12.2	*	*	*	9.0	16.8
11OHPdiol	*	*	*	10.3	7.7	*	*	*	18.0	20.0

Method C

Steroid	Accuracy (%RSD)					Precision (%RSD)				
	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	150 ng/mL	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	150 ng/mL
P4	9.1	2.0	6.3	6.2	4.1	11.2	8.8	14.4	5.6	6.0
A4	9.5	1.8	7.5	6.1	3.1	11.1	6.0	13.8	7.7	14.3
P5	*	*	12.4	9.1	7.8	*	*	8.9	11.2	8.7
E_1	*	*	*	4.0	6.8	*	*	*	12.1	9.5
DOC	15.9	15.9	5.5	4.8	6.7	19.3	20.1	12.6	7.7	16.6
DHEA	*	*	*	4.9	5.2	*	*	*	11.2	5.0
T	6.6	4.2	13.5	2.9	4.0	3.9	6.8	13.1	8.6	8.3
11OHA4	6.7	12.7	5.2	3.6	4.9	6.7	19.7	15.7	10.1	4.7
17OHP5	*	*	*	9.4	8.7	*	*	*	9.2	10.0
11-deoxycortisol	*	15.6	6.1	5.3	5.4	*	20.2	12.9	7.5	12.6
CORT	*	*	4.4	4.0	8.2	*	*	9.2	7.0	8.6
aldosterone	*	2.5	3.3	2.3	6.4	*	1.5	16.4	8.4	6.5
11OHT	*	10.1	6.2	4.1	3.5	*	19.5	14.3	10.1	5.6
18OHCORT	*	*	5.7	3.7	6.2	*	*	12.2	6.9	5.6
16OHP4	*	2.8	15.5	4.4	6.9	*	4.7	15.3	6.6	11.2
17OHP4	19.8	15.3	5.7	6.0	6.6	5.0	10.6	9.5	6.0	5.6
cortisol	15.3	2.3	3.7	5.1	5.6	10.2	3.3	3.7	10.6	8.7

This sample was used to prepare a suitable matrix for method validation. Prior to steroids being removed using dextran coated charcoal, the sample was extracted in the same manner as the samples from the four other rhinoceros and analysed for steroid hormones. All the steroids detected in the matrix sample (Table 8) were also present in the other samples except for 11βOHP4, 11αOHP4 and 21-deoxycortisone. *P*4 was lower in this sample compared to the concentration of *P*4 in the two male rhinoceros subjects from Amakala Game Reserve while 11-deoxycortisol was detected at a lower level compared to the faecal samples of all four of the other rhinoceros.

4. Discussion

This study successfully developed high-throughput UPC^2-MS/MS methods, A, B and C, to be used in the quantification of a panel of adrenal and gonadal steroids which include the C11-oxy C19 steroids and the C11-oxy C21 steroids in both faecal and serum matrices. Three optimised SCF chromatographic separations of 40 steroids and when carried out independently, each method can be utilised for specific and targeted analysis of steroid hormone panels. Specific gradients using mobile phase compositions of CO2 and a methanol:formic acid (99:1, v/

Table 5
Recovery, process efficiency and matrix effect in faecal matrix. * = <LOQ.

Method A

Steroid	Recovery					Process Efficiency					Matrix Effect				
	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL
5αdione	*	*	86.7	118	89.5	*	*	88.1	93.7	103	*	*	1.60	−20.2	15.5
A4	*	107	89.2	110	102	*	83.3	120	113	103	*	–22.2	34.7	3.30	0.50
11K5αdione	*	*	73.6	101	98.5	*	*	83.1	110	91.3	*	*	12.9	8.90	−7.40
DHT	*	*	79.7	86.5	85.7	*	*	102	97.9	95.2	*	*	27.4	13.2	11.1
11KA4	*	*	73.9	94.0	98.4	*	*	112	109	118	*	*	52.1	16.0	19.8
11OH5αdione	*	*	*	93.5	109	*	*	*	104	129	*	*	*	11.5	17.9
T	98.1	116	81.0	108	102	97.5	98.7	84.2	103	112	−0.70	−14.8	4.00	−5.30	9.90
11KAST	*	*	*	101	101	*	*	*	125	98.2	*	*	*	24.1	−3.00
11OHA4	*	115	76.3	101	105	*	77.9	102	92.6	103	*	–32.2	33.8	−8.70	−2.20
11KDHT	*	*	71.2	97.7	96.0	*	*	79.5	90.6	101	*	*	11.8	−7.30	4.80
11KT	*	97.4	79.6	107	118	*	85.3	97.3	91.9	104	*	−12.4	22.3	−13.8	−12.2
cortisone	*	*	54.2	86.1	76.4	*	*	74.0	94.2	92.0	*	*	36.6	9.50	20.5
11OHDHT	*	*	*	92.7	90.2	*	*	*	86.1	97.2	*	*	*	−7.10	7.80
11OHT	*	*	77.3	102	104	*	*	95.4	95.5	104	*	*	23.4	−6.00	0.70

Method B

Steroid	Recovery					Process Efficiency					Matrix Effect				
	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL
11KDHP4	*	*	90.6	87.8	94.8	*	*	96.5	74.5	82.8	*	*	6.50	−15.2	−12.6
11KP4	*	103	114	123	102	*	73.9	107	108	101	*	−28.2	28.6	−12.2	−1.00
11βOHDHP4	*	*	*	88.3	92.5	*	*	*	87.5	83.8	*	*	*	−1.00	−9.40
11KPdione	*	*	76.2	80.7	87.1	*	*	91.4	70.4	82.4	*	*	19.9	−12.7	−5.40
21-deoxycortisone	*	*	117	117	102	*	*	110	111	110	*	*	10.8	−5.30	−10.5
Alfaxalone	*	*	101	87.2	112	*	*	91.1	78.5	97.9	*	*	−9.80	−10.0	−12.9
11βOHP4	*	*	111	105	109	*	*	124	102	103	*	*	11.7	−2.80	−5.00
11αOHDHP4	*	*	121	94.5	108	*	*	95.8	88.4	102	*	*	−20.7	−6.50	−5.60
11OHPdione	*	*	*	77.7	115	*	*	*	74.0	97.2	*	*	*	−4.80	−15.8
11αOHP4	*	*	130	128	120	*	*	143	111	108	*	*	10.8	−13.3	−10.2
3,11diOHDP4	*	*	*	75.8	94.1	*	*	*	66.4	88.4	*	*	*	−12.4	−6.10
21-deoxycortisol	*	*	127	129	120	*	*	136	120	112	*	*	6.60	−6.70	−6.30
11KPdiol	*	*	*	86.4	101	*	*	*	92.6	90.5	*	*	*	7.20	−10.6
11OHPdiol	*	*	*	71.1	97.0	*	*	*	66.3	87.8	*	*	*	−6.80	−9.50

Method C

Steroid	Recovery					Process Efficiency					Matrix Effect				
	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL
P4	100	92.5	115	91.1	86.9	85.3	70.4	87.5	88.1	101	−14.8	–23.9	−23.8	−3.30	16.3
A4	108	112	90.9	78.4	90.5	97.1	82.0	108	80.1	97.3	−10.2	−26.4	19.0	2.20	7.60
P5	*	*	*	90.6	96.5	*	*	*	104	122	*	*	*	15.2	26.3
E_1	*	*	*	103	87.7	*	*	*	130	106	*	*	*	26.2	20.8
DOC	102	88.0	114	112	88.2	122	77.1	147	106	112	19.5	−12.4	28.6	−6.00	27.1
DHEA	*	*	147	96.1	103	*	*	189	126	125	*	*	28.8	31.0	20.8
T	102	105	129	101	102	87.8	85.6	107	109	103	−13.8	−18.6	31.9	7.50	0.90
11OHA4	105	97.7	120	77.4	105	46.2	70.9	136	80.7	118	−55.8	−27.4	13.1	4.30	12.3
17OHP5	*	*	*	90.3	96.2	*	*	*	107	102	*	*	*	18.3	5.80
11-deoxycortisol	*	111	121	98.7	98.1	*	91.9	137	105	121	*	−16.8	14.0	6.40	23.6
CORT	*	*	112	103	86.7	*	*	128	106	97.3	*	*	14.4	3.10	12.2
aldosterone	*	87.8	92.7	80.2	62.0	*	57.7	119	97.7	78.7	*	−34.3	28.7	21.8	26.8
11OHT	*	101	118	102	99.4	*	101	137	105	108	*	0.0	15.8	3.20	8.90
18OHCORT	*	*	98.7	101	93.2	*	*	102	108	99.5	*	*	3.20	7.10	6.80
16OHP4	*	96.4	126	103	87.2	*	110	136	103	91.0	*	13.7	8.40	0.30	4.30
17OHP4	110	102	113	95.8	89.8	109	102	111	101	98.1	−1.0	0.0	−1.30	5.60	9.30
cortisol	106	96	46.4	52.6	75.1	104	111	43.6	56.3	71.0	−2.0	15.5	−6.00	7.20	−5.60

Table 6
Recovery, process efficiency and matrix effect in serum matrix. * = <LOQ.

Method A

Steroid	Recovery					Process Efficiency					Matrix Effect				
	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL
5αdione	*	*	107	93.7	94.0	*	*	114	117	104	*	*	6.60	24.8	10.3
A4	*	117	97.5	86.8	85.1	*	103	112	99.2	118	*	−11.9	14.7	14.3	39.1
11K5αdione	*	*	80.5	79.0	89.4	*	*	90.7	106	109	*	*	12.6	33.7	21.6
DHT	*	*	61.5	87.8	78.9	*	*	78.3	74.9	74.4	*	*	27.4	−14.6	−5.80
11KA4	*	*	99.9	81.0	66.6	*	*	104	103	81.4	*	*	4.30	27.1	22.3
11OH5αdione	*	*	*	95.6	120	*	*	*	101	84.8	*	*	*	5.40	−29.1
T	102	109	95.9	106	97.2	90.9	94.5	112	101	92.4	−10.4	−12.9	17.0	−4.50	−4.90
11KAST	*	*	*	96.7	67.2	*	*	*	157	86.1	*	*	*	63.0	28.1
11OHA4	*	128	94.3	99.0	90.8	*	112	119	104	86.2	*	−12.6	26.6	5.00	−5.10
11KDHT	*	*	141	79.3	85.7	*	*	104	70.8	83.8	*	*	−26.4	−10.7	−2.30
11KT	*	105	102	80.5	80.4	*	86.1	139	84.3	82.8	*	−18.1	36.5	4.80	2.90
cortisone	*	*	84.1	77.4	81.4	*	*	113	81.3	81.0	*	*	34.3	5.00	−0.60
11OHDHT	*	*	*	83.5	86.6	*	*	*	81.8	81.6	*	*	*	−2.00	−5.70
11OHT	*	*	85.5	79.9	83.0	*	*	114	82.9	78.8	*	*	32.7	3.80	−5.10

Method B

Steroid	Recovery					Process Efficiency					Matrix Effect				
	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	100 ng/mL
11KDHP4	*	*	91.2	111	100	*	*	77.8	101	97.0	*	*	−14.8	−8.40	−3.00
11KP4	*	113	88.4	103	108	*	81.6	78.7	89.3	98.8	*	−27.5	−10.9	−13.3	−8.60
11βOHDHP4	*	*	*	97.1	93.2	*	*	*	94.9	94.8	*	*	*	−2.30	1.70
11KPdione	*	*	118	108	90.1	*	*	114	108	96.6	*	*	−2.60	−0.60	7.20
21-deoxycortisone	*	*	91.5	103	96.2	*	*	92.2	94.9	98.7	*	*	0.80	−7.70	2.60
Alfaxalone	*	*	98.1	106	86.7	*	*	90.3	104	88.2	*	*	−7.90	−2.00	1.70
11βOHP4	*	*	91.8	103	92.6	*	*	98.5	98.2	94.1	*	*	7.30	−4.70	1.70
11αOHDHP4	*	*	100	119	104	*	*	92.3	101	97.4	*	*	−7.70	−15.3	−5.90
11OHPdione	*	*	*	107	80.5	*	*	*	90.8	88.6	*	*	*	−14.8	10.1
11αOHP4	*	*	89.4	107	93.6	*	*	85.3	99.5	95.6	*	*	−4.60	−7.00	2.20
3,11diOHDP4	*	*	*	98.4	92.9	*	*	*	100	98.2	*	*	*	1.90	5.70
21-deoxycortisol	*	*	87.1	114	104	*	*	104	102	102	*	*	19.6	−11.0	−2.30
11KPdiol	*	*	*	98.2	102	*	*	*	92.0	92.3	*	*	*	−6.30	−9.30
11OHPdiol	*	*	*	118	107	*	*	*	123	110	*	*	*	4.20	2.80

Method C

Steroid	Recovery					Process Efficiency					Matrix Effect				
	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	150 ng/mL	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	150 ng/mL	0.05 ng/mL	0.25 ng/mL	5 ng/mL	50 ng/mL	150 ng/mL
P4	114	157	99.6	91.4	112	99.1	151	51.1	82.7	101	−12.8	−4.7	−48.7	−9.47	−9.91
A4	112	121	140	116	119	102	120	52.8	96.7	109	−9.0	−1.0	−45.9	−16.8	−8.15
P5	*	*	107	88.0	80.2	*	*	57.1	99.9	77.5	*	*	−46.3	13.6	−3.45
E_1	*	*	*	90.5	71.9	*	*	*	78.6	67.0	*	*	*	−13.1	−6.78
DOC	107	130	147	113	123	121	126	80.1	93.6	107	13.5	−4.0	−45.3	−16.8	−13.4
DHEA	*	*	*	103	90.7	*	*	*	96.7	89.5	*	*	*	−5.64	−1.32
T	92.1	109	131	131	124	82.6	107	59.8	109	113	−10.3	−2.0	−89.6	−16.5	−9.39
11OHA4	117	83.1	135	120	98.6	46.8	68.8	62.2	93.5	87.2	−60.1	−17.2	−54.1	−21.8	−11.5
17OHP5	*	*	*	84.5	78.1	*	*	*	84.8	73.4	*	*	*	0.37	−6.09
11-deoxycortisol	*	115	166	133	130	*	116	90.5	104	109	*	1.0	−45.4	–22.1	−16.2
CORT	*	*	157	140	118	*	*	95.3	101	97.0	*	*	−39.6	−27.7	−17.6
aldosterone	*	84.2	96.0	80.0	67.6	*	84.5	68.9	61.0	53.8	*	0.3	−28.3	–23.8	−20.5
11OHT	*	51.7	116	112	94.1	*	84.0	66.8	87.7	83.8	*	32.3	−42.3	–22.0	−11.0
18OHCORT	*	*	123	93.0	81.8	*	*	87.1	72.1	69.1	*	*	−28.9	–22.4	−15.5
16OHP4	*	116	170	104	113	*	123	69.1	81.9	94.0	*	6.0	−59.4	−21.5	−16.9
17OHP4	110	117	135	113	105	109	118	74.1	92.4	91.0	−1.0	1.0	−45.3	−18.4	−13.4
cortisol	128	83.0	83.8	66.0	57.8	103	84.1	54.6	48.8	43.5	−19.6	1.3	−34.8	−26.1	−24.6

Table 7
Hormones detected in matched serum (ng steroid/mL) and faecal (ng steroid/g faecal dry weight) southern white rhinoceros samples by UPC^2-MS/MS analysis. The age and sex (male, ♂; female, ♀) of the animal is also indicated.

Steroid	PND 18, ♂		SIP 5, ♂		KON 5, ♀		KTH 4, ♀	
	Serum (ng/mL)	Faeces (ng/g)	Serum (ng/mL)	Faeces (ng/g)	Serum (ng/mL)	Faeces (ng/g)	Serum (ng/mL)	Faeces (ng/g)
P4	–	80.6	–	90.4	–	–	–	–
11αOHDHP4	–	–	–	–	–	168	–	–
16OHP4	–	–	–	36.1	–	–	–	–
DOC	–	14.6	–	7.58	–	–	–	–
CORT	1.41	–	–	–	–	–	–	–
18OHCORT	14.6	–	–	–	–	–		–
aldosterone	7.62	–	–	–	–	–	0.54	–
11-deoxycortisol	1.29	45.1	–	67.3	–	53.7	–	27.1
cortisol	20.5	8.45	–	–	5.84	–	9.62	–
cortisone	1.86	–	–	–	–	–	–	–
A4	0.08	38.6	–	25.6	0.01	–	0.02	–
11OHA4	0.35	–	1.42	–	1.01	–	2.34	–
11KA4	–	3.37	–	3.32	–	9.88	–	19.6
T	0.41	9.17	–	–	–	–	–	–
11OHT	1.62	–	–	–	0.46	–	0.18	–
11KT	–	–	1.06	–	–	–	–	–

Table 8
Steroids detected in the faecal matrix sample of an unknown southern white rhinoceros prior to charcoal stripping.

Steroid	Concentration (ng steroid/g faecal dry weight)
P4	4.862
11-deoxycortisol	24.20
11KT	1.836
21-deoxycortisone	40.73
11βOHP4	210.2
11αOHP4	125.9

v) co-solvent were utilised for each of three methods and achieved excellent resolution without the need for derivatisation. Protocols were set up to separate either androgens, progestogens or adrenal steroids in each of the methods, which allowed for mass selectivity enabled by the tandem quadrupole mass spectrometer. A constant flow rate of 0.2 mL/min was maintained with an optimal back pressure of 1800 psi and a column temperature of 60 °C.

Baseline separation was achieved for all of the steroids which were identified by comparison of peak retention times with corresponding reference steroid standards. Steroids were distinguished from each other using their distinct MRM transitions which were meticulously chosen taking into account steroid cross-talk and co-elution of steroids. Co-elution of steroids in method B (11βOHP4 and alfaxalone, R_t 2.3; 21-deoxycortisol and 11KPdiol, R_t 2.99 and 3.00); and in method C (DOC and E1, R_t 1.60 and 1.61; 11-deoxycortisol and 16OHP4, R_t 2.16; 11OHT and cortisol, R_t 2.35 and 2.36) necessitated the selection of fragment ions unique to said steroids. Linearity was calculated over a concentration range spanning 0.01 to 350 ng/mL and subsequent linear regression analysis yielded r^2 greater than 0.975. The majority of the points in the linear regression analysis incorporated those at the lower end of the range (≤10 ng/mL) thus allowing greater weighting when fitting a regression curve and ensuring best fit. The r^2 value of all the steroids detected in serum and faecal concentrations ranged between 0.991 and 0.998 with the exception of faecal 11αOHDHP4 ($r^2 = 0.986$). The established LODs ranged from 0.01 to 25 ng/mL and LOQs were 0.01–50 ng/mL with a S/N ratio of 3 and 10, respectively. In a number of the serum samples four of the steroids, A4, 5αdione, 18OHCORT and 21-deoxycortisol, were detected below the LOQ and, while all other steroid concentrations in serum and faeces were well within their LOQs, 11βOHP4 was detected in the faecal matrix extraction was above the upper limit of the LOQ.

A number of regulatory documents, generally targeting pharmaceuticals regarding acceptance of method validation criteria exists. Nevertheless, the information from each of these sources are conflicting and lead to confusion when determining the appropriate criteria to which validation results are compared. We have therefore selected criteria, %RSD < 20 %, relevant to the field of study as well as those which are relevant to exploratory analysis over the constraints of pharmaceutical methods [31,39–43]. The methods presented in this study were validated as per the parameters of accuracy and precision, achieving a %RSD below 20 % for all metabolites in both faecal and serum matrices. Additional parameters such as matrix effect, recovery and process efficiency were determined thereby confirming the reliability and reproducibility of the methods.

This is the first time, to the best of our knowledge, that a comprehensive panel of glucocorticoids, progesterone and androgen sex steroids, which included the C11-oxy steroids and their metabolites has been investigated in southern white rhinoceros. 11OHT and 11OHA4, which is exclusively produced by the adrenal, were detected in male and female serum while 11KA4 was detected at noticeably higher concentrations than its 11OHA4 precursor in faecal samples. It is likely that the conversion of 11OHA4 to 11KA4 by 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) took place prior to defaecation due to the absence of 11OHA4 in faecal matter, ruling out faecal bacterial conversion. A4, which is produced by both gonads and the adrenal, was the predominant androgen detected in male faecal samples. T was only detected in serum and faecal samples of the older male. It can be presumed that age would influence sex steroid levels and our data corroborate this with higher A4 levels detected in the older male than in the younger male. A correlation between circulating T and fAMs have been reported in male white rhinoceros together with an increase in androgen metabolites at sexual maturity [44]. Steroids extracted from the matrix test sample, which was that of a sexually mature southern white rhinoceros, yielded a different profile. 11KT, representing sex steroids, was the only androgen detected in the matrix sample and was absent from all other samples, suggesting potential faecal bacterial conversion of 11KA4. Faecal progesterone metabolites detected were *P*4, 16OHP4 and 11αOHDHP4 at high concentrations. Interestingly, while low levels of *P*4 were detected in the matrix test sample, both 11βOHP4 and 11αOHP4 were the major steroids detected and present at high concentrations. These C11-hydroxy steroids are products of cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1), cytochrome P450 11β-hydroxylase (CYP11B) and hydroxysteroid dehydrogenase catalysed conversions. Oxidative, reductive and hydrolytic modifications of steroids in faeces have been reported [45,46] and, as such, conversions post-defaecation by faecal bacteria cannot be ruled out as the matrix sample had been exposed to

the environment for an unknown amount of time. Outdoor storage of faecal matter of black rhinoceros has been shown to result in increased progestogen concentrations. Assays were, however, conducted using a broad-spectrum progestogen antibody measuring fPMs [47] which would no doubt have cross reacted with C11-oxy C_{21} steroid metabolites. Faecal bacterial enzymes in southern white rhinoceros faeces and other wildlife species may catalyse the catabolism of faecal steroids and coupled with environmental bacteria can confound faecal steroid analysis. Furthermore, environmental bacteria may vary between regions suggesting that the catabolism of faecal steroids may differ between diverse environments [48] such as between the Eastern and Western Cape provinces. Our data nevertheless illustrate the complexity of steroid analyses of faecal samples and highlight the importance of basic research into the steroidogenic enzymes expressed in this species as well as the appropriate methods for field collection and storage of samples. Our study furthermore underscores the importance of faecal steroid analysis as a means to delineate the potential steroidogenic pathways which contribute to the pool of sex steroids. Although comparisons between the steroid hormones detected in male and female rhinoceros cannot be made due to the limited number of samples, it is clear that there are not only differences in individual profiles of steroid hormones detected, both in serum and faeces, but also the concentrations at which the steroids are present.

Non-invasive monitoring of reproductive function in females is mainly conducted by assessment of fPMs in estrous cycles and in pregnancy in Rhinocerotidae and other extant families belonging to the Perissodactyla order. IA have shown that progestogen profiles reflect reproductive activity when measuring various 20α-progestogens [6,47,49–54]. HPLC analyses of progesterone metabolism in the faeces of white rhinoceros has identified *P*4, albeit negligible, together with 4-pregnen-20α-ol-3-one constituting 90 % of the progesterone metabolites in this species [19]. Faecal 20α-hydroxypregnanes and 20-ketopregnanes have been detected using HPLC and are suggested to be reliable indicators of luteal function and pregnancy in rhinoceros [20]. Further insight has been gained into reproductive cycles in rhinoceros when employing a combination of IA, HPLC and GC–MS. Analyses of faecal progesterone metabolites using antibodies raised against 5α-pregnane-3β-ol-20-one and 5β-pregnane-3α-ol-20-one as well as HPLC facilitated the characterisation of estrous cycles with the identification of three 5α-reduced 20-oxopregnanes [51]. It was subsequently shown that the principal faecal *P*4 metabolite in the last two months of pregnancy was 5α-pregnan-3β,20α-diol, also a 5α-reduced steroid metabolite. While *P*4 was also not detected, a further 32 progesterone metabolites were identified by GC–MS, showing the majority of these to be present as 5α-reduced metabolites (81 %). The study furthermore showed that an antibody raised against a BSA-coupled 11βOHP4 antigen detected<15 % of the total faecal progesterone metabolites in rhinoceros [50]. Although we did not detect *P*4 in female samples, detection of 11αOHDHP4 indicates the 5α-reduction of 11αOHP4. Female rhinoceros first give birth between the ages of 6 and 8 [20,55] and the high 11αOHDHP4 levels may suggest that the 5 year old female is closer to sexual maturity. The pivotal role of 5α-reductase in the inactivation of *P*4 comes to the fore in female reproductive physiology in the context of aberrant gestation and parturition [56] with subsequent formation of downstream 5α-reduced metabolites. The formation of reduced *P*4 metabolites was recently highlighted in an investigation, by Conley *et* al., into the steroid metabolome of pregnancy in Perissodactyla, Cetartiodactyla and Carnivora species. LC-MS/MS analyses of circulating steroids at mid to late gestation showed 5α-reduced progestogens, dihydroprogesterone (DHP4), allopregnanolone, 20α- and 3β,20αOHDHP4 to be present in white and black rhinoceros [57]. It is likely that optimized analytical approaches to the improvement of assisted reproduction strategies in these species will require further investigations into the *P*4 metabolites incorporating technical strategies such as LC-MS or GC–MS with the former being more accessible.

A recent study reported that ovarian activity may be influenced by adrenal activity in eastern black rhinoceros (*Diceros bicornis michaeli*), a critically endangered species. IA of fGMs and fPMs showed that glucocorticoids were elevated during periods of irregular estrous cyclicity [7]. It is likely that elevated glucocorticoids (associated with abnormal adrenal steroidogenesis) coupled with increased adrenal androgens may contribute to the reproductive irregularities in these female black rhinoceros. The impact of stress on wildlife conservation and reproductive health is another topic of active investigation. Stress-linked translocation studies undertaken, in which CORT and sex steroid concentrations were measured in white rhinoceros faecal samples using RIA, reported supressed androgen levels associated with decreased glucocorticoids in males and, in females supressed progesterones and increased glucocorticoids. In female black rhinoceros, however, progesterones and glucocorticoid levels were decreased. It was concluded that the decline in glucocorticoids, in conjunction with supressed sex steroids, indicate that translocation resulted in distress and not stress [58].

Since the southern white rhinoceros samples available for this study were limited conclusions cannot be drawn regarding age and sex differences. However, our findings do show distinctive differences between in steroid profiles warranting further investigation. Our study demonstrates the specificity of UPC^2-MS/MS analysis for both glucocorticoids and sex steroids. Taken together, this data suggest that faecal 11-deoxycortisol, rather than faecal cortisol and CORT, should be an accurate indicator of adrenal cortical function and should rather be considered in physiologic stress/distress. The cortisol precursor, 11-deoxycortisol, was the predominant glucocorticoid detected as well as the most abundant steroid in all faecal samples. In addition, 11-deoxycortisol was the only glucocorticoid detected in the matrix test sample. It may be prudent when investigating stress and distress, to assess circulating cortisol and cortisone together with faecal 11-deoxycortisol levels.

5. Conclusion

Comprehensive steroid profiling and the accurate identification and quantification of glucocorticoids and sex steroids are essential to investigations into both physiological stress responses and the reproductive status in wildlife species. Studies to date reporting on gonadal and adrenal steroids highlight the importance of combined assessments of adrenal glucocorticoids and sex steroids when investigating reproductive health or when characterising stress. This cannot be determined by analysing glucocorticoids or sex steroids as single entities. Reports underpin the need for more accurate and comprehensive steroid hormone analyses in the management of both captive and free-ranging wildlife species. Techniques involving the use of antibodies fall short due to steroid cross reactivities which can only be circumvented by mass spectrophotometry. The C11-oxy metabolites detected in this study have not, to date, been measured in southern white rhinoceros or other wildlife mammalian species. The biological significance of C11-oxy C_{19} and C11-oxy C_{21} steroids in southern white rhinoceros or other species remains unknown. Furthermore, all pre-receptor C11-oxy C_{19} steroids were detected in either male or female rhinoceros and specifically in either serum or in faeces. Our findings suggest scant correlation between circulating steroids and faecal steroids and therefore, for faecal steroid analysis to contribute to the assessment of endocrinological status, all downstream steroid metabolites will need to be analysed to include comprehensive panels of pre- and post-receptor steroids. It may be possible that non-invasive measurement of steroid hormones could aid in the successful management of wildlife, however, it will require state-of-the-art analytical techniques.

CRediT authorship contribution statement

Rachelle Gent: Conceptualization, Data curation, Formal analysis, Methodology, Validation, Writing – original draft, Writing – review & editing. **Inge D. Barbier:** Conceptualization, Data curation, Formal

analysis, Methodology, Validation, Writing – original draft, Writing – review & editing. **Stephen L. Atkin:** Writing – review & editing. **Annie E. Newell-Fugate:** Conceptualization, Writing – review & editing. **Amanda C. Swart:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to thank Lisa Graham and the field monitors at Amakhala Game Reserve (South Africa) for the collection of faecal and serum samples and Brendan S. Wilhelmi (Rhodes University, South Africa) for sample storage. We would also like to thank Paul Loubscher from Buffelsfontein Game Reserve (South Africa) and Bodo Gent for the collection of faecal matrix for the purpose of method development and validation. Project supported by the National Research Foundation (IFR170125217588, CSUR160414162143, SFP180419322791) and Stellenbosch University

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2022.123576.

References

[1] B. Dantzer, Q.E. Fletcher, R. Boonstra, M.J. Sheriff, Measures of physiological stress: a transparent or opaque window into the status, management and conservation of species?, Conserv. Physiol. 2 (2014) 1-18 PMID: 27293644; PMCID: PMC4732472, https://doi.org/10.1093/conphys/cou023.

[2] D.C. Kersey, M. Dehnhard, The use of noninvasive and minimally invasive methods in endocrinology for threatened mammalian species conservation, Gen. Comp. Endocrinol. 203 (2014) 296–306, https://doi.org/10.1016/j.ygcen.2014.04.022.

[3] F. Schwarzenberger, The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species, Int. Zoo. Yearbook. 41 (2007) 52–74, https://doi.org/10.1111/j.1748-1090.2007.00017.x.

[4] L. Koren, H. Bryan, D. Matas, S. Tinman, Å. Fahlman, D. Whiteside, J. Smits, K. Wynne-Edwards, Towards the validation of endogenous steroid testing in wildlife hair, J. Appl. Ecol. 56 (2019) 547–561, https://doi.org/10.1111/1365-2664.13306.

[5] B. Pukazhenthi, V. Quse, M. Hoyer, H. van E. Gastelaars, O. Sanjur, J.L. Brown, A review of the reproductive biology and breeding management of tapirs, Integr Zool. 8 (2013) 18–34. https://doi.org/10.1111/J.1749-4877.2012.12008.X.

[6] L. Graham, F. Schwarzenberger, E. Möstl, W. Galama, A. Savage, A versatile enzyme immunoassay for the determination of progestogens in feces and serum, Zoo. Biol. 20 (2001) 227–236, https://doi.org/10.1002/ZOO.1022.

[7] K.L. Edwards, M. Pilgrim, J.L. Brown, S.L. Walker, Irregular ovarian cyclicity is associated with adrenal activity in female eastern black rhinoceros (Diceros bicornis michaeli), Gen. Comp. Endocrinol. 289 (2020), 113376, https://doi.org/10.1016/J.YGCEN.2019.113376.

[8] M. Badenhorst, M. Otto, A.C.V. der Goot, A. Ganswindt, Stress steroid levels and the short-term impact of routine dehorning in female southern white rhinoceroses (Ceratotherium simum simum), Afr. Zool. 51 (2016) 211–215, https://doi.org/10.1080/15627020.2016.1261002.

[9] K. Yarnell, S.L. Walker, Environmental impact on faecal glucocorticoid metabolite concentrations in Grevy's zebra (Equus grevyi), Afr. J. Wildl. Res. 47 (2017) 138–143, https://doi.org/10.3957/056.047.0138.

[10] P.A. Seeber, B. Quintard, F. Sicks, M. Dehnhard, A.D. Greenwood, M. Franz, Environmental stressors may cause equine herpesvirus reactivation in captive Grévy's zebras (Equus grevyi), PeerJ6:e5422(2018). https://doi.org/10.7717/peerj.5422.

[11] S.G. Penny, R.L. White, L. MacTavish, D.M. Scott, A.P. Pernetta, Negligible hormonal response following dehorning in free-ranging white rhinoceros (Ceratotherium simum), Conserv. Physiol. 8 (2020) 1–12. https://doi.org/10.1093/conphys/coaa117.

[12] R. Watson, C. Munro, K.L. Edwards, V. Norton, J.L. Brown, S.L. Walker, Development of a versatile enzyme immunoassay for non-invasive assessment of glucocorticoid metabolites in a diversity of taxonomic species, Gen. Comp. Endocrinol. 186 (2013) 16–24, https://doi.org/10.1016/j.ygcen.2013.02.001.

[13] N.J. Cook, Review: Minimally invasive sampling media and the measurement of corticosteroids as biomarkers of stress in animals, Can. J. Anim. Sci. 92 (2012) 227–259, https://doi.org/10.4141/CJAS2012-045.

[14] D.J. Handelsman, Mass spectrometry, immunoassay and valid steroid measurements in reproductive medicine and science, Hum. Reprod. 32 (2017) 1147–1150, https://doi.org/10.1093/HUMREP/DEX078.

[15] J. Abdel-Khalik, E. Björklund, M. Hansen, Simultaneous determination of endogenous steroid hormones in human and animal plasma and serum by liquid or gas chromatography coupled to tandem mass spectrometry, J. Chromatogr. B. Analyt. Technol. Biomed. Life. Sci. 928 (2013) 58–77, https://doi.org/10.1016/j.jchromb.2013.03.013.

[16] L. Denoroy, L. Zimmer, B. Renaud, S. Parrot, Ultra high performance liquid chromatography as a tool for the discovery and the analysis of biomarkers of diseases: A review, J. Chromatogr. B. Analyt. Technol. Biomed. Life. Sci. 927 (2013) 37–53, https://doi.org/10.1016/j.jchromb.2012.12.005.

[17] K. Jewgenow, A. Azevedo, M. Albrecht, C. Kirschbaum, M. Dehnhard, Hair cortisol analyses in different mammal species: Choosing the wrong assay may lead to erroneous results, Conserv. Physiol. 8 (2020) 1–17. https://doi.org/10.1093/conphys/coaa009.

[18] P.J. Monaghan, B.G. Keevil, P.M. Stewart, P.J. Trainer, Case for the wider adoption of mass spectrometry based adrenal steroid testing, and beyond, J. Clin. Endocrinol. Metab. 99 (2014) 4434–4437, https://doi.org/10.1210/JC.2014-2258.

[19] J.E. Hindle, J.K. Hodges, Metabolism of oestradiol-17β and progesterone in the white rhinoceros (Ceratotherium simum simum), Journal. of. Reprduction. and. Fertility. 90 (1990) 571–580. https://doi.org/10.1530/jrf.0.0900571.

[20] F. Schwarzenberger, W. Rietschel, J. Vahala, D. Holeckova, P. Thomas, J. Maltzan, K. Baumgartner, W. Schaftenaar, Fecal progesterone, estrogen, and androgen metabolites for noninvasive monitoring of reproductive function in the female Indian rhinoceros, Rhinoceros unicornis, Gen. Comp. Endocrinol. 119 (2000) 300–307, https://doi.org/10.1006/GCEN.2000.7523.

[21] J.W. Turner, P. Tolson, N. Hamad, Remote assessment of stress in white rhinoceros (Ceratotherium simum) and black rhinoceros (Diceros bicornis) by measurement of adrenal steroids in feces, J. Zoo. Wildl. Med. 33 (2002) 214–221, https://doi.org/10.1638/1042-7260(2002)033[0214:RAOSIW]2.0.CO;2.

[22] L. Koren, D. Whiteside, Å. Fahlman, K. Ruckstuhl, S. Kutz, S. Checkley, M. Dumond, K. Wynne-Edwards, Cortisol and corticosterone independence in cortisol-dominant wildlife, Gen. Comp. Endocrinol. 177 (2012) 113–119, https://doi.org/10.1016/j.ygcen.2012.02.020.

[23] L. Koren, E.S.M. Ng, K.K. Soma, K.E. Wynne-Edwards, Sample preparation and liquid chromatography-tandem mass spectrometry for multiple steroids in mammalian and avian circulation, PLoS. One 7 (2) (2012), e32496. https://doi.org/10.1371/journal.pone.0032496.

[24] T.M. Galligan, L.H. Schwacke, D.S. Houser, R.S. Wells, T. Rowles, A.S.P. Boggs, Characterization of circulating steroid hormone profiles in the bottlenose dolphin (Tursiops truncatus) by liquid chromatography–tandem mass spectrometry (LC–MS/MS), Gen. Comp. Endocrinol. 263 (2018) 80–91, https://doi.org/10.1016/j.ygcen.2018.04.003.

[25] N. Lübcker, L.M. Bloem, T. du Toit, P. Swart, P.J.N. de Bruyn, A.C. Swart, R. P. Millar, What's in a whisker? High-throughput analysis of twenty-eight C19 and C21 steroids in mammalian whiskers by ultra-performance convergence chromatography-tandem mass spectrometry, J. Chromatogr. B. Analyt. Technol. Biomed. Life. Sci. 1141 (2020), https://doi.org/10.1016/j.jchromb.2020.122028.

[26] J. Rege, S. Garber, A.J. Conley, R.M. Elsey, A.F. Turcu, R.J. Auchus, W.E. Rainey, Circulating 11-oxygenated androgens across species, J. Steroid. Biochem. Mol. Biol. 190 (2019) 242–249, https://doi.org/10.1016/j.jsbmb.2019.04.005.

[27] L. Barnard, T. du Toit, A.C. Swart, Back where it belongs: 11β-hydroxyandrostenedione compels the re-assessment of C11-oxy androgens in steroidogenesis, Mol. Cell. Endocrinol. 525 (2021), https://doi.org/10.1016/j.mce.2021.111189.

[28] S.A. Wudy, G. Schuler, A. Sánchez-Guijo, M.F. Hartmann, The art of measuring steroids: Principles and practice of current hormonal steroid analysis, J. Steroid. Biochem. Mol. Biol. 179 (2018) 88–103, https://doi.org/10.1016/j.jsbmb.2017.09.003.

[29] A.E. Taylor, B. Keevil, I.T. Huhtaniemi, Mass spectrometry and immunoassay: How to measure steroid hormones today and tomorrow, Eur. J. Endocrinol. 173 (2015) D1–D12, https://doi.org/10.1530/EJE-15-0338.

[30] J.M.W. van den Ouweland, I.P. Kema, The role of liquid chromatography-tandem mass spectrometry in the clinical laboratory, J. Chromatogr. B. Analyt. Technol. Biomed. Life. Sci. 883–884 (2012) 18–32, https://doi.org/10.1016/j.jchromb.2011.11.044.

[31] J.L. Quanson, M.A. Stander, E. Pretorius, C. Jenkinson, A.E. Taylor, K.-H. Storbeck, High-throughput analysis of 19 endogenous androgenic steroids by ultra-performance convergence chromatography tandem mass spectrometry, J. Chromatogr. B 1031 (2016) 131–138, https://doi.org/10.1016/j.jchromb.2016.07.024.

[32] T. du Toit, M.A. Stander, A.C. Swart, A high-throughput UPC^2-MS/MS method for the separation and quantification of C19and C21 steroids and their C11-oxy steroid metabolites in the classical, alternative, backdoor and 11OHA4 steroid pathways, J. Chromatogr. B. Analyt. Technol. Biomed. Life. Sci. 1080 (2018) 71–81. https://doi.org/10.1016/j.jchromb.2018.02.023.

[33] T. du Toit, D. van Rooyen, M.A. Stander, S.L. Atkin, A.C. Swart, Analysis of 52 C19 and C21 steroids by UPC^2-MS/MS: Characterising the C11-oxy steroid metabolome in serum, J. Chromatogr. B. Analyt. Technol. Biomed. Life. Sci. 1152 (2020) 122243. https://doi.org/10.1016/j.jchromb.2020.122243.
[34] K.L. Edwards, S.L. Walker, R.F. Bodenham, H. Ritchie, S. Shultz, Associations between social behaviour and adrenal activity in female Barbary macaques: Consequences of study design, Gen. Comp. Endocrinol. 186 (2013) 72–79, https://doi.org/10.1016/j.ygcen.2013.02.023.
[35] F. Hosseinkhani, A.C. Dubbelman, N. Karu, A.C. Harms, T. Hankemeier, Towards standards for human fecal sample preparation in targeted and untargeted lc-hrms studies, Metabolites 11 (2021) 364, https://doi.org/10.3390/METABO11060364/S1.
[36] N. de Kock, S.R. Acharya, S.J. Kumari, A. Ubhayasekera, J. Bergquist, A novel targeted analysis of peripheral steroids by ultra-performance supercritical fluid chromatography hyphenated to tandem mass spectrometry, Sci. Rep. 8 (2018) 16993, https://doi.org/10.1038/s41598-018-35007-0.
[37] P.J. Taylor, Matrix effects: The Achilles heel of quantitative high-performance liquid chromatography-electrospray-tandem mass spectrometry, Clin. Biochem. 38 (2005) 328–334, https://doi.org/10.1016/j.clinbiochem.2004.11.007.
[38] A. Kruve, R. Rebane, K. Kipper, M.L. Oldekop, H. Evard, K. Herodes, P. Ravio, I. Leito, Tutorial review on validation of liquid chromatography-mass spectrometry methods: Part II, Anal. Chim. Acta. 870 (2015) 8–28, https://doi.org/10.1016/j.aca.2015.02.016.
[39] 2002/657/EC: Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (Text with EEA relevance) (notified under document number C(2002) 3044) - Publications Office of the EU, (n.d.). OJ L 221 17.08.2002, p. 8, ELI. https://op.europa.eu/en/publication-detail/-/publication/ed928116-a955-4a84-b10a-cf7a82bad858/language-en (accessed June 19, 2022).
[40] S. Laforest, M. Pelletier, N. Denver, B. Poirier, S. Nguyen, B.R. Walker, F. Durocher, N.Z.M. Homer, C. Diorio, A. Tchernof, R. Andrew, Simultaneous quantification of estrogens and glucocorticoids in human adipose tissue by liquid-chromatography-tandem mass spectrometry, J. Steroid. Biochem. Mol. Biol. 195 (2019), 105476, https://doi.org/10.1016/J.JSBMB.2019.105476.
[41] M.V. Salvia, E. Vulliet, L. Wiest, R. Baudot, C. Cren-Olivé, Development of a multi-residue method using acetonitrile-based extraction followed by liquid chromatography–tandem mass spectrometry for the analysis of steroids and veterinary and human drugs at trace levels in soil, J. Chromatogr. A. 1245 (2012) 122–133, https://doi.org/10.1016/J.CHROMA.2012.05.034.
[42] T. Andrieu, T. du Toit, B. Vogt, M.D. Mueller, M. Groessl, Parallel targeted and non-targeted quantitative analysis of steroids in human serum and peritoneal fluid by liquid chromatography high-resolution mass spectrometry, Anal. Bioanal. Chem. 414 (2022) 7461–7472. https://doi.org/10.1007/s00216-022-03881-3.
[43] A. Dispas, P. Lebrun, P. Hubert. Chapter 11 - Validation of supercritical fluid chromatography methods, in: Supercritical fluid chromatography, Elsevier, 2017, pp. 317–344, https://doi.org/10.1016/B978-0-12-809207-1.00011-2.
[44] P. Kretzschmar, U. Ganslosser, M. Dehnhard, Relationship between androgens, environmental factors and reproductive behavior in male white rhinoceros (Ceratotherium simum simum), Horm. Behav. 45 (2004) 1–9, https://doi.org/10.1016/j.yhbeh.2003.08.001.
[45] E. García-Gómez, B. González-Pedrajo, I. Camacho-Arroyo, Role of sex steroid hormones in bacterial-host interactions, Biomed. Res. Int. 2013 (2013) 1, Article ID 928290. doi:10 10.1155/2013/928290.
[46] P. Lombardi, B. Goldin, E. Boutin, S.L. Gorbach, Metabolism of androgens and estrogens by human fecal microorganisms, J. Steroid. Biochem. 9 (1978) 795–801, https://doi.org/10.1016/0022-4731(78)90203-0.
[47] W.T. Galama, L.H. Graham, A. Savage, Comparison of fecal storage methods for steroid analysis in black rhinoceroses (Diceros bicornis), Zoo. Biol. 23 (2004) 291–300, https://doi.org/10.1002/zoo.20017.
[48] Z. Pasternak, A. Al-Ashhab, J. Gatica, R. Gafny, S. Avraham, D. Minz, O. Gillor, E. Jurkevitch, Spatial and temporal biogeography of soil microbial communities in arid and semiarid regions, PLoS. One 8 (2013) e69705.
[49] J.N. Garnier, D.I. Green, A.R. Pickard, H.J. Shaw, W. v. Holt, Non-invasive diagnosis of pregnancy in wild black rhinoceros (Diceros bicornis minor) by faecal steroid analysis, Reprod Fertil Dev. 10 (1998) 451–458. https://doi.org/10.1071/RD98118.
[50] V.A. Lance, M.L. Patton, L.R. Hagey, Identification of a series of $C_{21}O_2$ pregnanes from fecal extracts of a pregnant black rhinoceros (Diceros bicornis minor), Steroids 66 (2001) 875–881. https://doi.org/10.1016/S0039-128X(01)00126-X.
[51] F. Schwarzenberger, C. Walzer, K. Tomasova, J. Vahala, J. Meister, K.L. Goodrowe, J. Zima, G. Strauß, M. Lynch, Faecal progesterone metabolite analysis for non-invasive monitoring of reproductive function in the white rhinoceros (Ceratotherium simum), Anim. Reprod. Sci. 53 (1998) 173–190, https://doi.org/10.1016/S0378-4320(98)00112-2.
[52] F. Schwarzenberger, K. Tomášová, D. Holečková, B. Matern, E. Möstl, Measurement of fecal steroids in the black rhinoceros (Diceros bicornis) using group-specific enzyme immunoassays for 20-oxo-pregnanes, Zoo. Biol. 15 (1996) 159–171, https://doi.org/10.1002/(SICI)1098-2361(1996)15:2<159::AID-ZOO6>3.0.CO;2-A.
[53] J.L. Brown, S.B. Citino, J. Shaw, C. Miller, Endocrine profiles during the estrous cycle and pregnancy in the Baird's tapir (Tapirus bairdii), Zoo. Biol. 13 (1994) 107–117, https://doi.org/10.1002/zoo.1430130203.
[54] C.S. Asa, J.E. Bauman, E.W. Houston, M.T. Fischer, B. Read, C.M. Brownfield, J. F. Roser, Patterns of excretion of fecal estradiol and progesterone and urinary chorionic gonadotropin in Grevy's zebras (Equus grevyi): Ovulatory cycles and pregnancy, Zoo, Biol 20 (2001) 185–195. https://doi.org/10.1002/zoo.1019.
[55] A.K.K. Hillman-Smith, N. Owen-Smith, J.L. Anderson, A.J. Hall-Martin, J. P. Selaladi, Age estimation of the White rhinoceros (Ceratotherium simum), J. Zool. 210 (1986) 355–377, https://doi.org/10.1111/J.1469-7998.1986.TB03639.X.
[56] M.S. Mahendroo, D.W. Russel, Male and female isoenzymes of steroid 5α-reductase, Rev. Reprod. 4 (1999) 179–183. https://doi.org/10.1530/ror.0.0040179.
[57] A.J. Conley, S.C. Loux, E.L. Legacki, M.A. Stoops, B. Pukazhenthi, J.L. Brown, R. Sattler, H.M. French, A. Tibary, T.R. Robeck, The steroid metabolome of pregnancy, insights into the maintenance of pregnancy and evolution of reproductive traits, Mol. Cell. Endocrinol. 528 (2021) 111241, https://doi.org/10.1016/j.mce.2021.111241.
[58] W.L. Linklater, E.A. MacDonald, J.R.B. Flamand, N.M. Czekala, Declining and low fecal corticoids are associated with distress, not acclimation to stress, during the translocation of African rhinoceros, Anim. Conserv. 13 (2010) 104–111, https://doi.org/10.1111/j.1469-1795.2009.00308.x.