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Androgen and androgen metabolite levels in serum and urine of East African chimpanzees (*Pan troglodytes schweinfurthii*): Comparison of EIA and LC–MS analyses

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ABSTRACT

The primary male androgen testosterone (T) is often used as an endocrinological marker to investigate androgen-behaviour interactions in males. In chimpanzees and bonobos, studies investigating the relationship between T levels and dominance rank or aggressive behaviour have revealed contradictory results. The immunoassays used in these studies were originally developed for the measurement of steroids in serum. Their application to non-invasively collected samples, however, can lead to methodological problems due to cross-reacting metabolites, which might occur in urine or faeces but not in blood. The overall aim of this study, therefore, is to clarify whether a T enzyme immunoassay (EIA) is an applicable method to monitor testicular function in adult male chimpanzees. To estimate the impact of cross-reacting androgens on the used T EIA, we compared the results of an EIA measurement with a set of androgen metabolite levels measured by LC–MS. In urine from male chimpanzees, cross-reactivities appear to exist mainly with T and its exclusive metabolites, 5α-dihydrotestosterone (5α-DHT) and 5α-androstanediol (androstanediol). Both urinary and serum T levels of male chimpanzees were significantly higher than female T levels when measured with the T EIA, indicating a reliable measurement of testicular androgens and their exclusive metabolites with the used EIA. In urine from female chimpanzees, the comparison between LC–MS and T EIA results indicated a higher impact of cross-reactions with adrenal androgen metabolites. Therefore, the investigation of urinary T levels in female chimpanzees with a T EIA seems to be problematic. Overall our results show that a T EIA can be a reliable method to monitor testicular function in male chimpanzee urine and that LC–MS is a valuable tool for the validation of immunoassays.

1. Introduction

Testosterone (T) is the primary biologically active male androgen. It has modulating effects on morphology, physiology, and behaviour in many vertebrate species, including primates. Through its organizational effects during early ontogeny and puberty, T mediates the differentiation of the primary and a number of secondary male sexual characteristics [48]. Activational effects of T during adulthood are related to traits that enhance male reproductive success, such as mating behavior, male–male aggression, dominance relationships, and territory defence [5,12,22,67]. In a number of non-seasonal group-living primate species, dominance relationships and levels of aggression are positively correlated with T levels in unstable periods [4,55,58]. T is therefore used as an endocrinological marker to investigate androgen-behaviour interactions in males, which are related to reproductive effort and success.

For ethical and practical reasons, in most field studies on wild-living primates, endocrine profiles are monitored using non-invasively collected samples such as urine or faeces. Darting of free-living animals is risky, especially for arboreal species, and unpractical since the darting itself influences the steroid levels in the bloodstream [46,59]. Furthermore, measurements of steroids in serum or plasma are influenced by the pulsatile secretion of hormones [66]. Therefore, faeces and urine are easily collected sample mediums that represent an average of circulating hormone levels across days or hours, respectively. However, steroid measurements in these samples are subject to methodological problems since steroid hormones are highly metabolised before their excretion [49]. The resulting metabolites can differ enormously between species

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and even between sexes [41,51]. Furthermore, due to the metabolisation of steroids in the liver and kidneys prior to excretion, steroids in urine do not occur in the same ratios as they do in the circulation. For example, biologically active T occurs in serum of primate males in higher concentrations than other androgens and their metabolites while T as a urinary androgen is quantitatively of only minor importance [21,23,41,60]. Despite this, urinary T concentrations in humans are known to parallel biologically active T in serum [10,52]. Furthermore, although T is only a minor component of urinary androgen metabolites, it correlates best with serum T in adult male chimpanzees [24]. As in humans, two additional urinary androgen metabolites, 5α-dihydrotestosterone (5α-DHT) and 5α-androstanediol (androstanediol), seem to derive exclusively from circulating T in male chimpanzees [24,64,65]. Therefore, together with T, these androgens are likely to be suitable urinary markers for testicular function in male chimpanzees [24].

Immunoassays, which function through a specific antibody–antigen reaction, are a commonly used method for steroid analyses. The majority of these assays were originally developed and validated for the analysis of steroids in blood, where gonadal and adrenal steroids occur in high concentrations in their biologically active forms. Due to these high concentrations, the impact of cross-reactivities with structurally similar antigens (e.g., metabolites) that also bind to the specific antibody of the assay are generally negligible. When these assays are adapted to the measurement of steroids in urine and faecal extracts, where metabolites usually occur in much higher concentrations than the biologically active steroid, formerly negligible levels of cross-reactivities can reach significant dimensions [2,51,63]. Still, existing cross-reactivities might not negatively influence the accuracy of the measurement of gonadal functioning as long as the cross-reacting metabolites indicate the same function as the steroid of interest, such as a shared testicular origin. However, cross-reactions with structurally similar but physiologically different hormones can have a major impact. For example, for the assessment of testicular function and activity in males, the co-measurement of adrenal androgens and their metabolites has to be avoided and vice versa. Such a co-measurement has been shown for a group-specific glucocorticoid assay where a major faecal androgen metabolite cross-reacted with the group-specific antibody [17,26].

Thus, before an immunoassay is used for the measurement of steroids in urine or faecal extracts, a careful validation is of paramount importance [25,43,49,51,63].

In male chimpanzees and bonobos, studies investigating the relationship between urinary T levels, aggression, and dominance relationships have revealed contradictory results. While T levels in wild-living East African male chimpanzees are positively correlated with rank and aggression, irrespective of periods of instability, this pattern was absent in a group of captive chimpanzees [32,45]. Although the observed differences can be related to environmental and group size differences between the two study groups (11 males vs. 4 males; wild vs. zoo) [32], methodological problems in the T measurement could not be excluded. In a carefully conducted radio metabolism study, a 17β-OH EIA (T EIA) did not produce significant differences between the urinary androgen levels of male and female chimpanzees [41]. It was assumed that a high co-measurement of adrenal androgens (DHEA) with the T assay led to falsely elevated androgen levels in females [41].

Significant differences in urinary androgen levels between male and female chimpanzees and bonobos were found using a 17a-OH EIA [56]. However, another study using the same assay could not confirm these results for male and female bonobos [11]. Finally, while a positive correlation between urinary T concentrations and dominance rank of the long-term resident males was found in a group of wild-living bonobos [36], urinary T levels were not related to dominance rank in either of the two sexes in captive bonobos [57]. In the aforementioned studies, a number of different immunoassays for assessing testicular activity in males and T levels in females were used producing partly contradictive results.

In light of these opposing results, there is a need for further investigation to distinguish testicular and adrenal androgens and their metabolites in chimpanzee urine and to identify a reliable method to measure them. The overall aim of this study, therefore, is to investigate whether a T EIA reliably monitors testicular function in adult male chimpanzees. To estimate the impact of other androgens and androgen metabolites in serum and urine of male and female chimpanzees on the results of the T assay, we compared two different analytic methods. We used a T EIA system obtained from the Institute of Biochemistry and Ludwig Boltzmann Institute of Veterinary Endocrinology in Vienna and liquid chromatography–tandem mass spectrometry (LC–MS). The latter method is highly specific and allows one to analyse a number of metabolites in parallel [23].

To test if the measurement of chimpanzee urine samples with a T EIA can indeed reliably measure testicular activity, we aimed to answer the following questions:

(1) How do T levels differ between the two methods?
(2) How do serum and urinary T levels differ between males and females when measured with LC–MS and EIA?
(3) How do urinary androgen levels measured with a T EIA correlate with testicular androgens and their metabolites measured with LC–MS?
(4) How do urinary T levels correlate between EIA and LC–MS measurements in sexually mature female chimpanzees?

2. Materials and methods

2.1. Animals

Urine and serum samples were obtained from chimpanzees living in the Ngamba Island chimpanzee sanctuary in Uganda. A total of 42 chimpanzees live in this sanctuary and all are orphans that were confiscated from poachers and bush meat dealers. At the time of sample collection, all individuals were kept in one social group except for two males, who were kept separate from the others. Altogether there were 16 adult and adolescent males (8–24 years of age), two of which were castrated, three juvenile males (3 years of age), and 23 adolescent and adult females (6–24 years of age). During the day, the chimpanzees were kept outside and were able to range freely in a forested fenced-in area. During the night they stayed in indoor enclosures. They were provisioned with fruit, vegetables, and water.

2.2. Sample collection

Serum samples were collected during a yearly health check in February and March 2008. For the health check the chimpanzees were anaesthetised with a combination of ketamine (3 mg/kg) and meditomidine (0.03 mg/kg). Sedation was carried out with a blowgun or by hand when animals were familiar with the keepers. One 7.5 ml blood sample was taken from each chimpanzee 10–15 min after the beginning of the anesthetisation. The samples were centrifuged immediately and serum was stored at −80 °C.

A total of 89 urine samples were collected from the anesthetised chimpanzees 30–45 min after the onset of anesthetisation and also at different times during the 2 month health check period. During anaesthesia, urine samples for some of the chimpanzees were obtained by catheterisation or abdominal manipulation. The other urine samples were collected in the indoor enclosures before the chimpanzees were released to the outdoor enclosures. Sampling was carried out with a collecting vessel and normally took

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place between 7:00 and 8:00 am. The collected urine was stored in 2 ml tubes at −80 °C. The samples were transported from Uganda to the MPI-EVA in Leipzig, Germany, on dry ice. At the MPI-EVA, the samples were stored at −20 °C until extraction.

2.3. Extraction of steroids

2.3.1. Serum

The extraction of steroids from serum followed the protocol of Hauser et al. (2011) [24]. We used the same extraction procedure for the enzyme immunoassay (EIA) as well as for the high-performance liquid chromatography–tandem mass spectrometry (LC–MS) measurements. To 200 µl of serum, we added 400 µl acetonitrile and 50 µl internal standard mixtures, which contained 10 pg/µl each of prednisolon, d4-estrone, d3-testosterone, and d9-progesterone (Dr. Ehrenstorfer, Augsburg, Germany). The internal standard mixture was only added to the serum extracts for LC–MS measurements to control for losses during extraction and purification, and matrix effects on ionisation of MS measurements. Samples were vortexed for 1 min and centrifuged for 10 min at 18000*g*. The supernatant of the precipitated protein pellet was aspirated and diluted with 3 ml deionised water. This was followed by a solid phase extraction with C 18 cartridges (Chromabond C 18ec, 200mg, Macherey and Nagel, Dueren, Germany). Conditioning was carried out with 3 ml methanol and 3 ml deionised water (HPLC grade, Mallinckrodt Baker, Phillipsburg, NJ, USA). After the serum extract was applied, the cartridge was washed with 5 ml deionised water (HPLC grade) and 3 ml 20% methanol and dried for 30 min. Elution of steroids was carried out with 3 ml methanol. The eluate was evaporated to dryness and reconstituted with 300 µl acetonitrile and was then vortexed and centrifuged for 2 min. The extract was then transferred to a 650 µl Eppendorf tube and evaporated in a Speedvac. The residue was redissolved in 50 µl 30% acetonitrile and transferred to a 150 µl insert of a HPLC vial. Extracts for the EIA measurement were stored in Eppendorf tubes until further use.

2.3.2. Urine

The steroids from the urine samples were extracted as described in detail by Hauser et al. [23,25]. We used 200 µl urine for steroid extraction and added 50 µl internal standard mixture only to extractions for the LC–MS measurement. The internal standard mixtures contained the same deuterated standards as described for serum above but with different concentrations (100 ng/µl). Steroid glucuronides were hydrolysed using β-glucuronidase from *Escherichia coli* (activity: 200 U/40 µl) (Sigma Chemical Co., St. Louis, MO, USA). β-Glucuronidase from *E. coli* was used for hydrolysis and a separate solvolysis was conducted in order to avoid enzymatic conversion of androgens as described for β-glucuronidase/sulphatase of *Helix pomatia* juice [25,37,38,64]. Extracts were purified by solid phase extractions (Chromabond C18ec, 200 mg, Macherey-Nagel, Dueren, Germany). Afterwards, steroid sulphates were cleaved by solvolysis with ethyl acetate/sulphuric acid (200 mg sulphuric acid, 98% in 250 ml ethyl acetate). Extraction of steroids was carried out with tert. butyl methyl ether, evaporated and reconstituted in 30% acetonitrile. Extraction efficiencies ranged from 60.4% to 103% [23].

2.4. Analytical methods

2.4.1. LC–MS

LC measurements were carried out using a Waters Alliance 2695 separation module equipped with a quaternary pump and a column oven (Waters, Milford, MA, USA) and separation was performed on a reverse phase C-18 material (Gemini; 150 × 2mm, 3 µm) with gradient elution (acetonitrile/water and 0.1% formic acid) [23]. MS analyses were carried out on a Quattro Premier XE tandem mass spectrometer (Micromass, Manchester, UK) with an electro spray interface (ESI) in positive mode [23].

The quantitative analysis of 11 urinary and five serum androgens by LC–MS was realised in the range of 0.3 – 1000 ng/µl [23,25] (Table 1). We excluded samples that had an internal standard recovery of more than ±50% from the expected values from our analysis. We examined LC–MS data with MassLynx (Version 4.1; QuanLynx-Software).

2.4.2. EIA

For the measurement of urinary and serum androgens with EIA, we used a T EIA, which detects 17β-OH-androgens and their metabolites. Antisera for this T assay were raised in rabbits against testosterone-3-CMO [41,50]. As label we used 5α-androstane-3β, 17β-diol-3-Hemisuccinat-DADOO-Biotin, and T (0.31-40 pg/well) as standard [40,41,50]. Incubation of strepatvidin and substrate solution took place for 45 min at 4 °C.

Assay procedures and cross-reactivities have been described in detail [40,41,50] (Table 2). In addition, we tested the cross reactivity of three more androgen metabolites, DHEA (androst-5-ene-3β-ol-17-one), androstenedione (androst-4-ene-3,17-dione; A^4dione), and androstenediol (androst-5-ene-3β,17β-diol; A^5diol). Cross-reactivity was determined by detecting the concentration of the tested substance at 50% binding in the T EIA through a duplicate dilution series. This concentration was then compared with the corresponding concentration of the T standard at 50% binding and expressed in percentages [40]. Cross-reactivities of the T antibody were 0.01% for DHEA, 1.6% for A^4dion, and 1.9% for A^5diol (Table 2).

The inter-assay coefficients of variation (CV) of high and low concentration quality controls were 2.7% and 11.9% for serum extracts and 12.4% and 13.3% for urine extracts, while intra-assay CVs were 9.5% and 12.3%, respectively.

Urinary androgens and their metabolites were indexed against creatinine to compensate for variations in urine concentration [2,14]. Therefore, urinary steroids are expressed as ng/mg creatinine (ng/mg crea). Creatinine was measured by microtiter plate analysis based on the Jaffé reaction [23,62].

2.5. Statistical analysis

For the comparison of serum T levels between the two methods and sexes, 16 serum samples from 16 females between 7 and 24 years of age and 12 serum samples from 12 males between 8 and 22 years of age were used. For urine analysis, we used a total of 24 samples from 12 males and 22 samples from 15 females. We only included sexually mature individuals in our statistical analyses. Captive chimpanzees reach sexual maturity at around 7 years of age [7,42].

We used only urine samples that were collected between 6:00 and 8:00 a.m. in order to avoid diurnal effects of hormone concentrations in urine [44,45]. To compare T levels between the two methods and sexes, we used mean values per individual. All statistical analyses were carried out with non-parametric tests. When sample sizes were small, we used exact tests [47].

To investigate the comparability of T levels measured with EIA and with LC–MS in urine of male and female chimpanzees and to detect cross-reactivities of the T EIA, we calculated Spearman rank correlations [61].

Since we had different numbers of urine samples from each of the study individuals (ranging from 1 to 5), we had to avoid pseudo-replication [30]. Therefore, we used a program written in R (Version 2.8.1) by Mundry to choose one urine sample per individual by chance. With these random selections, a Spearman rank correlation between different measures of T levels was calculated.

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Table 1
Investigated androgens and their metabolites measured in serum (S) and urine (U) with LC–MS. QL = quantitation limits of investigated steroids.

Trivial name	Systematic name (IUPAC)	Abbreviation	QL (ng/ml)	Sample medium
Testosterone	17β-Hydroxyandrost-4-ene-3-one	T	0.3	S, U
Epitestosterone	17α-Hydroxyandrost-4-ene-3-one	–	0.3	U
Androstenedione	Androst-4-ene-3,17-dione	A^4dione	0.3	S, U
Androstenediol	Androst-5-ene-3β,17β-diol	A^5diol	3	S, U
Dehydroepiandrosterone	3β-Hydroxyandrost-5-ene-17-one	DHEA	1	S, U
5α-Dihydrotestosterone	17β-Hydroxy-5α-androstane-3-one	5α-DHT	0.5	S, U
5α-Androstanediol	5α-Androstane-3α,17β-diol	Androstanediol	0.5	U
Etiocholanolon	3α-Hydroxy-5β-androstane-17-one	–	1	U
Androsterone	3α-Hydroxy-5α-androstane-17-one	–	1	U
Epietiocholanolone	3β-Hydroxy-5β-androstane-17-one	–	1	U
Epiandrosterone	3β-Hydroxy-5α-androstane-17-one	–	1	U

Table 2
Cross-reactivities of the used testosterone antibody.

Systematic name	Trivial name	Percentage (%)	Tested by
17β-Hydroxyandrost-4-ene-3-one	Testosterone	100	Palme and Möstl [50]
7β-Hydroxy-5α-androstane-3-one	5α-Dihydrotestosterone	23.7	Palme and Möstl [50]
17β-Hydroxy-5β-androstane-3-one	5β-Dihydrotestosterone	12.3	Palme and Möstl [50]
Androst-4-ene-3β,17β-diol	4-Androstenediol	7.6	Palme and Möstl [50]
5α-Androstane-3α,17β-diol	Androstanediol	5.5	Palme and Möstl [50]
5α-Androstane-3β,17β-diol	3β-Androstanediol	1.3	Palme and Möstl [50]
5β-Androstane-3α,17β-diol	5β-Androstanediol	1.1	Palme and Möstl [50]
17α-Hydroxyandrost-4-ene-3-one	Epitestosterone	<0.1	Palme and Möstl [50]
3α-Hydroxy-5α-androstane-17-one	Androsterone	<0.1	Palme and Möstl [50]
5α-Androstane-17α-ol-3-one		<0.1	Palme and Möstl [50]
3β-Hydroxyandrost-5-ene-17-one	DHEA	<0.1	Palme and Möstl [50]
Androst-4-ene-3,17-dione	A^4dione	1.6	Ourselves
Androst-5-ene-3β,17β-diol	A^5diol	1.9	Ourselves

This procedure was repeated 1000 times in order to avoid results being dependent on a single random selection. If there was only one sample for an individual, this was used for each correlation. At the end, the program calculated mean statistical parameters such as *r* and *p* values derived from all random selections, which were then used as final results.

Testing the same null-hypothesis (e.g., no correlation between T (EIA) and any of the androgens and their metabolites (LCMS)) several times required some correction for multiple testing. We achieved this using Fischer's omnibus test. This procedure combines a number of *P*-values into a single chi-square distributed variable with degrees of freedom equalling twice the number of *P*-values [20,54]. If this is significant, it means that the collective of correlations is significant. However, since several correlations between variables obtained from the same set of individuals are not independent, an essential assumption of this procedure was not met. We hence established significance based on a permutation procedure [1,35]. For this we randomised the variable to be correlated with all others, derived all its correlations with the other variables and combined the obtained *P*-values into a chi-square as for the original data [53]. We repeated this 1000 times and, from this, obtained the expected distribution of the chi-square under the assumption that the null-hypothesis was true (no correlation between the variable and any of the others). Since this distribution was derived with our original, non-independent data, it is an appropriate sampling distribution. We then determined the summarised *P*-value of all correlations by determining the number of permutations revealing a chi-square at least as large as that derived for the original data (which we included as one permutation). Since for male chimpanzees, we assumed we would only find significant correlations between T and its exclusive metabolites (DHT and androstanediol), we decided to split the correction for multiple testing into two separate sets: one set with testicular androgens and their metabolites (T, DHT and androstanediol) for which, based on data from humans, our prediction was that their levels should correlate with testosterone and the other set with adrenal androgen metabolites or metabolites that could derive from both testis and adrenal gland (DHEA, epitestosterone, androstenedione, epiandrosterone, epietiocholanolone, etiocholanolone, androsterone and androstenediol) for which we did not predict a correlation with testosterone. Because of these conflicting predictions, testing for multiple testing with all correlations pooled did not make sense. For females we could not perform such a splitting due to the less known metabolism of circulating testosterone.

3. Results

3.1. T levels in serum

T levels measured with EIA in male serum were significantly higher than measured with LC–MS (Wilcoxon signed-rank test: $T = 77.0$, $N = 12$, $p < 0.001$). The EIA produced 1.4-fold higher T levels than the LC–MS (Table 3). In female serum, the mean T level was 0.89 ± 0.33 ng/ml measured with EIA and 0.11 ± 0.06 ng/ml measured with LC–MS. In female serum, T levels of the EIA measurement were significantly higher than T levels measured with LC–MS (Wilcoxon signed-rank test: $T = 136.0$, $N = 15$, $p < 0.001$); in our study, the EIA produced 8.9-fold higher T levels than the LC–MS measurement.

With both methods, T levels of males were significantly higher than those of females (Mann–Whitney U test, males $N = 12$, females $N = 16$; EIA: $U = 0.0$, $p < 0.001$; LC–MS: $U = 0.0$, $p < 0.001$).

Of all androgens and androgen metabolites measured with LC–MS in male serum, T, together with DHEA, had the highest proportions of 36% each (Table 3). In female serum, DHEA had the highest

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Table 3
Androgen and androgen metabolite concentrations in serum. The proportion (%) of each urinary androgen or androgen metabolite measured with LC–MS was calculated from the total concentration of androgens and their metabolites analysed with LC–MS.

Males ($N = 12$)				Females ($N = 16$)		
Androgens (ng/ml)	Mean	SD (±)	Proportion (%)	Mean	SD (±)	Proportion (%)
T (EIA)	5.37	2.21		0.89	0.33	
T (LC–MS)	3.86	1.86	35.94	0.11	0.06	1.80
A^5diol (LC–MS)	1.98	1.25	18.39	0.56	0.28	9.33
DHEA (LC–MS)	3.92	2.27	36.43	4.55	2.72	75.99
A^4dione (LC–MS)	0.43	0.14	3.95	0.45	0.28	7.60
5α-DHT (LC–MS)	0.57	0.16	5.29	0.32	0.12	5.28
Total (LC–MS)	10.75		100	5.98		100

Table 4
Androgen and androgen metabolite concentrations in urine. The proportion (%) of each urinary androgen or androgen metabolite measured with LC–MS was calculated from the total concentration of androgens and their metabolites analysed with LC–MS.

Males ($N = 12$)				Females ($N = 16$)		
Androgens (ng/mg crea)	Mean	SD (±)	Proportion (%)	Mean	SD (±)	Proportion (%)
T (EIA)	104.30	38.52		47.25	26.78	
T (LC–MS)	39.36	25.68	1.59	6.11	2.91	0.27
DHEA (LC–MS)	938.38	689.25	37.90	1436.77	1048.20	64.15
Epitestosterone (LC–MS)	15.15	9.17	0.61	5.63	3.88	0.25
A^4dione (LC–MS)	19.55	25.05	0.79	11.56	14.84	0.52
Epiandrosterone (LC–MS)	53.07	40.45	2.14	24.58	15.76	1.10
Epietiocholanolone (LC–MS)	16.60	11.15	0.67	10.26	11.15	0.46
Etiocholanolone (LC–MS)	738.54	255.57	29.83	287.90	109.62	12.85
Androsterone (LC–MS)	426.75	178.77	17.24	184.27	89.79	8.23
A^5diol (LC–MS)	203.10	113.59	8.20	261.62	144.79	11.68
Androstanediol (LC–MS)	19.50	10.09	0.79	8.20	3.96	0.37
5α-DHT (LC–MS)	6.04	3.16	0.24	2.83	1.67	0.13
Total (LC–MS)	2476.05		100	2239.73		100

proportion of all androgens and their metabolites measured with LC–MS whereas T was only a minor component (1.8%). A^4 dione in males and 5α-DHT in females had the lowest proportions of all androgens and androgen metabolites measured in serum.

3.2. T levels in urine

Male urinary T levels were significantly higher when measured with EIA than with LC–MS (Wilcoxon signed-rank test: $T = 78.0$, $N = 12$, $p < 0.001$). Overall, the EIA detected 2.7-fold higher urinary T levels as the LC–MS (Table 4). In female urine, the EIA detected 7.4-fold higher T levels than the LC–MS, by this the EIA measurement revealed significantly higher T levels than the LC–MS (Wilcoxon signed-rank test: $T = 120.0$, $N = 15$, $p < 0.001$). Within both methods, male urinary T levels were significantly higher than female urinary T levels (Mann–Whitney U test, males $N = 12$, females $N = 15$; EIA: $U = 13.0$, $p < 0.001$; LC–MS: $U = 0.0$, $p < 0.001$).

Of all urinary androgens and androgen metabolites measured with LC–MS, T had a proportion of 1.59% in males and 0.27% in females. In the urine of both sexes, DHEA had the highest proportion of all androgens and their metabolites measured with LC–MS, whereas 5α-DHT had the lowest.

3.3. Correlations between male urinary T levels measured with EIA and urinary androgen metabolites measured with LC–MS

Overall, the collective of correlations between T EIA and putative testicular androgens of the LC–MS measurement was significant (chi-square combining P-values of spearman correlations = 22.10, permutation test: $p = 0.008$). The collective of the correlations between T EIA and adrenal androgen metabolites or metabolites that could derive from both the testis and adrenal gland was not significant (chi-square combining P-values of spearman correlations = 10.71, permutation test: $p = 0.71$).

Table 5
Spearman rank correlations between urinary T concentrations measured with EIA and urinary androgen concentrations of the LC–MS analysis of 12 males between 8 and 22 years of age. Correlations of T levels measured with LC–MS with other LC–MS androgens and androgen metabolites were used as reference method in order to control for physiological connections between androgen metabolites which are excreted into urine. Significant correlations are highlighted with bold script.

Androgens (LC–MS)	N	T (EIA)		T (LC–MS)	
		r	p	r	p
T	12	0.74	**0.011**		
DHEA	12	−0.37	0.27	−0.25	0.47
Epitestosterone	11	0.25	0.47	0.17	0.56
A^4dione	12	0.30	0.35	−0.03	0.71
Epiandrosterone	12	0.06	0.69	−0.10	0.60
Epietiocholanolone	12	−0.02	0.56	−0.21	0.44
Etiocholanolone	12	0.02	0.69	0.11	0.52
Androsterone	12	0.25	0.44	0.41	0.22
A^5diol	12	0.13	0.64	−0.08	0.64
Androstanediol	12	0.59	0.06	0.65	**0.04**
5α-DHT	12	0.70	**0.02**	0.26	0.46
T + Androstanediol + 5α-DHT	12	0.76	**0.008**		

There was a significantly positive correlation between the T levels of both methods ($r = 0.74$, $N = 12$, $p = 0.011$) (Table 5). Furthermore, T levels measured with EIA correlated significantly with the sum of the two androgens and one androgen metabolite (T + 5α-DHT + androstanediol) of the LC–MS measurement ($r = 0.76$, $N = 12$, $p = 0.008$). There was only one additional significant correlation between T levels of the EIA with 5α-DHT levels of the LC–MS ($r = 0.7$, $N = 12$, $p = 0.02$). No significant correlations between T EIA and adrenal androgens were found for all other tested androgens and androgen metabolites (Table 5). To account for physiological

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connections between T and other androgens and androgen metabolites, correlations between T and androgen metabolites within the LC–MS measurement were used as a control (Table 5). Only one of these correlations between T and androstanediol was significant ($r = 0.65$, $N = 12$, $p = 0.04$). Androstanediol also showed a nearly significant correlation with T measured with EIA ($r = 0.59$, $N = 12$, $p = 0.06$). There was no significant correlation between T LC–MS and 5α-DHT ($N = 12$, $r = 0.26$, $p = 0.46$). Furthermore, T LC–MS did not correlate significantly with any of the adrenal androgens and their metabolites measured with LC–MS (Table 5). Correction for multiple testing revealed that the collectives of both sets of correlations within the LC–MS measurement were not significant. However, there was a trend toward significance in the collective of correlations between testicular androgens and their metabolites (chi-square combining *P*-values of spearman correlations = 8.15, permutation test: $p = 0.085$), as opposed to the collection of correlations between T and adrenal androgen metabolites or metabolites of both steroid sources (chi-square combining *P*-values of spearman correlations = 12.29, permutation test: $p = 0.61$).

3.4. Correlations between female urinary T levels measured with EIA and urinary androgens and androgen metabolites measured with LC–MS

Overall, the collective of correlations between T EIA and LC–MS androgens and androgen metabolites was significant (chi-square combining *P*-values of spearman correlations = 49.13, permutation test: $p = 0.016$).

In female urine, there were several significantly positive correlations between T EIA and adrenal gland androgens such as DHEA ($r = 0.61$, $N = 15$, $p = 0.02$), A^4dione ($r = 0.63$, $N = 15$, $p = 0.02$) and A^5diol ($r = 0.63$, $N = 15$, $p = 0.02$) measured with LC–MS (Table 6). We found no significant correlation between T levels measured with EIA and T levels of the LC–MS analysis ($r = 0.45$, $N = 15$, $p = 0.11$). Within the LC–MS measurements, there were also many significant or nearly significant correlations between T and other androgens and androgen metabolites, for which a significant correlation was also found for T levels measured with EIA. There was only one exception where a significant correlation between T EIA and A^4dione (LC–MS) did not show a significant correlation with the T concentrations measured with LC–MS. The collective of correlations within the LC–MS measurement was not significant, but revealed a trend towards significance (chi-square combining *P*-values of spearman correlations = 35.53, permutation test: $p = 0.087$).

4. Discussion

The data presented in this study provide a detailed analysis of the T EIA measurement in urine and serum of male and female chimpanzees validated and compared with LC–MS analyses. T levels were significantly higher in both matrices and sexes when measured with the T EIA than with the LC–MS. Males had significantly higher T levels than females in serum and urine in both measurement types. When comparing urinary T measured with EIA and LC–MS androgens, we found that only androgens of the LC–MS measurement, which are known to exclusively derive from T in humans, correlated significantly with the T measured with EIA and no indications of co-measurements of adrenal androgens were found [24,64,65]. Taken together, these results indicate that the tested T EIA is a reliable method for monitoring testicular function in male chimpanzees. However, T levels measured with the T EIA in female urine showed indications of considerable co-measurements of adrenal androgens and their metabolites, which is probably due to the much lower T concentrations in relation to these cross-reacting metabolites than in male urine. No significant correlation between the T levels of the EIA and the LC–MS was found, indicating that the tested T EIA is not a reliable method to measure urinary T in female chimpanzees.

Table 6
Spearman rank correlations between urinary T concentrations measured with EIA and urinary androgen concentrations of the LC–MS analysis of 15 females between 7 and 24 years of age. Significant correlations are highlighted with bold script [58].

Androgens (LC–MS)	*N*	T (EIA)		T (LC–MS)	
		r	*p*	*r*	*p*
T	15	0.45	0.11		
DHEA	15	0.61	**0.02**	0.52	0.06
Epitestosterone	14	0.20	0.48	0.11	0.70
A^4dione	15	0.63	**0.02**	0.08	0.66
Epiandrosterone	15	0.66	**0.01**	0.63	**0.01**
Epietiocholanolone	13	0.56	0.06	0.43	0.15
Etiocholanolone	15	0.21	0.45	0.72	**0.01**
Androsterone	15	0.61	**0.02**	0.55	0.07
A^5diol	15	0.63	**0.02**	0.51	0.06
Androstanediol	13	0.35	0.26	0.65	**0.03**
5α-DHT	15	0.73	**0.004**	0.66	**0.01**

4.1. T levels in serum and urine

T levels measured with the EIA were generally higher than measured with LC–MS. This was true for both matrices as well as for both males and females. These results are in concordance with other comparative studies in which immunoassays generally revealed higher steroid levels than mass spectrometry measurements [3,8,15,29] but see [13]. Most of the former studies related the higher steroid levels measured with immunoassays to the co-measurement of additional steroids by the used antibody. This is consistent with our findings in male chimpanzees. Differences between the two T measurements were higher in urine than in serum. Since, in contrast to in urine, steroids in serum primarily occur in their natural form and are not highly metabolised, the higher proportion of metabolites in male urine cross-reacting with the T antibody led to more elevated T readings. However, for female chimpanzees, the differences between T levels within urine and serum measurements of the two methods were more similar. This could be due to the already very low concentration of T and the relatively higher influence of cross-reacting metabolites in female serum. Alternatively, the low T concentrations in female serum might have been insufficient for a reliable measurement. Both explanations seem to be possible since T levels in female serum were extremely low (detection limits of the LC–MS for T was reached) and cross-reacting steroids, such as A^4dione and DHEA, had much higher concentrations in relation to T than in male serum.

Despite this, we found highly significant differences between the T levels of males and females with both methods and in both serum and in urine. These results are in contrast to an earlier validation study that did not find significant differences in urine with the same T immunoassay [41]. Mean urinary T levels of males and females measured with the T EIA were not in the same range as measured in the study from Möhle and colleagues [41]. Indeed they found much higher T levels in male and especially female chimpanzees than we found in our study. Since the aforementioned study used the same T antibody, higher levels of cross-reacting metabolites could not explain the opposing results. We assume that the reason for the elevated urinary T levels and the non-significant differences between the T levels of males and females in the study by Möhle and colleagues [41] was the use of a special enzyme from *H. pomatia* (*H. p.*) juice, β-glucuronidase/sulphatase. *H. p.* juice has the additional ability to artificially increase the original T levels in samples by converting A^5diol to T through a cholesterol oxidase which is also present in the juice [25,28,37,64]. It has been shown that even a slow conversion rate of the *H. p.* juice enzymes can have a strong impact due to significantly increasing the origi-

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nal urinary T levels in chimpanzee males [25]. In our study, chimpanzee females had 43 times higher urinary A^5diol concentrations compared to T. The ratio in male urine was 5:1 A^5diol to T, respectively. Thus the additional activity of the *H. p.* juice enzyme might have a much stronger impact on modifying T concentrations in female urine than in male urine, which would explain why female chimpanzees in the study by Möhle and colleagues [41] had much higher T levels. The use of *H. p.* juice enzyme might also explain patterns in a study on the European stonechat where male T levels in plasma were significantly higher than female T levels whereas there was no difference in T levels in excreta treated with *H. p.* juice [18,19]. Therefore, as already proposed by Hauser et al. [25], β-glucuronidase from *E. coli* and an additional solvolysis step should be applied instead of β-glucuronidase/sulphatase from *H. p.* juice to cleave glucuronides and sulphates from steroids in order to avoid the artificial increase of T during urine extraction. Alternatively, cholesterol oxidase activity within *H. p.* juice can be inhibited by concomitant use of ascorbic acid [9].

4.2. Correlations between male urinary T levels measured with EIA and urinary androgens and androgen metabolites measured with LC–MS

Correlations were calculated to investigate the comparability of T levels measured with EIA and with LC–MS in urine of male chimpanzees, and to detect possible cross-reactivities of androgen metabolites with the T EIA. Correlations between T and other androgens and androgen metabolites within the LC–MS measurement were used to control for physiological coherences between T and other androgen metabolites due to metabolic origin. There was a strong correlation between T concentrations measured with EIA and LC–MS. The correlation was even stronger when all main urinary testicular androgens known for humans, which could be measured with the LC–MS [24], were summed up and correlated with the T EIA measurement. No significant correlation between adrenal androgens of the LC–MS measurement and T levels measured with EIA was found. There was an additional significant correlation between T measured with EIA and 5α-DHT of the LC–MS measurement, which was probably due to the known cross-reactivity of 5α-DHT with the T antibody [50]. Among the correlations within the LC–MS measurement, there was only one significant correlation between T and androstanediol. Androstanediol is known to be derived from T through 5α-DHT, thus there is a known metabolic association between these two androgens [24,64]. The measurement of 5α-DHT in our LC–MS method can be confounded by epietiocholanolone, as both compounds coelute and have the same retention time [23]. This could explain the non-significant correlation between T and 5α-DHT within the LC–MS measurements as well as the absence of an overall significance in the collective of correlations of the testicular androgens (T-androstanediol and T-5α-DHT) within the LC–MS measurement.

But besides that, our results indicate that significant cross-reactivities of the tested EIA mainly occur with testicular androgens and their metabolites and that existing cross-reactivities of the T EIA with adrenal androgens seem to have a negligible impact on the measurement of testicular function in male chimpanzees. Therefore, contrary to results from earlier studies [21,41], T immunoassays can be a reliable method to investigate male gonadal activity and to monitor male reproductive state in urine of chimpanzees.

4.3. Correlations between female urinary T levels measured with EIA and urinary androgens and androgen metabolites measured with LC–MS

To investigate whether the T EIA is a reliable method to measure T levels in urine of female chimpanzees, we conducted correlations between T measured with EIA and T and other androgens and androgen metabolites measured with LC–MS, as described for males above. We did not find a significant correlation between the T levels of the two methods. Instead there were significant correlations between T measured with EIA and adrenal androgens measured with LC–MS, such as A^4dione, A^5diol, and DHEA. In addition, T measured with EIA correlated with two other androgen metabolites, androsterone and epiandrosterone, from the LC–MS measurement, which are not directly produced by the adrenal gland or gonads but are known to be converted from both types of androgens through the adrenal androgen A^4dione in humans [16,39]. These metabolites were also significantly or nearly significantly correlated to T within the LC–MS measurement, indicating an association with T through metabolic pathways rather than detecting cross-reacting metabolites. There was one exception where T levels of the EIA measurement did significantly correlate with A^4dione measured with LC–MS, but no significant or nearly significant correlation was observed within the LC–MS measurement. Since no cross-reactivity with the tested T antibody for A^4dione had been described we conducted a cross-reactivity test which revealed a cross-reactivity of 1.6% (unpublished data). This finding could explain the significant correlation between T measured with EIA and A^4dione of the LC–MS measurement. Furthermore, we tested A^5diol and DHEA for cross-reactivities with the T antibody and found cross-reactivities of 1.9% and 0.01%, respectively. The significant correlations between T measured with EIA and DHEA and A^5diol of the LC–MS measurement can thus partly be explained by cross-reactions with the T antibody. However, the cross-reactivity of DHEA with the antibody is on the low side and although DHEA concentrations are high in female urine, this cannot fully explain the strong correlation between T measured with EIA and DHEA (LC–MS), nor can it explain the nearly significant correlation between T and DHEA within the LC–MS measurement.

In contrast to the production of T in males, T in females is not produced from the gonads in high proportions [34,64]. In human females, the production of T occurs equally in the gonads and adrenal glands, each contributing around 20%, and the majority of the T originates from the peripheral conversion of adrenal androgens such as A^4dione (60 %) and DHEA (1–8%) [6,27,31,34]. Since the main precursor of T in human females is A^4dione, we expected to find a significant correlation between urinary T and A^4dione levels in female chimpanzees within the LC–MS measurement as well. The absence of a significant correlation between these two androgens within the LC–MS measurement could indicate a difference between human and chimpanzee females in terms of sources of T production, however, this issue needs further investigation. In addition the non-significance of the collective of correlations within the LC–MS measurement makes the interpretation of these results even more difficult. Nevertheless, there was a trend towards significance within the collective of correlations that cannot be ignored. Further investigation would be important to clarify these results.

Furthermore, the correlations between the T EIA and the LC–MS measurement of male and female chimpanzees were highly diverse, which indicates that T production and metabolism differs between the two sexes, which would agree with results from studies on humans [33,34,64]. In summary, due to the numerous correlations between T (EIA) and various androgens and androgen metabolites from the LC–MS measurement, including adrenal androgens, which are not all known to be converted into T in peripheral tissue in humans and the low concentration of T in female chimpanzee urine in relation to other cross-reacting androgens and androgen metabolites, the T EIA does not seem to be a reliable method to investigate female urinary T levels.

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4.4. Conclusion

Our results showed that the tested T EIA is a reliable method to assess testicular activity and function in male chimpanzees. This was verified with the strong correlation between urinary T levels measured with EIA and LC–MS and with the lack of indications for influential cross-reactivities of adrenal androgens with the T antibody. Furthermore, we found highly significant differences between T levels measured with EIA of male and female chimpanzees, which indicates that the T EIA can discriminate between urinary T levels of males and females, despite the fact that T is only a minor component among urinary androgens [21,24,41,60]. Contradictory results between this study and the previous study from Möhle and colleagues [41] could be explained by the use of the *H. p.* juice, which should therefore not be used for the extraction of urine samples. To some extent, contradictory results from other studies that have investigated urinary androgen levels in chimpanzees in relation to behavioral patterns could also be explained as artifacts of the used *H. p.* juice.

In contrast to males, the performance of the T EIA in measuring urinary T levels in females was not reliable. This was due to the low concentration of T in female chimpanzee urine and due to the high proportion of cross-reacting adrenal androgens. Considering all possible validation procedures and methods, the LC–MS seems to be a valuable option for a detailed validation of immunoassays.

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References

[1] D.C. Adams, C.D. Anthony, Using randomisation techniques to analyse behavioural data, Anim. Behav. 51 (1996) 733–738.
[2] N.I. Bahr, R. Palme, U. Möhle, J.K. Hodges, M. Heistermann, Comparative aspects of the metabolism and excretion of cortisol in three individual nonhuman primates, Gen. Comp. Endocrinol. 117 (2000) 427–438.
[3] S.K. Baid, N. Sinaii, M. Wade, D. Rubino, L.K. Nieman, Radioimmunoassay and tandem mass spectrometry measurement of bedtime salivary cortisol levels: a comparison of assays to establish hypercortisolism, J. Clin. Endocrinol. Metab. 92 (2007) 3102–3107.
[4] J.C. Beehner, T.J. Bergman, D.L. Cheney, R.M. Seyfarth, P.L. Whitten, Testosterone predicts future dominance rank and mating activity among male chacma baboons, Behav. Ecol. Sociobiol. 59 (2006) 469–479.
[5] I. Bernstein, T. Gordon, R. Rose, The interaction of hormones, behavior and social context in nonhuman primates, Hormones and aggressive behavior. B. B. Svare. Plenum Press [MLC, DdC, CTS] (1983).
[6] K.L. Boulton, D.U. Hudson, S.W. Coppack, K.N. Frayn, Steroid hormone interconversions in human adipose tissue in vivo, Metabolism 41 (1992) 556–559.
[7] Carlsen, European studbook for the chimpanzee *Pan troglodytes*, Copenhagen, Denmark, 2007.
[8] Y.-C. Chang, M.-C. Li, L.-A. Li, S.-B. Jong, P.-C. Liao, L.W. Chang, Quantitative measurement of male steroid hormones using automated on-line solid phase extraction-liquid chromatography–tandem mass spectrometry and comparison with radioimmunoassay, Analyst 128 (2003) 363–368.
[9] S. Christakoudi, D.A. Cowan, N.F. Taylor, Sodium ascorbate improves yield of urinary steroids during hydrolysis with *Helix pomatia* juice, Steroids 73 (2008) 309–319.
[10] L. Dehennin, A.M. Matsumoto, Long-term administration of testosterone enanthate to normal men: alterations of the urinary profile of androgen metabolites potentially useful for detection of testosterone misuse in sport, J. Steroid Biochem. Mol. Biol. 44 (1993) 179–189.
[11] J. Dittami, S. Katina, E. Möstl, J. Eriksson, I. Machatschke, G. Hohmann, Urinary androgens and cortisol metabolites in field-sampled bonobos (*Pan paniscus*), Gen. Comp. Endocrinol. 155 (2007) 552–557.
[12] A.F. Dixson, Primate Sexuality, Oxford University Press, Oxford, 1998.
[13] J.F. Dorgan, T.R. Fears, R.P. McMahon, L. Aronson Friedman, B.H. Patterson, S.F. Greenhut, Measurement of steroid sex hormones in serum: a comparison of radioimmunoassay and mass spectrometry, Steroids 67 (2002) 151–158.
[14] R.E. Erb, S.A. Tillson, G.D. Hodgen, E.D. Plotka, Urinary creatinine as an index compound for estimating rate of excretion of steroids in the domestic sow, J. Anim. Sci. 30 (1970) 79–85.
[15] R.T. Falk, M.H. Gail, T.R. Fears, S.C. Rossi, F. Stanczyk, H. Adlercreutz, P. Kiura, K. Wahala, J.L. Donaldson, J.B. Vaught, C.-M. Fillmore, R.N. Hoover, R.G. Ziegler, Reproducibility and validity of radioimmunoassays for urinary hormones and metabolites in pre- and postmenopausal women, Cancer Epidemiol. Biomarkers Prev. 8 (1999) 567–577.
[16] P. Felig, J.D. Baxter, L.A. Frohmann, Endocrinology and Metabolism, third ed., McGraw-Hill Inc., New York, 1995.
[17] A. Ganswindt, R. Palme, M. Heistermann, S. Borragan, J.K. Hodges, Non-invasive assessment of adrenocortical function in the male African elephant (*Loxodonta africana*) and its relation to musth, Gen. Comp. Endocrinol. 134 (2003) 156–166.
[18] W. Goymann, Noninvasive monitoring of hormones in bird droppings: physiological validation, sampling, extraction, sex differences, and the influence of diet on hormone metabolite levels, Ann. NY Acad. Sci. 1046 (2005) 35–53.
[19] W. Goymann, E. Möstl, E. Gwinner, Non-invasive methods to measure androgen metabolites in excrements of European stonechats, *Saxicola torquata* rubicola, Gen. Comp. Endocrinol. 129 (2002) 80–87.
[20] P. Haccou, E. Meelis, Statistical Analyses of Behavioural Data, University Press, Oxford, 1994.
[21] L.R. Hagey, N.M. Czekala, Comparative urinary androstanes in the great apes, Gen. Comp. Endocrinol. 130 (2003) 64–69.
[22] M. Hau, Regulation of male traits by testosterone: implications for the evolution of vertebrate life histories, BioEssays 29 (2007) 133–144.
[23] B. Hauser, T. Deschner, C. Boesch, Development of a liquid chromatography–tandem mass spectrometry method for the determination of 23 endogenous steroids in small quantities of primate urine, J. Chromatogr. B 862 (2008) 100–112.
[24] B. Hauser, L. Mugisha, A. Preis, T. Deschner, LC–MS analysis of androgen metabolites in serum and urine from east African chimpanzees (*Pan troglodytes schweinfurthii*), Gen. Comp. Endocrinol. 170 (2011) 92–98.
[25] B. Hauser, D. Schulz, C. Boesch, T. Deschner, Measuring urinary testosterone levels of the great apes – Problems with enzymatic hydrolysis using *Helix pomatia* juice, Gen. Comp. Endocrinol. 158 (2008) 77–86.
[26] M. Heistermann, R. Palme, A. Ganswindt, Comparison of different enzymeimmunoassays for assessment of adrenocortical activity in primates based on fecal analysis, Am. J. Primatol. 68 (2006) 257–273.
[27] R. Horton, J.F. Tait, In vivo conversion of dehydroisoandrosterone to plasma androstenedione and testosterone in man, J. Clin. Endocrinol. Metab. 27 (1967) 79–88.
[28] E. Houghton, L. Graingerl, M.C. Dumasia, P. Teale, Applications of gas chromatography/mass spectrometry to steroid analysis in equine sports: problems with enzyme hydrolysis, Org. Mass Spectrom. 27 (1992) 1061–1070.
[29] A.W. Hsing, F.Z. Stanczyk, A. Belanger, P. Schroeder, L. Chang, R.T. Falk, T.R. Fears, Reproducibility of serum sex steroid assays in men by RIA and mass spectrometry, Cancer Epidemiol. Biomarkers Prev. 16 (2007) 1004–1008.
[30] S.H. Hurlbert, Pseudoreplication and the design of ecological field experiments, Ecol. Monogr. 54 (1984) 187–211.
[31] M.A. Kirschner, S. Sinhamahapatra, I.R. Zucker, L. Loriaux, E. Nieschlag, The production, origin and role of dehydroepiandrosterone and 5-androstenediol as androgen prehormones in hirsute women, J. Clin. Endocrinol. Metab. 37 (1973) 183–189.
[32] E. Klinkova, M. Heistermann, J.K. Hodges, Social parameters and urinary testosterone level in male chimpanzees (*Pan troglodytes*), Horm. Behav. 46 (2004) 474–481.
[33] F. Labrie, A. Bélanger, V. Luu-The, C. Labrie, J. Simard, L. Cusan, J.-L. Gomez, B. Candas, DHEA and the intracrine formation of androgens and estrogens in peripheral target tissues: its role during aging, Steroids 63 (1998) 322.
[34] C. Longcope, Adrenal and gonadal androgen secretion in normal females, Clin. Endocrinol. Metab. 15 (1986) 213–228.
[35] B.F.J. Manly, Randomization, Bootstrap and Monte Carlo Methods in Biology, Chapman & Hall, New York, 1997.
[36] A.J. Marshall, G. Hohmann, Urinary testosterone levels of wild male bonobos (*Pan paniscus*) in the Lomako Forest, Democratic Republic of Congo, Am. J. Primatol. 65 (2005) 87–92.
[37] R. Massé, C. Ayotte, R. Dugal, Studies on anabolic steroids. Integrated methodological approach to the gas chromatographic–mass spectrometric analysis of anabolic steroid metabolites in urine, J. Chromatogr. Biomed. Appl. 489 (1989) 23–50.
[38] G. Messeri, G. Cugnetto, G. Moneti, M. Serio, *Helix pomatia* induced conversion of some 3[beta]-hydroxysteroids, J. Steroid Biochem. Mol. Biol. 20 (1984) 793–796.
[39] G. Michael, Biochemical Pathways, Spektrum, Heidelberg, 1999.
[40] U. Möhle, Metabolismus und Ausscheidung von Testosteron bei männlichen Primaten und deren Bedeutung für die Entwicklung nicht-invasiver enzymimmunologischer Testverfahren zur Erfassung der endokrinologischen Gonadenaktivität Fachbereich Biologie, Universität Hamburg, Hamburg, 2001.
[41] U. Möhle, M. Heistermann, R. Palme, J.K. Hodges, Characterization of urinary and fecal metabolites of testosterone and their measurement for assessing

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gonadal endocrine function in male nonhuman primates, Gen. Comp. Endocrinol. 129 (2002) 135–145.
[42] Y. Mori, K. Shimoda, H. Kobayashi, I. Hayasaka, Y. Hamada, Development of the sexual skin with pubertal maturation in female chimpanzees, Primates 48 (2007) 97–101.
[43] E. Möstl, S. Rettenbacher, R. Palme, Measurement of corticosterone metabolites in birds' droppings: an analytical approach, Ann. N.Y. Acad. Sci. 1046 (2005) 17–34.
[44] M.N. Muller, S.F. Lipson, Diurnal patterns of urinary steroid excretion in wild chimpanzees, Am. J. Primatol. 60 (2003) 161–166.
[45] M.N. Muller, R.W. Wrangham, Dominance, aggression and testosterone in wild chimpanzees: a test of the 'challenge hypothesis', Anim. Behav. 67 (2004) 113–123.
[46] M.N. Muller, R.W. Wrangham, The reproductive ecology of male hominoids. in: P.T. Ellison (Ed.), Reproductive Ecology and Human Evolution, Aldinde, New York, 2001.
[47] R. Mundry, J. Fischer, Use of statistical programs for nonparametric tests of small samples often leads to incorrect *P* values: examples from animal behaviour, Anim. Behav. 56 (1998) 256–259.
[48] R. Nelson, An Introduction to Behavioral Endocrinology, third ed., Sinauer Associates Inc., Sunderland, Massachusetts, 2005.
[49] R. Palme, Measuring Fecal Steroids: Guidelines for Practical Application, Ann. N.Y. Acad. Sci. 1046 (2005) 75–80.
[50] R. Palme, E. Möstl, Biotin-streptavidin enzyme immunoassay for the determination of oestrogens and androgens in boar faeces (1994) 111–117.
[51] R. Palme, S. Rettenbacher, C. Touma, S.M. El-Bahr, E. Möstl, Stress hormones in mammals and birds: comparative aspects regarding metabolism, excretion, and noninvasive measurement in fecal samples, Ann. N.Y. Acad. Sci. 1040 (2005) 162–171.
[52] E. Palonek, C. Gottlieb, M. Garle, I. Björkhem, K. Carlström, Serum and urinary markers of exogenous testosterone administration, J. Steroid Biochem. Mol. Biol. 55 (1995) 121.
[53] D.M. Potter, D.J. Griffiths, Omnibus permutation tests of the overall null hypothesis in datasets with many covariates, J. Biopharm. Stat. 16 (2006) 327–341.
[54] G.P. Quinn, M.J. Keough, Experimental Designs and Data Analysis for Biologists, Cambridge University Press, Cambridge, 2002.
[55] R.M. Rose, T.P. Gordon, I.S. Bernstein, Plasma testosterone levels in the male rhesus: influences of sexual and social stimuli, Science 178 (1972) 643–645.
[56] A. Sannen, M. Heistermann, L. Van Elsacker, U. Möhle, M. Eens, Urinary testosterone metabolite levels in bonobos: a comparison with chimpanzees in relation to social system, Behaviour 140 (2003) 683.
[57] A. Sannen, L. Van Elsacker, M. Heistermann, M. Eens, Urinary testosterone-metabolite levels and dominance rank in male and female bonobos (*Pan paniscus*), Primates 45 (2004) 89.
[58] R.M. Sapolsky, Endocrine aspects of social instability in the olive baboon (*Papio anubis*), Am. J. Primatol. 5 (1983) 365–379.
[59] R.M. Sapolsky, The endocrine stress–response and social status in the wild baboon, Horm. Behav. 16 (1982) 279.
[60] K.D.R. Setchell, B. Almé, M. Axelson, J. Sjövall, The multicomponent analysis of conjugates of neutral steroids in urine by lipophilic ion exchange chromatography and computerised gas chromatography–mass spectrometry, J. Steroid Biochem. 7 (1976) 615.
[61] S. Siegel, N.J. Castellan, Nonparametric Statistics for the Behavioral Sciences, second ed., McGraw-Hill, New York, 1988.
[62] H.H. Taussky, With the technical assistance of Gloria, A microcolorimetric determination of creatinin in urine by the Jaffé reaction, J. Biol. Chem. 208 (1954) 853–862.
[63] C. Touma, R. Palme, Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation, Ann. N. Y. Acad. Sci. 1046 (2005) 54–74.
[64] E. Venturelli, A. Cavalleri, G. Secreto, Methods for urinary testosterone analysis, J. Chromatogr. B: Biomed. Sci. Appl. 671 (1995) 363.
[65] A. Vermeulen, L. Verdonck, Some studies on the biological significance of free testosterone, J. Steroid Biochem. 3 (1972) 421–426.
[66] P.L. Whitten, D.K. Brockman, R.C. Stavisky, Recent advances in noninvasive techniques to monitor hormone-behavior interactions, Am. J. Phys. Anthropol. 107 (1998) 1–23.
[67] J.C. Wingfield, R.E. Hegner, M. Alfred, J. Dufty, G.F. Ball, The "challenge hypothesis": theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies, Am. Nat. 136 (1990) 829–846.

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