Physiology & Behavior 105 (2012) 510–521

Contents lists available at SciVerse ScienceDirect

Physiology & Behavior

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

Simultaneous measurement of endogenous steroid hormones and their metabolites with LC–MS/MS in faeces of a New World primate species, *Cebus capucinus*

Anja Weltring [a,*], Franka S. Schaebs [a], Susan E. Perry [b], Tobias Deschner [a]

[a] Max Planck Institute for Evolutionary Anthropology, Department of Primatology, Deutscher Platz 6, 04103 Leipzig, Germany
[b] University of California, Los Angeles, Department of Anthropology, 375 Portola Plaza, Los Angeles, CA 90095-1553, USA

ARTICLE INFO

Article history:
Received 28 April 2011
Received in revised form 16 August 2011
Accepted 6 September 2011
Available online 14 September 2011

Keywords:
LC–MS/MS
Steroids
Feces
New World primates
White-faced capuchins
Validation

ABSTRACT

We developed and validated a method to measure steroid hormones with liquid chromatography–tandem mass spectrometry (LC–MS/MS) in faecal samples of white-faced capuchins. The method includes the measurement of adrenal and gonadal hormones such as cortisol, testosterone, estrone, progesterone and a number of their faecal metabolites. This method can be used for simultaneous routine measurements of steroids in faecal samples and provides a reference method for the validation of new immunoassays in this matrix. The optimised method consists of an extraction of the dried faecal samples with 80% methanol followed by purification of the extracts by solid phase extraction, solvolytic cleavage of conjugates and liquid–liquid extraction. Extracts were measured by LC–MS/MS with an electrospray interface in positive ionisation mode. Out of 19 steroids spiked into methanol extracts, 14 showed a recovery of 79.8–118.5% with an intra-day precision of 2.5–13.0% and an inter-day precision of 7.2–15.1%. Detection limits for these steroids ranged from 0.3 to 27.0 ng/mL of extract. Five steroids did not fulfil our requirements concerning precision and accuracy and we therefore considered these to not be reliably measurable with this method. While there was no indication of considerable amounts of conjugated forms for most metabolites, 87% of the testosterone was found in the solvolysis fraction, which indicates that the majority of testosterone was conjugated. Therefore, solvolysis turned out to be crucial, especially for the quantification of the total amount of testosterone. The physiological validation of this LC–MS/MS method confirmed known physiologically caused differences in faecal steroid concentrations. This indicates the usefulness of the method in investigating variation in the levels of major steroid hormones in faeces of white-faced capuchins. The possibility to simultaneously measure hormones of the hypothalamic–pituitary–adrenal (HPA) axis and the hypothalamic–pituitary–gonadal (HPG) axis makes this method suitable for dealing with questions concerning the cross talk between those axes.

1. Introduction

Non-invasive sampling is an important technique in the field of behavioural endocrinology since it provides data that are not influenced by potential changes in hormonal levels resulting from invasive collection [1,2]. Urine and faecal matrices are easily accessible by non-invasive sampling and are therefore often used for hormonal studies in free-ranging animals [3]. Increased interest in the monitoring of non-habituated primate populations leads to a greater demand for the use of these matrices in hormone analysis. In New World primates compared to Old World primates, glucocorticoid and androgen levels are much higher in plasma as well as urine and faeces [4], which is probably caused by an elevated expression of special genes that leads to a resistance against these steroids [5,6]. In addition, secretion into plasma is higher and the capacity and affinity of cortisol-binding globulin are reduced in plasma [4,7,8]. Furthermore, the effects of high cortisol levels may be minimised by an increased metabolism of active into inactive forms [9]. Thus, in New World primates, faecal steroid concentrations are reasonably high [10–12]. The use of faecal samples has a number of advantages over that of urine when focusing on the hormonal status of animals and their long-term endocrine profiles. In faecal samples, diurnal effects and effects of single events are dampened by the longer lag-time compared with urine [13,14]. Furthermore, faeces from white-faced capuchins are easier to collect than urine. Faecal samples have been conclusively shown to be a suitable matrix for monitoring hormonal profiles in New World monkeys such as white-faced capuchins and have been used with immunoassays in a number of studies [e.g., 10,11,15–17].

However, while steroid analyses of faecal samples with LC–MS methods have already been used to investigate a range of topics, such as steroid abuse in livestock [e.g., 18,19] and cortisol metabolism in ruminants [20], studies of hormone profiles in faeces of free-living primates have not yet profited from the advantages of this method.

* Corresponding author. Tel.: +49 341 3550 231; fax: +49 341 3550 299.
E-mail address: anja_weltring@eva.mpg.de (A. Weltring).

0031-9384/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.physbeh.2011.09.004

Until now, steroid analysis in faecal samples of capuchins has been carried out by immunoassays [15–17]. Immunoassays are sensitive, allow high sample throughput, and are often less expensive than LC–MS techniques. On the other hand, LC–MS techniques are more specific in distinguishing similar compounds [21]. Cross reactivity in immunoassays may cover metabolites of the hypothalamic–pituitary–adrenal (HPA) and the hypothalamic–pituitary–gonadal (HPG) axes. This can be highly disadvantageous when it is reasonable to analyse metabolites of only one axis [22–25]. Thus, LC–MS is an important reference technique in addition to analyses with immunoassays and has been applied in matrices other than faeces as a reference method to evaluate the suitability of specific enzyme immunoassays (urine: [26], saliva: [27]). Moreover, endocrine interactions with behaviour are recognised to be more complex than the relationship of a single hormone to a given behaviour. In recent years, an increasing number of studies analysing behavioural patterns or environmental influences on behaviour have investigated interactions of hormones of both axes [28–30]. Since hormones and metabolites of both axes can be measured simultaneously, LC–MS provides a valuable approach when interactions between the HPG and HPA axes are investigated. Thus, LC–MS/MS can be advantageous despite its higher costs and necessity of well-trained personnel when it is essential to measure several analytes simultaneously, while immunoassays can be more time- and cost-effective when analysing only a single hormone.

To optimise the purification of the samples and the chromatographic separation of the target analytes, it is important to estimate influences of matrix compounds that can interfere with the final measurement. Compared to urine, faeces contain a higher proportion of solid material. Components of the dry matter are indigestible material such as fibre, intestinal bacteria, proteins, and fat [31–33]. Some exogenous compounds such as sterols, cholesterol, and other steroids like ecdysteroids reflect the diet and are excreted via faeces [34]. These compounds as well as bile acids are structurally related to endogenous steroid hormones and may interfere with their measurements. This necessitates an appropriate sample purification prior to the measurement of steroid hormones in faecal samples.

Steroid hormones are metabolised and conjugated primarily in the liver, and conjugated steroids can then be easily excreted via either the kidneys or bile [35]. Steroids excreted via the bile into the faeces are to some extent further metabolised and deconjugated in the intestinal tract [35,36]. Heistermann [37] concluded that steroids are excreted in faeces of most species mainly in the unconjugated form but that there are exceptions to this. Species may vary according to whether steroids are excreted in faeces as conjugates or in the free form, with different steroids being differently conjugated. In callitrichids, for example, one-fifth of the excreted faecal amount of estradiol and two-thirds of that of progesterone were unconjugated [38]. In contrast, in baboons about one-fifth of the faecal oestrogens were excreted as conjugates [39]. Furthermore, a comparison showing differences between the conjugation of steroids of several New World primates and one Old World primate species is given by Ziegler and Wittwer [40]. In conclusion, a necessary step in the development and validation of an LC–MS/MS method to measure steroid concentrations in faeces, therefore, is the investigation of faecal conjugation patterns in the target species.

In this study, a method to measure steroid hormones and a number of their metabolites in faecal samples of white-faced capuchins was developed and validated. In principle, this method is built on the one used for extracting and measuring steroids in urine of great apes described by Hauser et al. [21]. The development of the faecal method included an optimisation of the purification steps to effectively separate target analytes from endogenous and exogenous compounds that are structurally related to them. Additional compounds such as corticosterone, which have been used in other studies as faecal target glucocorticoids [41,42], were screened as possible faecal metabolites and target analytes, and their LC–MS measurement was optimised.

The development and validation of the method consisted of four steps:

1. Main steroid metabolites in white-faced capuchin faeces were identified and their concentration ranges estimated by scanning faecal extracts of different individuals with a method validated for measuring steroids in primate urine [21]. Existing interferences with the faecal matrix were detected simultaneously.
2. Conjugation patterns of the target analytes in faecal samples were investigated. The information obtained was used to determine the necessity of different deconjugation steps to be integrated in the sample preparation.
3. The purification of the faecal extracts was optimised and the resulting method analytically validated using recoveries, coefficients of variation of repeated measurements and detection limits to identify metabolites that could be measured reliably.
4. The method was then physiologically validated by testing its capacity to indicate known physiologically occurring differences in specific steroid levels.

2. Materials and methods

2.1. Samples and animals

S. Perry and her research assistants collected faecal samples from nine groups of free ranging white-faced capuchins (*Cebus capucinus*) that are part of S. Perry's long-term study at Lomas Barbudal Biological Reserve, Costa Rica, between 2006 and 2010. For the analytical and physiological validation, faecal samples from this project were used (Table 1). Samples were collected during the day in latex gloves. They were transported on cooling aids to the camp and then stored in a freezer until further processing. Samples were dried at 80–115 °C in an oven for 2–3 h. After drying, samples were homogenised in a mortar. Seeds, plant material, and insect parts were discarded. The samples were then stored in sealable plastic bags on silica gel until they could be shipped to the lab at the Max Planck Institute of Evolutionary Anthropology in Leipzig, Germany.

In addition, fresh faecal samples were collected at the "Serengeti-Park Hodenhagen", Germany from one breeding group consisting of one male and two females, and one bachelor group (two individuals). Samples were collected in the morning in latex gloves and directly transported on dry ice to our lab where they were further processed according to the same protocol used for field samples. These samples were used to investigate conjugation patterns and to examine recoveries and repeatability of the method including the extraction step (Table 1).

All samples were stored at −20 °C in our lab until analysis.

2.2. Chemicals and reagents

Steroid reference materials were obtained from Steraloids (Newport, Rhode Island, USA): 5β-pregnane-3α,11β,17,20α,21-pentol (α-cortol); 5β-pregnane-3α,11β,17α,21-tetrol-20-one (tetrahydrocortisol); 5α-pregnane-3α,11β,17,21-tetrol-20-one (allotetrahydrocortisol); 5β-pregnane-3α,17α,21-triol-11,20-dione (tetrahydrocortisone); 5β-androstane-3α-ol-11,17-dione (11-oxoetiocholanolone); 5β-androstane-3α,11β-diol-17-one (11β-hydroxyetiocholanolone); androst-4-ene-3,17-dione (4-androstenedione); 5α-androstane-3,17-dione (androstanedione); 5α-androstane-17α-ol-3-one (epiallodihydrotestosterone); 5α-androstane-3α-ol-17-one (androsterone); 5α-androstane-3β-ol-17-one (epiandrosterone); androst-4-ene-17α-ol-3-one (epitestosterone); 5β-androstane-3α-ol-17-one (etiocholanolone); 5β-androstane-3β-ol-17-one (epietiocholanolone); androst-5-ene-3β-ol-17-one (dehydroepiandrosterone, DHEA); 5α-androstane-3α,17β-

Table 1
An overview of samples that were used for analytical and physiological validation. An "unknown" in the column "samples per individual" refers to samples that could not be attributed to a distinct individual. Samples came from either the Lomas Barbudal field site in Costa Rica or the "Serengeti Park Hodenhagen" in Germany.

Sampled individuals	Samples per individual	Samples total	Subsamples	Kind of samples	Sample site	Used for
10	1	10	1	Adult alpha males	Lomas Barbudal	Physiological validation
10	1	10	1	Juvenile males (1–5 years)	Lomas Barbudal	Physiological validation
10	1	10	1	Lactating females	Lomas Barbudal	Physiological validation
10	1	10	1	Pregnant females	Lomas Barbudal	Physiological validation
10	1	10	1	Females (2–21 years)	Lomas Barbudal	Analyte screening
10	1	10	1	Males (5–29 years)	Lomas Barbudal	Analyte screening
29	Pool	1	28	Males (2–29 years) with low expected hormone levels	Lomas Barbudal	Analytical validation of purification step
19	Pool	1	28	Females (2–21 years) with low expected hormone levels	Lomas Barbudal	Analytical validation of purification step
3	Unknown	8	1	Breeding group (1 male, 2 females)	Hodenhagen	Conjugation patterns
2	Unknown	2	1	Bachelor group (2 males)	Hodenhagen	Conjugation patterns
3	Unknown	9	2	Breeding group (1 male, 2 females)	Hodenhagen	Recovery including extraction step
2	Unknown	1	2	Bachelor group (2 males)	Hodenhagen	Recovery including extraction step
1	1	1	10	Male of bachelor group	Hodenhagen	Repeatability including extraction step

diol (androstanediol); 5α-androstane-17β-ol-3-one (dihydrotestosterone); androst-4-ene-17α-methyl-17β-ol-3-one (methyl testosterone); 1,3,5(10)-estratriene-3-ol-17-one (estrone); 1,3,5(10)-estratriene-3,16α,17β-triol (estriol); pregn-1,4-diene-11β,17α,21-triol-3,20-dione (prednisolone); 5β-pregnane-3α,20α-diol (pregnanediol); pregn-4-ene-3,20-dione (progesterone) and Sigma-Aldrich (Steinheim, Germany): pregn-4-ene-11β,17,21-triol-3,20-dione (cortisol); pregn-4-ene-17α,21-diol-3,11,20-trione (cortisone); pregn-4-ene-11β,21-diol-3,20-dione (corticosterone); androst-4-ene-17β-ol-3-one (testosterone); 1,3,5(10)-estratriene-3,17β-diol (17β-estradiol). Deuterated internal standards were purchased from Sigma-Aldrich (Steinheim, Germany): 1,3,5(10)-estratriene-3-ol-17-one-2,4,16,16-d4 (estrone-d4); androst-4-ene-17β-ol-3-one-16,16,17-d3 (testosterone-d3) and C/D/N-Isotopes Inc. (Pointe-Claire, Quebec, Canada) pregn-4-ene-3,20-dione-2,2,4,6,6,17α,21,21,21-d9 (progesterone-d9). Stock solutions of each standard (1 mg/mL) were prepared in methanol and stored at −20 °C. A standard mix was prepared in methanol containing each analyte at a concentration of 10 μg/mL and working solutions (0.1–2000 ng/mL) were obtained from this mixture by diluting each with 30% acetonitrile in water.

LC–MS grade methanol and acetonitrile were supplied by Roth (Karlsruhe, Germany), water in HPLC gradient grade quality was purchased from Baker (Phillipsburg, NJ, USA), and formic acid was purchased from Sigma-Aldrich (Chromasolv, Steinheim, Germany). Deionised water for extraction and purification was prepared using a MilliQ® water purification system (Millipore, Bedford, MA, USA). Extraction was carried out using methanol (gradient grade, Roth, Karlsruhe, Germany), *tert*-butyl methyl ether (TBME; VWR, Darmstadt, Germany), and ethyl acetate (Roth, Karlsruhe, Germany). Potassium hydroxide, potassium carbonate, sodium dihydrogen phosphate, disodium hydrogen phosphate, and sulphuric acid (98%) were obtained from VWR (Darmstadt, Germany).

2.3. Extraction of faecal samples

The first extraction step of the dried samples followed the method described by Heistermann et al. [43]. Briefly, we extracted 100 mg of each dried sample by twice adding 3 mL of a methanol/water mixture (80 + 20, v/v) and thoroughly shaking for 15 min. Supernatants were decanted after centrifugation and the methanol extracts were stored at −20 °C until further purification and measurement.

2.4. Analyte screening and investigation of conjugation patterns

As a first screening step intended to identify possible target analytes, faecal samples of free-living white-faced capuchins were extracted as described in Section 2.3 and then purified and analysed with the urine method described by Hauser at al. [21]. In short, extracts were hydrolysed with glucuronidase from *Escherichia coli* to deconjugate glucuronides. Free steroids were then extracted by liquid–liquid extraction with *tert*-butyl methyl ether and aqueous layers were purified by solid-phase extraction (SPE) on a silica based C-18 material. The SPE extracts were solvolysed with a mixture of ethyl acetate/sulphuric acid at 55 °C to break sulphates and multiple conjugates. Free steroids were then extracted again by liquid–liquid extraction with *tert*-butyl methyl ether and all organic fractions were combined for measurement. Single samples from 10 males and 10 females were analysed (Table 1). To cover a wide range of naturally occurring steroid concentrations, individuals of different age were chosen. For positive identification of possible target analytes, retention times and ion ratios of compounds in the samples were compared to those of reference compounds summarised in Table 2. Those that could be positively identified were then quantified to evaluate their concentration ranges.

In a second experiment, 10 methanol extracts of non-spiked faecal samples of free-living white-faced capuchins were purified to investigate the proportions of conjugated and free steroids. These samples were also extracted as described in Section 2.3. In contrast to the method described by Hauser et al. [21], an additional liquid–liquid extraction step was carried out before enzymatic hydrolysis. The organic fractions of each of the resulting three liquid–liquid extraction steps – before hydrolysis, after hydrolysis, and after solvolysis – were collected and measured separately to get the proportions of free steroids, steroids after hydrolysis, and steroids after solvolysis.

2.5. Optimisation of the purification step

The purification of the methanol extracts of faecal samples was optimised with respect to matrix influences by compounds structurally related to the target analytes, and adapted to the metabolic profiles and conjugation patterns present in faeces. In contrast to extraction protocols used for urine samples, enzymatic hydrolysis was omitted based on the results of the investigation of conjugation patterns (Fig. 1). Free and conjugated steroids were extracted from 500 μL methanol extracts by solid phase extraction. In the optimised procedure, the solid phase extraction was applied before the first liquid–liquid extraction to obtain better purification of the extracts by covering both the fraction containing the free and the conjugated steroids. A polymer-based reversed phase material (Chromabond® HR-X, 30 mg, Macherey-Nagel) was used for solid phase extraction. In contrast to silica based C-18 materials, polymer-based solid phase materials show higher retention of more polar compounds in addition to their retention of non-polar compounds [44]. This property provides the possibility of obtaining pure extracts containing both free and conjugated forms of steroids with high recovery. Cartridges were conditioned with 1 mL of methanol followed successively by the same volume of water and phosphate buffer (pH 6.9) and were washed after sample application with 3 mL of water

Table 2
Compound specific MRM parameters of target steroids modified from Hauser et al. [21]. IS = internal standard.

Class of analytes	Analyte	Retention time (min)	Precursor ion m/z	Product ions (1/2)	Cone voltage (V)	Collision energy (eV)	Polarity
Corticoids	α-Cortol	6.0	333	273/255	25	26/20	ESI+
	Cortisol	6.4	363	121/97	30	26/30	ESI+
	Cortisone	6.6	361	91/163	41/17	59/10	ESI+
	Tetrahydrocortisol	8.2	349	301/295	17	13/13	ESI+
	Tetrahydrocortisone	9.1	365	347/329	19	9/13	ESI+
	Corticosterone	10.8	347	121/91	29	25/51	ESI+
	11β-Hydroxyetiocholanolone	13.1	307	271/253	14	9/13	ESI+
	11-Oxoetiocholanolone	14.4	287	229/91	30	21/57	ESI+
Oestrogens	Estriol	5.0	271	133/157	25	26/20	ESI+
	17β-Estradiol	15.9	255	159/133	25	19/20	ESI+
	Estrone	19.2	271	159/157	25	20/20	ESI+
Androgens	Androstenediol	16.0	273	255/159	18	12/21	ESI+
	Testosterone	17.0	289	97/109	33	26/26	ESI+
	4-Androstenedione	19.4	287	97/109	30	22/26	ESI+
	Dehydroepiandrosterone	19.9	289	271/253	17	9/9	ESI+
	Epitestosterone	21.0	289	109/97	33	26/26	ESI+
	Epiandrosterone	22.3	273	255/91	26	13/42	ESI+
	Dihydrotestosterone	23.4	291	255/105	32	15/41	ESI+
	Androstanediol	23.6	275	257/95	24	11/25	ESI+
	Epietiocholanolone	23.7	273	255/147	26/30	13/20	ESI+
	Etiocholanolone	26.3	273	255/91	26	13/42	ESI+
	Androstanedione	26.3	289	271/253	30	13/17	ESI+
	Androsterone	27.8	273	255/147	26/30	13/20	ESI+
Gestagens	Progesterone	30.9	315	97/109	30	25/25	ESI+
	Pregnanediol	31.9	285	175/189	30	18/18	ESI+
IS	Prednisolone	6.1	361	343	17	10	ESI+
	Testosterone-d3	16.8	292	97	33	26	ESI+
	Estrone-d4	19.1	275	135	25	26	ESI+
	Methyl testosterone	20.0	303	97	33	28	ESI+
	Progesterone-d9	30.6	324	100	30	25	ESI+

followed by 1 mL of 40% methanol. Steroids were eluted with 1 mL of methanol followed by 1 mL of ethyl acetate collected in a single fraction. Free steroids were separated from conjugated ones by liquid–liquid extraction with *tert*-butyl methyl ether. The aqueous layer containing the conjugates was solvolysed using a mixture of ethyl acetate/sulphuric acid followed by liquid–liquid extraction to extract the free steroids. The combined steroid extracts were measured by LC–MS/MS. For more details on the solvolysis and liquid–liquid extraction, see Hauser et al. [21].

2.6. LC–MS measurements

Analytes and matrix compounds were separated at 50 °C on a reversed phase C18 column (Gemini C18; 150 mm × 2 mm, 3 µm, Phenomenex Torrance, CA, USA) covered by a guard cartridge of the same material (Security Guard; 4 mm × 2 mm, 5 µm, Phenomenex Torrance, CA, USA). The composition of eluent A and eluent B was water/acetonitrile 95 + 5 (v/v) and water/acetonitrile 5 + 95 (v/v), respectively, and both contained 0.1% formic acid. The gradient was 30% B (0 min), linear increase to 45% B (0–20 min), linear increase to 78% B (20–32 min), 100% B (32–37 min), 30% B (37–47 min). 20 µL of extract was injected while the auto sampler was tempered to 5 °C (Waters Alliance 2695 separation module, equipped with quaternary pump and column oven, Waters, Milford, MA, USA). The retention times of the analytes are given in Table 2.

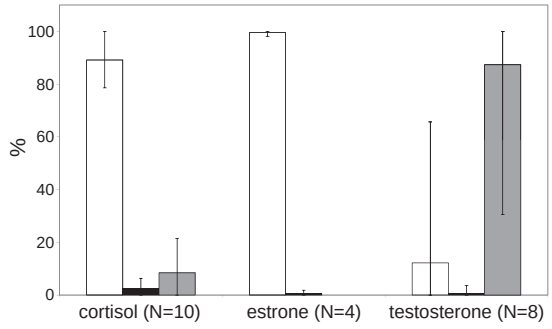

Fig. 1. Conjugation pattern of main target steroids in capuchin faeces, percentage of total amount found in free fraction (white), hydrolysis fraction (black) and solvolysis fraction (grey), error bars indicate minimal and maximal percentages.

The MS equipment (Quattro Premier XE tandem mass spectrometer with Z spray ESI interface, Micromass, Manchester, UK) and settings were the same as in the method described by Hauser et al. [21]. In short, nitrogen (NGM-11 nitrogen generator, CMC Instruments, Eschborn, Germany) was used as desolvation and cone gas (flow rate: 900 L/h and 250 L/h, respectively). Argon was used as collision gas at 5.1×10^{-3} mbar. The potential applied to the capillary was set to 3.8 V. The sample cone voltage and the collision energy were set individually for each compound. Steroids were detected in positive ion mode with multiple reaction monitoring (MRM) of the two most abundant product ions per analyte. Dwell time was 50 ms for each transition. The ion ratio of the two transitions was used as an identification criterion, allowing a maximal deviation of 30% compared to that of standards. In addition to the compounds analysed with the urine method, two further cortisol metabolites, cortisone and corticosterone [41,42], were added to the existing mixture of calibration standards and included in the method. The retention times and multiple reaction monitoring (MRM) parameters are given in Table 2. MRM parameters have been optimised for cortisone and corticosterone; for all other analytes they are similar to those described by Hauser et al. [21].

The internal standards prednisolone (for glucocorticoids), estrone-d4 (for oestrogens), testosterone-d3 (for androgens), and progesterone-d9 (for gestagens) were used for quantification. Methyl testosterone was used as internal standard when hydrolysis and solvolysis fractions were measured separately. Calibration standards

were prepared at 13 concentration levels between 0.1 and 2000 ng/mL containing a mixture of all target analytes and spiked with a mixture of the internal standards each at 50 ng/mL. Calibration curves were then obtained from the ratios of analyte to internal standard peak areas using linear regression with 1/x weighting. Samples were spiked before purification with the same mixture of internal standards as used for the calibration curves at 50 ng/mL final extract. If internal standard concentrations measured in a sample deviated more than 50% from its known concentration, samples were excluded from statistical analysis. Final faecal steroid concentrations were given as ng/g dry weight.

2.7. Analytical validation

To validate the purification step of faecal methanol extracts and their successive LC–MS/MS measurement, two sample pools were created, one with methanol extracts of female samples and one with methanol extracts of male samples. Samples of low steroid concentrations were pooled (see Table 1) to keep the proportion of endogenous concentrations as low as possible. The male and female pools were each spiked at five different concentration levels between 2 and 400 ng/mL extract (for details see Table 3). Subsamples of level 0 were spiked with analyte-free solvent to obtain matrix blanks. The concentration levels were chosen considering the expected sex specific concentration ranges of the analytes in faecal extracts of white-faced capuchins.

All subsamples were purified and measured repeatedly (levels 0–4 five times, level 5 three times) according to the method described in Sections 2.5 and 2.6.

Recoveries were calculated by comparing the differences of spiked samples and matrix blanks with the amounts spiked. The effectively spiked amounts were determined in advance by analysing the spiking solutions, thus specifically allowing an investigation of the effectiveness of the purification step and the influence of possible matrix effects on the measurements. Regression curves of spiked to measured concentrations were calculated, and the linearity of the measurements in matrix samples was used to estimate matrix influences. The limit of detection (LOD) in matrix samples was calculated on the most intense transition based on a signal to noise ratio of three, the limit of quantification (LOQ) on a signal to noise ratio of ten [45]. In this study, working ranges were defined as the range that was covered by the validation, with the limit of quantification as the minimal and the highest spiking level reliably measurable as the maximal concentration.

As the same extraction step was used and validated for immunoassay measurements already, we only included the purification step into the validation in the first experiment in this study. The recoveries and repeatability of the method, including the extraction step, were examined in an additional experiment. The drying step was not included in these experiments, since steroids can be distributed unevenly in faecal samples [37]. The within-sample variance can be markedly reduced when homogenising previously dried instead of fresh material [46]. Since we were exclusively interested in the repeatability of the extraction and measurement in this validation step and all samples were entirely collected, dried, and homogenised as a whole, previously dried samples instead of wet fresh samples were used.

For the recovery experiments including the extraction step, dried faecal samples of ten individuals were divided into two subsamples. One of the two subsamples was spiked with a mixture of target analytes at concentration levels expectable in this matrix, and the other subsample was left unmodified. Recoveries were calculated as described above. To determine inter- and intra-day precision of the method including the extraction step, one sample was extracted and purified five times on one day, and one sample was extracted and purified on five different days. All samples were extracted and purified as described in Sections 2.3 and 2.5.

2.8. Physiological validation

Due to animal welfare regulations, it is difficult or even impossible to carry out studies in free-living animals that include pharmacological administration to induce changes in hormonal levels. Thus, in this study, the method's ability to indicate known naturally occurring differences in hormone levels was investigated for physiological validation.

Single faecal samples of ten adult alpha males, ten juvenile males (age classification following Fedigan et al. [47]), ten pregnant females, and ten lactating females were extracted and measured following the method analytically validated before. A possible diurnal variation in steroid levels in these faecal samples was expected to be considerably smaller [48–50] than the effect that was to be studied [2,17,51] and was therefore regarded as negligible in this study. Nonetheless, morning samples were preferred whenever available.

In addition to testosterone, total androgen concentrations were calculated by summing the concentrations of testosterone and the metabolites epitestosterone, 4-androstenedione, androsterone, epiandrosterone, and etiocholanolone.

The hormone levels of individuals of different subgroups were compared to test the following predictions:

a) Adult alpha males have higher faecal testosterone and androgen levels than juvenile males [17,52,53].
b) Adult alpha males have higher faecal testosterone and androgen levels than lactating females [54].
c) Pregnant females have higher faecal estrone and estradiol levels than lactating females [2,55,56].
d) Pregnant females have higher faecal progesterone levels than lactating females [55].
e) Pregnant females have higher faecal cortisol levels than lactating females [57–60].

Table 3
Spiking levels for male and female pools in ng/mL extract. According to the estimated naturally-occurring concentrations, analytes are summarised in groups with comparable concentrations. Amounts given refer to nominal concentrations. Level 0 is not listed in the table as it represents non-spiked samples.

Pool	Analytes	Spiked amount in ng/mL extract				
		Level 1	Level 2	Level 3	Level 4	Level 5
Male	17β-Estradiol	4	8	12	16	20
	Corticosterone; 11-oxoetiocholanolone; estriol; progesterone; epitestosterone; epiandrosterone	4	8	20	40	80
	Cortisol; tetrahydrocortisol; tetrahydrocortisone; α-cortol; 11β-hydroxyetiocholanolone; androsterone	4	20	40	80	200
	Cortisone; estrone; pregnanediol; testosterone; 4-androstenedione; etiocholanolone	4	20	40	200	400
Female	Tetrahydrocortisone; testosterone; epiandrosterone	2	4	6	8	10
	Tetrahydrocortisol; estriol; epitestosterone; etiocholanolone	2	4	8	12	16
	Cortisol; 11-oxoetiocholanolone; cortisone; 17β-estradiol; 4-androstenedione	2	8	16	24	32
	Corticosterone; 11β-hydroxyetiocholanolone; estrone; progesterone; androsterone	4	20	40	80	160
	α-Cortol	4	40	80	160	200
	Pregnanediol	4	40	80	160	400

2.9. Statistics

To obtain information on the quality of the method, recoveries and coefficients of variation (CV) for each concentration level as well as linear regression equations, method coefficients of variation (method CV) [61] and coefficients of determination (R^2) were calculated for the target analytes in the pooled samples of the analytical validation. For linear regressions, the spiked steroid concentration was used as the predictor variable and the measured steroid concentration as the response variable.

The physiological validation was carried out by testing each prediction separately using exact Mann–Whitney-U tests. Therefore, pregnant and lactating females were compared regarding their faecal cortisol, estrone, estradiol, and progesterone concentrations. Juvenile and adult alpha males were compared regarding their faecal testosterone and androgen levels, and adult alpha males and lactating females were compared regarding their faecal testosterone and androgen levels as well.

R (version 2.10.0, R Development Core Team 2009) was used for statistical analyses. All analyses of the physiological validation were performed using nonparametric tests. Correlations between analytes were calculated with the function cor.test using the method = "spearman". Differences between groups were examined with the Mann–Whitney-U test using the function wilcox.exact of the R-package 'exactRankTests' (version 0.8-19, Hothorn and Hornik 2010).

3. Results and discussion

3.1. Selection of target analytes

Of 25 tested steroids, all but androstenediol and estradiol were detectable in the faeces of adult male white-faced capuchins in median concentrations higher than 10 ng/g dry faeces. In faecal samples of adult female white-faced capuchins, tetrahydrocortisol, estradiol, estriol, and all androgens were detected in median concentrations lower than 10 ng/g dry faeces (see Table 4).

Analytes with the highest median concentrations (e.g., cortisol, estrone, testosterone) were selected as target analytes after additionally considering their metabolic relevance (Table 4). Androstanediol was not selected as a target analyte despite its acceptable median concentration in faecal samples of males. This was based on its smaller concentration range found between individuals (not detectable — 428 ng/g dry weight) compared to that of epitestosterone (not detectable — 1124 ng/g dry weight), a selected target analyte with a comparable median concentration.

Higher median estrone and estriol levels were found in the faecal samples from males compared to females. For horses, it is known that urinary oestrogen levels in stallions are extremely high, even compared to those of mares [62]. Some urinary oestrogen concentrations in males of different species were summarised by Velle [63] and male oestrogen levels of New World primate species were determined in different matrices [64,65]. Oestrogens were studied in either males or females (e.g., females: [66], males: [64]) and sexes were not compared within the same study. However, male and female faecal oestrogen levels of common marmosets were compared between two studies using the same assay [67], indicating the probability that at least in some New World primate species, males have oestrogen levels comparable or higher to those of females at the time of ovulation. A radio-metabolism study could help to identify the metabolic origin of the oestrogens in male faecal samples. Aromatase which is expressed in the placenta, ovaries, testes, and adipose tissue, for example, catalyses the irreversible conversion of androgens into oestrogens [68]. Testosterone in this reaction is converted into estradiol, whereas 4-androstenedione is converted into estrone [68]. Furthermore, a strain of *E. coli* was reported to be able to aromatise steroids [69]. So it is also possible that in faecal samples, at least part of the oestrogens might derive from some kind of intestinal activity.

Table 4
Screening of target analytes (females N = 10, males N = 10), DW = dry weight, n.d. = not detectable, n.a. = not analysable due to matrix interferences, + selected as target analyte.

Class of analytes	Analyte	Median concentration ng/g DW			
		Male		Female	
Corticoids	α-Cortol	197	+	975	+
	Cortisol	661	+	203	+
	Allo-/tetrahydrocortisol[a]	103	+	n.d.	+
	Cortisone	288	+	113	+
	Tetrahydrocortisone	74	+	21	+
	11β-Hydroxyetiocholanolone	432	+	349	+
	Corticosterone	233	+	138	+
	11-Oxoetiocholanolone	197	+	106	+
Oestrogens	Estrone	189		41	+
	17β-Estradiol	n.d.		8.4	+
	Estriol	170		n.d.	+
Androgens	Testosterone	840	+	3.6	+
	4-Androstenedione	n.a.	+	n.a.	+
	Epitestosterone	122	+	n.d.	+
	Epiandrosterone	301	+	n.d.	
	Etiocholanolone	1719	+	6	+
	Androsterone	955	+	n.d.	+
	Androstanedione	47		n.d.	
	Androstenediol	1.8		10.0	
	Epietiocholanolone	16		n.d.	
	Dehydroepiandrosterone	n.a.		n.a.	
	Androstanediol	171		n.d.	
	Dihydrotestosterone	19		n.d.	
Gestagens	Pregnanediol	136		548	+
	Progesterone	1700		12,574	+

[a] Coeluting compounds.

Our findings of generally higher estrone than 17β-estradiol concentrations in the faeces of white-faced capuchins is in agreement with a study by Hodges et al. [70] on urinary oestrogen excretion in four New World monkey species (*Cebus albifrons*, *Saguinus fuscicollis*, *Saimiri sciureus*, and *Ateles fusciceps robustus*).

The main androgens detected in faeces of male white-faced capuchins were testosterone, etiocholanolone, and androsterone. Contrary to these results, no authentic testosterone could be detected in a radiometabolism study of faeces of three different primate species (chimpanzee, macaque, marmoset) [24]. Similar to our findings, this study found that androsterone seemed to be one of the testosterone metabolites found in the marmoset [24].

In line with previous studies of marmosets, dehydroepiandrosterone was not detectable in our samples from male and female individuals due to low concentrations and matrix interferences. For marmosets, it is known that circulating levels of dehydroepiandrosterone or its sulphate are low due to the absence of, or an only rudimentarily functional, adrenal zona reticularis [71,72]. In contrast, faecal dehydroepiandrosterone or dehydroepiandrosterone sulphate levels in chimpanzees are comparable to other androgen levels [73]. Dehydroepiandrosterone could also be detected in a radiometabolism study in faecal samples of macaques [24].

Cortisol and a number of its metabolites could be detected and quantified in samples from both sexes. In previous studies, cortisol and corticosterone were identified in the faeces of a New World primate species (marmosets) but not in those of two Old World primate species (macaques and chimpanzees) [41]. The high number of glucocorticoid metabolites found in faecal extracts in our study is consistent with the results from a radiometabolism study on three nonhuman primate species (macaques, chimpanzees, marmosets) [41]. In that study, multiple radioactive metabolites were detected in faecal extracts of all three species after the administration of radiolabelled cortisol.

This suggests that, depending on the question addressed, it can be advantageous to either measure levels of single glucocorticoid metabolites or calculate more group specific values. For this purpose, glucocorticoid metabolites that are reliably measurable can additionally be summed up as has been shown in studies assessing overall cortisol secretion in human urine [74,75]. The same consideration that is necessary for LC–MS/MS measurements regarding the use of single or group values has to be made when analysing glucocorticoids in faecal extracts with immunoassays. Here, the use of either more specific antibodies or those with higher cross reactivities towards a number of metabolites has to be evaluated [23]. Furthermore, studies correlating results from immunoassays with those of LC–MS/MS measurements can help to decide which technique is more suitable to address specific questions [26,27].

3.2. Conjugation pattern

Less than or equal to 10% of the total amount of each target analyte in this study was found in the fraction after enzymatic hydrolysis.

The solvolysis fraction added an average of 87% testosterone and 44% epiandrosterone to the total amount of these compounds. For all other finally selected target analytes, solvolysis added an average of less than 10% to the total amount (Fig. 1).

These results fit well with findings from faecal samples of other primate species where steroids were found to be excreted in faeces mainly in the free or sulphated form [11,38,39,41,76–78], especially in tufted capuchins, for which 95% of faecal testosterone and 50% of faecal cortisol were found to be conjugated [17].

In the human gut, sulphates and glucuronides are deconjugated in the colon by enzymes of either intestinal or bacterial origin, with sulfatases being exclusively of bacterial origin [36]. For human faeces, this indicates that a higher proportion of steroids are unconjugated and that deconjugation of sulphates is dependent on the intestinal flora.

Our results stress the necessity of assessing the need for hydrolysis of samples of each matrix and species. An enzymatic hydrolysis of glucuronides can be omitted for faecal samples of white-faced capuchins. In contrast the solvolysis step is necessary to capture most of the testosterone but also much of the epiandrosterone. An alternative to solvolysis would be the simultaneous measurement of the sulphated steroids, which is possible with LC–MS/MS as long as reference standards are available. Solvolysis covers sulphates of all target analytes as well as multiple sulphated analytes, whereas expanding the LC–MS/MS method only covers the sulphates specifically integrated into the measurement. While the direct measurement of conjugated steroids by LC–MS/MS can be of interest to metabolism studies, for studies focusing on hormone–behaviour interactions, no detailed data on conjugation patterns is needed and solvolysis is the more efficient approach.

3.3. Analytical validation

3.3.1. Precision and recovery of the purification of methanol extracts

Mean recoveries and coefficients of variation (CV) of repeated measurements are summarised in Table 5. These parameters were determined generally following the Commission Decision 2002/657/EC [79], with modifications made based on a consideration of the sample material properties and the required performance of the method with respect to the questions that should be addressed. Thus, recoveries of 50–120% and coefficients of variation (CVs) of up to 15% were considered acceptable in this study. Whether or not the CVs reached with

Table 5
Results of analytical validation. Precision, accuracy, statistics of calibration curves, limits of detection (LOD) and quantification (LOQ) and working range of target analytes in matrix, CV = coefficient of variation, R^2 = coefficient of determination.

		CV level 0–5 (%)	Recovery level 1–5 (%)	Linearity			Method CV (%)	LOD (ng/mL)	LOQ (ng/mL)	Working range (ng/mL)
		Mean (range)	Mean (range)	Slope	Interception	R^2				
Cortisol	Females	3.7 (1.0–5.1)	85.7 (80.8–90.5)	0.88	−0.27	0.9937	6.4	0.30	0.90	0.9–34.7
	Males	3.5 (2.5–4.5)	104.8 (98.7–109.9)	0.98	1.40	0.9985	5.6			0.9–66.3
Tetrahydrocortisol	Females	7.4 (5.5–9.8)	102.0 (74.9–114.4)	1.11	−0.31	0.9828	10.2	1.26	3.77	3.8–22.5
	Males	4.2 (1.5–5.9)	97.3 (63.5–114.0)	1.14	−2.81	0.9972	7.1			3.8–87.4
Tetrahydrocortisone	Females	5.7 (1.5–9.7)	93.4 (84.0–99.4)	0.96	−0.05	0.9848	8.3	0.40	1.19	1.2–10.4
	Males	5.7 (1.8–8.6)	95.6 (90.2–100.5)	0.95	−0.26	0.9950	9.7			1.2–64.3
α-Cortol	Females	6.1 (3.6–9.6)	97.0 (71.1–105.4)	1.03	−0.16	0.9916	8.5	4.16	12.47	12.5–226.2
	Males	4.7 (3.4–6.9)	96.8 (83.8–112.4)	0.96	1.82	0.9946	10.3			12.5–74.9
Cortisone	Females	5.0 (2.5–9.1)	90.4 (72.9–101.0)	0.98	−0.36	0.9953	7.0	1.53	4.60	4.6–28.2
	Males	6.2 (2.8–8.9)	80.5 (75.7–84.8)	0.80	1.14	0.9968	7.2			4.6–325.4
Corticosterone	Females	6.3 (2.8–10.9)	130.9 (124.4–139.0)	1.30	0.47	0.9964	6.1	1.65	4.95	5.0–186.0
	Males	4.2 (1.9–6.1)	108.4 (103.4–112.9)	1.06	0.49	0.9973	5.3			5.0–77.8
11β-Hydroxyetiocholanolone	Females	7.1 (5.3–11.9)	148.6 (139.9–157.0)	1.51	−1.25	0.9921	9.0	2.29	6.88	6.9–201.4
	Males	5.9 (2.3–14.4)	107.0 (90.9–120.8)	1.20	−3.19	0.9929	11.1			6.9–74.3
11-Oxoetiocholanolone	Females	6.7 (3.7–11.0)	158.0 (139.0–164.8)	1.59	−0.30	0.9844	10.3	0.89	2.67	2.7–50.9
	Males	6.5 (3.1–14.0)	106.4 (97.8–114.7)	1.06	0.42	0.9948	7.5			2.7–84.8
Estrone	Females	6.4 (2.8–9.1)	100.1 (94.8–108.4)	0.95	1.44	0.9948	8.4	1.47	4.42	4.4–191.3
17β-Estradiol	Females	5.9 (3.0–11.8)	84.6 (80.8–88.9)	0.83	0.17	0.9941	6.4	0.59	1.76	1.8–29.1
Estriol	Females	9.8 (4.6–18.2)	58.4 (53.7–66.7)	0.55	0.14	0.9749	13.1	0.32	0.96	1.0–9.7
Progesterone	Females	5.4 (3.9–6.9)	117.4 (115.5–119.5)	1.16	0.53	0.9974	5.2	0.84	2.53	2.5–173.1
Pregnanediol	Females	19.8 (8.6–30.5)	65.9 (55.8–72.3)	0.70	−0.52	0.9444	27.7	2.42	7.26	7.3–82.3
	Males	5.7 (3.7–9.6)	94.8 (87.8–97.9)	0.95	0.43	0.9978	6.2			7.3–284.1
Testosterone	Females	3.5 (1.7–4.6)	86.2 (82.6–93.6)	0.85	0.07	0.9862	8.1	0.91	2.73	2.7–11.0
	Males	5.6 (1.8–21.3)	85.9 (80.7–89.7)	0.90	−0.77	0.9995	2.9			2.7–317.4
4-Androstenedione	Females	5.0 (4.0–6.2)	106.8 (102.6–112.4)	1.03	0.23	0.9953	5.7	0.79	2.38	2.4–31.0
	Males	6.8 (1.6–25.4)	100.7 (93.5–105.6)	1.05	−0.96	0.9989	4.1			2.4–373.0
Epitestosterone	Females	3.4 (1.6–5.6)	84.6 (80.8–87.6)	0.83	0.06	0.9973	4.1	1.09	3.26	3.3–13.4
	Males	3.2 (1.2–6.4)	97.1 (93.7–101.3)	1.01	−0.36	0.9995	2.2			3.3–63.8
Etiocholanolone	Females	6.1 (1.8–9.8)	86.2 (76.3–95.3)	0.77	0.35	0.9857	9.6	2.11	6.34	6.3–14.7
	Males	6.2 (4.0–7.4)	77.4 (64.3–81.9)	0.81	0.13	0.9966	7.2			6.3–275.9
Androsterone	Females	5.6 (3.6–9.6)	85.0 (80.4–88.1)	0.81	1.04	0.9939	8.0	3.51	10.52	10.5–130.5
	Males	9.2 (5.2–13.2)	74.9 (71.2–80.2)	0.74	0.27	0.9958	9.3			10.5–41.4
Epiandrosterone	Males	6.3 (4.0–9.26)	80.5 (75.3–84.1)	0.80	0.17	0.9961	6.4	9.01	27.02	27.0–62.9

this method allow one to distinguish between levels representing a specific effect has to be decided individually for each study.

In the pooled male samples used for validation of the purification, mean recoveries of spiked standards were between 50% and 120% except for 11β-hydroxyetiocholanolone at level 4 (c = 210 ng/mL, recovery = 120.8%). CVs of repeated measurements were below 15% at all concentration levels except for testosterone and 4-androstenedione at level 0 (CV (testosterone, c = 0.9 ng/mL) = 21.3% and CV (4-androstenedione, c = 0.4 ng/mL) = 25.4%).

In the samples from the female pool, recoveries for 11β-hydroxyetiocholanolone, 11-oxoetiocholanolone and corticosterone were clearly above 120% at all concentration levels, while recoveries of the other target analytes were between 50% and 120%. For pregnanediol, CVs were >15% at most concentration levels and the CV of estriol was 18.2% at level 3 (c = 4.6 ng/mL). All other CVs of analytes were <15% in the female pool samples.

Slopes and intercepts of linear regressions as well as the coefficient of determination are given in Table 5. The weakest correlation was found for pregnanediol in the samples of the female pool with an $R^2 = 0.9444$. The quality of the method, expressed as the method coefficient of variation (method CV, see Table 5), was below 15% for most target analytes. Only pregnanediol has a method CV (females) of 27.7%.

3.3.2. Precision and recovery including the extraction step

Intra-day CVs of the procedure, including the extraction step (n = 5) of dried samples, were ≤15% for all tested analytes (Table 6). Inter-day CVs were >15% for 11β-hydroxyetiocholanolone, 11-oxoetiocholanolone, estriol, α-cortol, and cortisone. Measurements were regarded as reliable for inter- and intra-day CVs ≤ 15%. For CVs > 15%, the value of the corresponding concentration level calculated following Horwitz [80] was compared to the experimentally determined one, and those with an experimentally determined CV smaller than the calculated one were considered to be reliable. Accordingly, estriol measurements were regarded as reliable since the experimentally determined CV was within the range given by calculation (calculated CV (c = 30.7 ng/g) = 27%).

Extreme recoveries above 120% and below 70% indicate that the measurements of these corticoids were not reliable. Mean recoveries of standards in ten spiked samples of dried faeces ranged between 58% and 369% for the different analytes. Mean recoveries above 120% were calculated for 11β-hydroxyetiocholanolone (277%), 11-oxoetiocholanolone (369%), THF (148%), and α-cortol (198%) and below 70% for cortisone (58%) (Table 6).

In this study, we intended to identify a number of reliably measurable steroids of each of the following classes: glucocorticoids, androgens, oestrogens, and gestagens. Cortisol, tetrahydrocortisone, and corticosterone fulfilled this criterion so that the corticoids with unacceptable recoveries could be discarded. Thus, this method is suitable for all studies addressing questions that can be answered by determining one of the glucocorticoids reliably measureable or their sums within the limits defined by the analytical validation. Which glucocorticoid or combination of glucocorticoids allow for the most adequate measurement of adrenocortical activity can, for example, be estimated on the basis of an appropriate biological validation. However, this was beyond the scope of this study.

Changing faecal glucocorticoid levels have been described when samples were stored over longer time periods [81,82]. Hunt and Wasser [81] suggest that the changes they found in glucocorticoid levels after treating fresh faecal samples with ethanol might be due to a gradual release of free hormones from lipid micelles over time, a mechanism described by Yalkowsky [83]. However, this mechanism cannot explain our recoveries. First, samples were in contact with methanol during extraction only for relatively short periods of time. Second and more importantly, spiked and non-spiked samples were treated comparably, so a release of free hormones from matrix should occur to a similar extent in both subsamples. Another explanation for extreme recoveries might be transformations. Storing and drying techniques are known to influence faecal steroid concentrations, possibly by enzymatic degradation and conversions into other metabolites [84,85].

In the human intestinal tract, transformations of steroids such as hydrolysis, aromatisation, dehydroxylation, hydrogenations, and dehydrogenations are described and reviewed by MacDonald et al. and Groh et al. [36,86]. The described transformations could explain changes in glucocorticoid levels of fresh faecal samples, but in this study, previously dried faecal samples were spiked and directly extracted with 80% methanol for recovery experiments. Thus, more detailed investigations of the metabolism and potentially occurring post excretory conversions are necessary to clarify and eliminate the causes of the extreme recoveries of a number of glucocorticoid metabolites, which might then allow for the inclusion of the affected metabolites as additional adrenocortical markers.

3.3.3. Summary of analytical validation

By means of the parameters discussed above (recoveries 70–120%, CVs ≤ 15% or for estriol, CV ≤ calculated CV [80]), the measurement of

Table 6
Precision of extraction, clean-up and measurement, S.D. = standard deviation, DW = dry weight, CV = coefficient of variation.

Analyte	Recovery (%)		Precision			
	No matrix (n = 1)	Matrix (n = 10) Mean (+/−S.D.)	Mean (ng/g DW)	Intra-day CV (%)	Mean (ng/g DW)	Inter-day CV (%)
Cortisol	101.4	104.7 +/− 5.5	319.3	5.5	6879.3	10.0
Tetrahydrocortisol	111.4	148.2 +/− 19.1	–	–	6406.4	13.4
Tetrahydrocortisone	90.4	79.8 +/− 8.0	294.7	8.1	4080.7	8.1
α-Cortol	106.4	197.6 +/− 47.0	403.6	7.6	1140.0	20.7
Cortisone	75.5	58.3 +/− 22.5	163.0	9.1	14567.4	24.6
Corticosterone	106.6	118.5 +/− 28.4	40.9	5.6	1523.9	13.1
11β-Hydroxyetiocholanolone	137.9	276.5 +/− 70.3	292.8	7.3	321.0	47.1
11-Oxoetiocholanolone	160.9	368.8 +/− 105.9	59.9	12.8	777.1	48.9
Estrone	103.0	102.6 +/− 3.9	241.1	2.5	282.3	10.6
17β-Estradiol	108.4	106.1 +/− 4.7	16.6	13.0	8.4	13.3
Estriol	123.0	102.2 +/− 10.5	35.3	12.0	30.7	15.1
Progesterone	100.5	108.2 +/− 6.1	45.4	9.8	9.1	14.3
Pregnanediol	112.8	100.2 +/− 8.9	326.2	9.1	196.1	9.4
Testosterone	99.1	108.1 +/− 4.8	226.2	9.6	471.0	8.2
4-Androstenedione	97.3	108.8 +/− 7.1	195.9	9.4	214.3	7.2
Epitestosterone	101.3	116.3 +/− 8.7	44.5	5.5	254.7	7.9
Etiocholanolone	95.2	102.8 +/− 4.7	199.5	5.4	2117.6	7.3
Androsterone	90.2	95.7 +/− 11.2	276.5	7.7	1166.7	9.2
Epiandrosterone	93.6	89.5 +/− 4.7	43.3	7.5	–	–

the following steroids in the faeces of white-faced capuchins can be regarded as reliable in the concentration ranges given in Table 5:

cortisol, tetrahydrocortisone, corticosterone, estrone (females), 17β-estradiol (females), estriol (females), testosterone, 4-androstenedione, epitestosterone, etiocholanolone, androsterone, epiandrosterone (males), pregnanediol and progesterone (females).

For tetrahydrocortisol, α-cortol, 11β-hydroxyetiocholanolone, and 11-oxoetiocholanolone, recoveries were too high to be acceptable (>120%). Inter-day repeatability was too high (CV>15% and CV>calculated CV [80]) for reliable measurements of α-cortol, cortisone, 11β-hydroxyetiocholanolone, and 11-oxoetiocholanolone. These analytes were therefore excluded from this method.

Although it was not possible to measure cortisone, 11β-hydroxyetiocholanolone, and 11-oxoetiocholanolone reliably, their estimated amounts in the analysed samples were considerably high. For 11-oxoetiocholanolone, this also corroborates the findings of Wallner et al. [87], who used an immunoassay measuring 11,17-dioxoandrogens to detect stress responses in female Barbary macaques. Because of the high amounts of these analytes estimated in our study, it would be desirable to establish these metabolites as additional faecal stress markers in white-faced capuchins.

3.4. Physiological validation

The physiological validation in this study was intended to support the results of the analytical validation by showing that some known physiologically induced differences in faecal steroid concentrations of white-faced capuchins in general can be detected with the method. The median faecal testosterone and androgen concentrations of alpha males were significantly higher than those of juvenile males (testosterone, see Fig. 2). The median testosterone concentrations of the lactating females and their median androgen concentrations were significantly lower than those of the alpha males (Table 7).

Testosterone and androgen concentrations correlated significantly in adult males and juvenile males but only showed a trend in lactating females (adult males: $r_S = 0.783$, $N = 10$, $P = 0.02$; juvenile males: $r_S = 0.898$, $N = 9$, $P < 0.001$; lactating females: $r_S = 0.596$, $N = 10$, $P = 0.07$). The slightly weaker correlation in lactating females might reflect a different origin for androgens in males and females. In male marmosets, no physiologically functional adrenal zona reticularis was detectable, whereas in female marmosets, a rudimentarily functional one was found [71,72]. Thus, androgens might be at least partially of adrenal origin in female New World primates, while they are of gonadal origin in males.

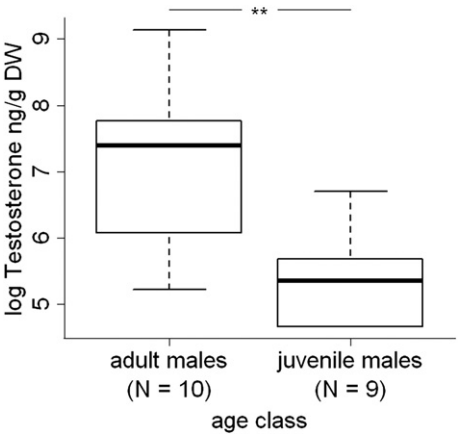

Fig. 2. Comparison of testosterone concentrations in faeces of adult alpha males and juvenile males displayed as their natural logarithms (Mann–Whitney-U-test), **P<0.01, DW = dry weight.

Table 7
Results of physiological validation (Mann–Whitney-U test, exact), DW = dry weight.

		N	Median (ng/g DW)	U	P
Testosterone	Adult males	10	1620	10	0.003
	Juvenile males	9	210		
	Adult males	10	1620	0	<0.001
	Lactating females	10	10.8		
Androgens	Adult males	10	12,552	10	<0.001
	Juvenile males	9	484		
	Adult males	10	12,552	10	<0.001
	Lactating females	10	208		
Cortisol	Pregnant females	10	3115	9	0.001
	Lactating females	10	341		
Estrone	Pregnant females	10	7089	0	<0.001
	Lactating females	10	17.5		
Estradiol	Pregnant females	10	1019	0	<0.001
	Lactating females	10	6.96		
Progesterone	Pregnant females	10	873	0	<0.001
	Lactating females	9	9.96		

Pregnant females had significantly higher estrone and estradiol concentrations than lactating ones (for estrone, see Fig. 3a). However, estrone and estradiol concentrations did not correlate within the two groups, although a trend was present in lactating females (lactating: $r_S = 0.602$, $N = 10$, $P = 0.066$; pregnant: $r_S = -0.055$, $N = 10$, $P = 0.892$). For practical application, this means that both hormones should be monitored because they might be affected in different ways.

Progesterone concentrations were significantly higher in pregnant females compared to lactating ones (Fig. 3b). This is in agreement with findings by French et al. [55] that plasma concentrations of progesterone in baboons significantly dropped after parturition. In our study, pregnant females had approximately 80 times higher progesterone values than lactating ones. Albuquerque et al. [51] found a five-fold increase in faecal progesterone concentrations during the latter part of pregnancy in marmosets compared to values before pregnancy. This difference in effect size compared to our findings might be due to both the comparison of different species and pregnant and lactating instead of pregnant and cycling females. As different species are studied and results are referenced to either dry or wet weight, a direct comparison of absolute values is difficult (pregnants: our study: 873 ng/g dry weight, Albuquerque et al. [51] 500 ng/g wet weight). Differences in mean faecal progesterone concentrations might, for example, be due to species differences in excretion rates or to differences in cross reactivities of the antibodies of the immunoassays used towards progesterone metabolites.

Mean cortisol concentrations of pregnant females were also significantly higher than those of lactating females (Fig. 3c). This is in agreement with several studies' findings on a number of New World primate species (e.g., golden lion tamarin, black tufted-ear marmoset, cotton-top tamarin), which reported elevated cortisol levels during the third trimester of pregnancy [57–60]. We found 10 times higher cortisol concentrations in pregnant compared to lactating female white-faced capuchins while Albuquerque et al. [51] found a 40-fold increase in faecal cortisol concentrations in pregnant marmosets. As outlined for progesterone, however, a direct comparison of absolute values is problematic.

In summary, all expected physiologically occurring differences in hormone concentrations could be detected in faeces with this LC–MS/MS method. Thus, the physiological validation of the measurements of faecal samples of white-faced capuchins can be regarded as successful within the scope of this validation.

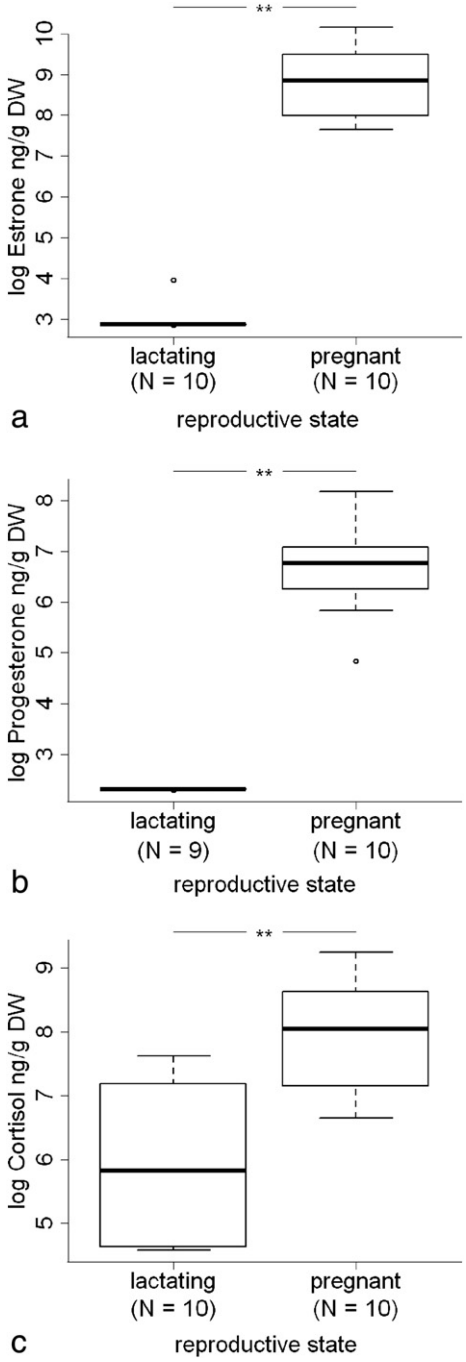

Fig. 3. Comparison of a. estrone, b. progesterone, and c. cortisol concentrations in faeces of lactating and pregnant white-faced capuchin females displayed as their natural logarithms (Mann–Whitney-U-test), **P<0.01, DW = dry weight.

4. Conclusion

The aim of this study was to develop and validate an LC–MS/MS method to measure steroids in faecal samples of white-faced capuchins.

As shown in the physiological validation, the method can detect known physiologically occurring differences in faecal steroid concentrations of white-faced capuchins.

With the LC–MS/MS method developed in this study, the main hormones of the HPA and HPG axes as well as a number of their metabolites can be reliably measured in faeces of white-faced capuchins. Furthermore, the method can be used in the validation of new immunoassays for this matrix.

Acknowledgements

We thank the Costa Rican park service (MINAE), the Area de Conservación Tempisque-Arenal, Hacienda Pelon de la Bajura, Hacienda Brin D'Amor and the community of San Ramón de Bagaces for permission to work in the study area. The following field assistants of the Lomas Barbudal Monkey Project are gratefully acknowledged for assisting SEP with sample collection: B. Barrett, R. Berl, L. Blankenship, M. Corrales, C. Dillis, R. Dower, K. Feilen, A. Fuentes J., C. Gault, I. Gottlieb, S. Hyde, W. Lammers, S. Leinwand, E. Johnson, S. Koot, W. Krimmel, M. Mayer, W. Meno, M. Milstein, C. Mitchell, A. Neyer, C. O'Connell, J.C. Ordoñez J., J. Rottman, E. Rothwell, M. Saul, I. Schamberg, S. Schulze, J. Vandermeer, J. Verge, A. Walker-Bolton, and E. Williams. W. Lammers, C. Mitchell, C. Dillis and M. Corrales assisted in the processing of samples in the field. We gratefully acknowledge the "Serengeti-Park Hodenhagen" (Germany) for providing fresh faecal samples of white-faced capuchins. We wish to thank Dr. Roger Mundry (Primatology, Max Planck Institute for Evolutionary Anthropology) for help with the statistical analysis and Carolyn Rowney for helpful comments on the manuscript. Vera Schmeling is thanked for extracting faecal samples. This research was supported by the Max Planck Society and the following grants to SEP: NSF (grant numbers SBR-0613226 and 6848360), National Geographic Society, and Leakey Foundation. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation or other funding agencies.

References

[1] Möstl E, Palme R. Hormones as indicators of stress. Domest Anim Endocrinol 2002;23:67–74.
[2] Risler L, Wasser SK, Sackett GP. Measurement of excreted steroids in *Macaca nemestrina*. Am J Primatol 1987;12:91–100.
[3] Schwarzenberger F. The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species. Int Zoo Yb 2007;41:52–74.
[4] Pugeat MM, Chrousos GP, Nisula BC, Loriaux DL, Brandon D, Lipsett MB. Plasma cortisol transport and primate evolution. Endocrinology 1984;115:357–61.
[5] Hubler TR, Denny WB, Valentine DL, Cheung-Flynn J, Smith DF, Scammell JG. The FK506-binding immunophilin FKBP51 is transcriptionally regulated by progestin and attenuates progestin responsiveness. Endocrinology 2003;144:2380–7.
[6] Scammell JG, Denny WB, Valentine DL, Smith DF. Overexpression of the FK506-binding immunophilin FKBP51 is the common cause of glucocorticoid resistance in three New World primates. Gen Comp Endocrinol 2001;124:152–65.
[7] Cassorla FG, Albertson BD, Chrousos GP, Booth JD, Renquist D, Lipsett MB, et al. The mechanism of hypercortisolemia in the squirrel monkey. Endocrinology 1982;111:448–51.
[8] Siiteri PK, Murai JT, Hammond GL, Nisker JA, Raymoure WJ, Kuhn RW. The serum transport of steroid hormones. Recent Prog Horm Res 1982;38:457–503.
[9] Scammell JG, Westberry JM, Sadosky PW, Hubler TR, Williams LE, Gibson SV, et al. Cortisol metabolism in the bolivian squirrel monkey (*Saimiri boliviensis boliviensis*). Comp Med 2006;56:128–35.
[10] Cristóbal-Azkarate J, Chavira R, Boeck L, Rodríguez-Luna E, Vea JJ. Glucocorticoid levels in free ranging resident mantled howlers: a study of coping strategies. Am J Primatol 2007;69:866–76.
[11] Heistermann M, Tari S, Hodges JK. Measurement of faecal steroids for monitoring ovarian function in New World primates, Callitrichidae. J Reprod Fert 1993;99: 243–51.
[12] Romano MC, Rodas AZ, Valdez RA, Hernández SE, Galindo F, Canales D, et al. Stress in wildlife species: noninvasive monitoring of glucocorticoids. Neuroimmunomodulation 2010;17:209–12.
[13] Touma C, Palme R. Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. Ann N Y Acad Sci 2005;1046:54–74.
[14] Whitten PL, Brockman DK, Stavisky RC. Recent advanced in noninvasive techniques to monitor hormone–behavior interactions. Yearbk Phys Anthropol 1998;41:1–23.
[15] Carnegie SD, Fedigan LM, Ziegler TE. Behavioral indicators of ovarian phase in white-faced capuchins (*Cebus capucinus*). Am J Primatol 2005;67:51–68.
[16] Carosi M, Heistermann M, Visalberghi E. Display of proceptive behaviors in relation to urinary and fecal progestin levels over the ovarian cycle in female tufted capuchin monkeys. Horm Behav 1999;36:252–65.
[17] Lynch JW, Ziegler TE, Strier KB. Individual and seasonal variation in fecal testosterone and cortisol levels of wild male tufted capuchin monkeys, *Cebus apella nigritus*. Horm Behav 2002;41:275–87.
[18] Poelmans S, De Wasch K, Noppe H, Van Hoof N, Van De Wiele M, Courtheyn D, et al. Androstadienetrione, a boldenone-like component, detected in cattle faeces with GC-MS*n* and LC-MS*n*. Food Addit Contam 2005;22:798–807.

[19] Van de Wiele M, De Wasch K, Vercammen J, Courtheyn D, De Brabander H, Impens S. Determination of 16beta-hydroxystanozolol in urine and faeces by liquid chromatography–multiple mass spectrometry. J Chromatogr A 2000;904: 203–9.
[20] Möstl E, Maggs JL, Schrötter G, Besenfelder U, Palme R. Measurement of cortisol metabolites in faeces of ruminants. Vet Res Commun 2002;26:127–39.
[21] Hauser B, Deschner T, Boesch C. Development of a liquid chromatography–tandem mass spectometry method for the determination of 23 endogenous steroids in small quantities of primate urine. J Chromatogr B 2008;862:100–12.
[22] Ganswindt A, Palme R, Heistermann M, Borragan S, Hodges JK. Non-invasive assessment of adrenocortical function in the male African elephant (*Loxodonta africana*) and its relation to musth. Gen Comp Endocrinol 2003;134:156–66.
[23] Heistermann M, Palme R, Ganswindt A. Comparison of different enzymeimmunoassays for assessment of adrenocortical activity in primates based on fecal analysis. Am J Primatol 2006;68:257–73.
[24] Möhle U, Heistermann M, Palme R, Hodges JK. Characterisation of urinary and fecal metabolites of testosterone and their measurement for assessing gonadal endocrine function in male nonhuman primates. Gen Comp Endocrinol 2002;129:135–45.
[25] Schatz S, Palme R. Measurement of faecal cortisol metabolites in cats and dogs: a non-invasive method for evaluating adrenocortical function. Vet Res Commun 2001;25:271–87.
[26] Preis A, Mugisha L, Hauser B, Weltring A, Deschner T. Androgen and androgen metabolite levels in serum and urine of east African chimpanzees (*Pan troglodytes schweinfurthii*). Comparison of EIA and LC-MS analyses. J Comp Endocrinol in press.
[27] Kutsukake N, Ikeda K, Honma S, Teramoto M, Mori Y, Hayasaka I, et al. Validation of salivary cortisol and testosterone assays in chimpanzees by liquid chromatography–tandem mass spectrometry. Am J Primatol 2009;71:696–706.
[28] Gesquiere LR, Onyango PO, Alberts SC, Altmann J. Endocrinology of year-round reproduction in a highly seasonal habitat: environmental variability in testosterone and glucocorticoids in baboon males. Am J Phys Anthropol 2011;144:169–76.
[29] Girard-Buttoz C, Heistermann M, Krummel S, Engelhardt A. Seasonal and social influences on fecal androgen and glucocorticoid excretion in wild male long-tailed macaques (*Macaca fascicularis*). Physiol Behav 2009;98:168–75.
[30] Mehta PH, Josephs RA. Testosterone and cortisol jointly regulate dominance: evidence for a dual-hormone hypothesis. Horm Behav 2010;58:898–906.
[31] Chame M. Terrestrial mammal feces: a morphometric summary and description. Mem Inst Oswaldo Cruz 2003;98(Suppl. 1):71–94.
[32] Jacobsen R, Lorenzen JK, Toubro S, Krog-Mikkelsen I, Astrup A. Effect of short-term high dietary calcium intake on 24-h energy expenditure, fat oxidation, and fecal fat excretion. Int J Obes 2005;29:292–301.
[33] Meillet D, Raichvarg D, Tallet F, Savel J, Yonger J, Gobert JG. Measurement of total, monomeric and polymeric IgA in human faeces by electroimmunodiffusion. Clin Exp Immunol 1987;69:142–7.
[34] Lin DS, Connor WE. Fecal steroids of the coprolite of a Greenland Eskimo mummy, AD 1475: a clue to dietary sterol intake. Am J Clin Nutr 2001;74:44–9.
[35] Taylor W. The excretion of steroid hormone metabolites in bile and feces. Vitam Horm 1971;29:201–85.
[36] MacDonald IA, Bokkenheuser VD, Winter J, McLernon AM, Mosbach EH. Degradation of steroids in the human gut. J Lipid Res 1983;24:675–700.
[37] Heistermann M. Non-invasive monitoring of endocrine status in laboratory primates: methods, guidelines and applications. Adv Sci Res 2010;1:1–9.
[38] Ziegler TE, Scheffler G, Wittwer DJ, Schultz-Darken N, Snowdon CT, Abbott DH. Metabolism of reproductive steroids during the ovarian cycle in two species of callitrichids, *Saguinus oedipus* and *Callithrix jacchus*, and estimation of the ovulatory period from fecal steroids. Biol Reprod 1996;54:91–9.
[39] Wasser SK, Monfort SL, Southers J, Wildt DE. Excretion rates and metabolites of oestradiol and progesterone in baboon (*Papio cynocephalus cynocephalus*) faeces. J Reprod Fert 1994;101:213–20.
[40] Ziegler TE, Wittwer DJ. Fecal steroid research in the field and laboratory: improved methods for storage, transport, processing, and analysis. Am J Primatol 2005;67:159–74.
[41] Bahr NI, Palme R, Möhle U, Hodges JK, Heistermann M. Comparative aspects of the metabolism and excretion of cortisol in three individual nonhuman primates. Gen Comp Endocrinol 2000;117:427–38.
[42] Volin P. High-performance liquid chromatographic analysis of corticosteroids. J Chromatogr B 1995;671:319–40.
[43] Heistermann M, Finke M, Hodges JK. Assessment of female reproductive status in captive-housed hanuman langurs (*Presbytis entellus*) by measurement of urinary and fecal steroid excretion patterns. Am J Primatol 1995;37:275–84.
[44] Hennion M-C. Solid-phase extraction: method development, sorbents, and coupling with liquid chromatography. J Chromatogr A 1999;856:3–54.
[45] MacDougall D, Crummett WB. Guidelines for data acquisition and data quality evaluation in environmental chemistry. Anal Chem 1980;52:2242–9.
[46] Wasser SK, Papageorge S, Foley C, Brown JL. Excretory fate of estradiol and progesterone in the African elephant (*Loxodonta africana*) and patterns of fecal steroid concentrations throughout the estrous cycle. Gen Comp Endocrinol 1996;102:255–62.
[47] Fedigan LM, Rose LM, Avila RM. See how they grow. Tracking capuchin monkey (*Cebus capucinus*) populations in a regenerating Costa Rican dry forest. In: Norconk MA, Rosenberger AL, Garber PA, editors. Adaptive Radiations of Neotropical Primates. New York: Plenum Press; 1996. p. 289–307.
[48] Castro DC, Sousa MBC. Fecal androgen levels in common marmoset (*Callithrix jacchus*) males living in captive family groups. Braz J Med Biol Res 2005;38:65–72.
[49] Raminelli JLF, de Sousa MBC, Cunha MS, Barbosa MFV. Morning and afternoon patterns of fecal cortisol excretion among reproductive and non-reproductive male and female common marmosets, *Callithrix jacchus*. Biol Rhythm Res 2001;32:159–67.
[50] Sousa MBC, Ziegler TE. Diurnal variation on the excretion patterns of fecal steroids in common marmoset (*Callithrix jacchus*) females. Am J Primatol 1998;46: 105–17.
[51] Albuquerque ACSR, Sousa MBC, Santos HM, Ziegler TE. Behavioral and hormonal analysis of social relationships between oldest females in a wild monogamous group of common marmosets (*Callithrix jacchus*). Int J Primatol 2001;22:631–45.
[52] Beehner JC, Gesquiere L, Seyfarth RM, Cheney DL, Alberts SC, Altmann J. Testosterone related to age and life-history stages in male baboons and geladas. Horm Behav 2009;56:472–80.
[53] Coe CL, Chen J, Lowe EL, Davidson JM, Levine S. Hormonal and behavioral changes at puberty in the squirrel monkey. Horm Behav 1981;15:36–53.
[54] Hauser B, Mugisha L, Preis A, Deschner T. LC–MS analysis of androgen metabolites in serum and urine from east African chimpanzees (*Pan troglodytes schweinfurthii*). Gen Comp Endocrinol 2011;170:92–8.
[55] French JA, Koban T, Rukstalis M, Ramirez SM, Bardi M, Brent L. Excretion of urinary steroids in pre- and postpartum female baboons. Gen Comp Endocrinol 2004;137:69–77.
[56] Hodgen GD, Stouffer RL, Barber DL, Nixon WE. Serum estradiol and progesterone during pregnancy and the status of the corpus luteum at delivery in cynomolgus monkeys (*Macaca fascicularis*). Steroids 1977;30:295–301.
[57] Bales KL, French JA, Hostetler CM, Dietz JM. Social and reproductive factors affecting cortisol levels in wild female golden lion tamarins (*Leontopithecus rosalia*). Am J Primatol 2005;67:25–35.
[58] Saltzman W, Maestripieri D. The neuroendocrinology of primate maternal behavior. Prog Neuropsychopharmacol Biol Psychiatry 2011;35:1192–204.
[59] Smith TE, French JA. Social and reproductive conditions modulate urinary cortisol excretion in black tufted-ear marmosets (*Callithrix kuhli*). Am J Primatol 1997;42: 253–67.
[60] Ziegler TE, Scheffler G, Snowdon CT. The relationship of cortisol levels to social environment and reproductive functioning in female cotton-top tamarins, *Saguinus oedipus*. Horm Behav 1995;29:407–24.
[61] Kromidas S. Validierung in der Analytik. Weinheim: Wiley-VCH; 1999.
[62] Zondek B. Mass excretion of oestrogenic hormone in the urine of the stallion. Nature 1934;133:209–10.
[63] Velle W. Urinary oestrogens in the male. J Reprod Fert 1966;12:65–73.
[64] Nunes S, Brown C, French JA. Variation in circulating and excreted estradiol associated with testicular activity in male marmosets. Am J Primatol 2002;56:27–42.
[65] Ziegler TE, Carlson AA, Ginther AJ, Snowdon CT. Gonadal source of testosterone metabolites in urine of male cotton-top tamarin monkeys (*Saguinus oedipus*). Gen Comp Endocrinol 2000;118:332–43.
[66] Hodges JK, Eastman SAK. Monitoring ovarian function in marmosets and tamarins by the measurement of urinary estrogen metabolites. Am J Primatol 1984;6: 187–97.
[67] Ziegler TE, Peterson LJ, Sosa ME, Barnard AM. Differential endocrine responses to infant odors in common marmoset (*Callithrix jacchus*) fathers. Horm Behav 2011;59:265–70.
[68] Sohl CD, Guengerich FP. Kinetic analysis of the three-step steroid aromatase reaction of human cytochrome P450 19A1. J Biol Chem 2010;285:17734–43.
[69] Goddard P, Hill MJ. Degradation of steroids by intestinal bacteria: IV. The aromatisation of ring A. Biochim Biophys Acta 1972;280:336–42.
[70] Hodges JK, Gulick BA, Czekala NM, Lasley BL. Comparison of urinary oestrogen excretion in South American primates. J Reprod Fert 1981;61:83–90.
[71] Abbott DH, Bird IM. Nonhuman primates as models for human adrenal androgen production: function and dysfunction. Rev Endocr Metab Disord 2009;10: 24–33.
[72] Pattison JC, Saltzman W, Abbott DH, Hogan BK, Nguyen AD, Husen B, et al. Gender and gonadal status differences in zona reticularis expression in marmoset monkey adrenals: cytochrome b5 localization with respect to cytochrome P450 17,20-lyase activity. Mol Cell Endocrinol 2007;265–266:93–101.
[73] Seraphin SB, Whitten PL, Reynolds V. The influence of age on fecal steroid hormone levels in male Budongo Forest chimpanzees (*Pan troglodytes schweinfurthii*). Am J Primatol 2008;70:661–9.
[74] Remer T, Maser-Gluth C, Boye KR, Hartmann MF, Heinze E, Wudy SA. Exaggerated adrenarche and altered cortisol metabolism in Type 1 diabetic children. Steroids 2006;71:591–8.
[75] Wudy SA, Hartmann MF, Remer T. Sexual dimorphism in cortisol secretion starts after age 10 in healthy children: urinary cortisol metabolite excretion rates during growth. Am J Physiol Endocrinol Metab 2007;293:E970–6.
[76] Adlercreutz H, Järvenpää P. Assay of estrogens in human feces. J Steroid Biochem 1982;17:639–54.
[77] Eriksson H, Gustafsson J-A. Excretion of steroid hormones in adults. Eur J Biochem 1971;18:146–50.
[78] Ziegler TE, Sholl SA, Scheffler G, Haggerty MA, Lasley BL. Excretion of estrone, estradiol, and progesterone in the urine and feces of the female cotton-top tamarin (*Saguinus oedipus oedipus*). Am J Primatol 1989;17:185–95.
[79] Commission Decision 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (notified under document number C(2002) 3044). Off J Eur Communities 2002;L221:8–36.
[80] Horwitz W. Evaluation of analytical methods used for regulation of foods and drugs. Anal Chem 1982;54:67A–76A.

[81] Hunt KE, Wasser SK. Effect of long-term preservation methods on fecal glucocorticoid concentrations of Grizzly bear and African elephant. Physiol Biochem Zool 2003;76:918–82.
[82] Kahn MZ, Altmann J, Isani SS, Yu J. A matter of time: evaluating the storage of fecal samples for steroid analysis. Gen Comp Endocrinol 2002;128:57–64.
[83] Yalkowsky SH. Solubility and solubilization in aqueous media. Washington, D. C.: American Chemical Society & Oxford University Press; 1999
[84] Terio KA, Brown JL, Moreland R, Munson L. Comparison of different drying and storage methods on quantifiable concentrations of fecal steroids in the cheetah. Zoo Biol 2002;21:215–22.
[85] Wasser SK, Risler L, Steiner RA. Excreted steroids in primate feces over the menstrual cycle and pregnancy. Biol Reprod 1988;39:862–72.
[86] Groh H, Schade K, Hörhold-Schubert C. Steroid metabolism with intestinal microorganisms. J Basic Microbiol 1993;33:59–72.
[87] Wallner B, Möstl E, Dittami J, Prossinger H. Fecal glucocorticoids document stress in female Barbary macaques (*Macaca sylvanus*). Gen Comp Endocrinol 1999;113:80–6.