General and Comparative Endocrinology xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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

LC–MS analysis of androgen metabolites in serum and urine from east African chimpanzees (*Pan troglodytes schweinfurthii*)

Barbara Hauser [a], Lawrence Mugisha [b,c], Anna Preis [a], Tobias Deschner [a,*]

[a] Max Planck Institute for Evolutionary Anthropology, Department of Primatology, Deutscher Platz 6, D-04103 Leipzig, Germany
[b] Ngamba Island Chimpanzee Sanctuary, Uganda
[c] Department of Wildlife and Animal Resources Management, Faculty of Veterinary Medicine, P.O. Box 7062, Kampala, Uganda

ARTICLE INFO

Article history:
Received 6 November 2009
Revised 9 September 2010
Accepted 13 September 2010
Available online xxxx

Keywords:
Testosterone
Metabolism
Urine
Hydrolysis
Chimpanzee
Gonadal activity
LC–MS
Serum

ABSTRACT

Testosterone regulates a wide variety of behavioral and physiological traits in male vertebrates. It influences reproductive and aggressive behaviors and is used as a marker of gonadal activity. While testosterone is the primary biologically active male gonadal steroid in the blood, it is metabolized into a variety of related steroids when excreted via urine and feces. To monitor endocrinological profiles studies on wild-living animals primarily rely on non-invasively collected samples such as urine or feces. Since a number of androgen metabolites that are found in high concentrations in these matrices do not stem exclusively from gonadal production, but are also produced by the adrenal cortex, the metabolism and excretion pattern of testosterone and its characteristic metabolites have to be investigated. Here, we compare the levels of 11 androgens and their metabolites in serum and urine (after hydrolytic/solvolytic cleavage of conjugates) from female, and intact and castrated male chimpanzees to investigate whether they were of testicular or adrenal origin. For serum, significant differences in concentrations were found only for native testosterone. For urine, testosterone concentrations showed the largest differences between intact and castrated males, and intact males and females, while no differences were seen between females and castrated males. Epitestosterone levels revealed the same pattern. These differences in urinary concentrations could also be seen for 5α-androstane-3α,17β-diol (androstanediol), and less clearly for 5α-dihydrotestosterone (5α-DHT), etiocholanolone, and androsterone. In urine of males, significant correlations were found between the levels of testosterone and 5α-androstane-3α,17β-diol, as well as between testosterone and epitestosterone. Therefore, the clearest urinary markers of gonadal activity in male chimpanzees seems to be testosterone itself.

1. Introduction

Testosterone is used as an endocrinological marker in many behavioral studies on primates and other vertebrates, including studies on reproductive strategies, dominance, and aggression. It regulates many aspects of male reproductive physiology, such as the development of reproductive anatomy, the production of sperm, and the onset of secondary sexual characteristics (Dixson, 1998). Furthermore, it is associated with many behaviors related to mate competition, territoriality, mate guarding, aggression, and displays (Wingfield et al., 1990, Dixson, 1998). Sample collection from free-living animal populations for the analysis of testosterone levels, however, is challenging in several ways. For example, in order to minimize the influence of the observer on the animal, the use of non-invasively collected samples, either urine or feces, is preferred. Studies on behavioral correlates of urinary testosterone levels have produced inconsistent results in chimpanzees. Some report positive associations between aggressive behavior and dominance rank and testosterone concentrations in urine (Muller and Wrangham, 2004; Anestis, 2006), while others do not confirm these findings (Klinkova et al., 2004). The antibodies used in these assays were however characterized through different sets of cross reactivities. Since in matrices like urine and feces, the composition of steroids causing the analytical response of the immunoassay remains unknown and the metabolite of interest often only represents a minor fraction, the use of different antibodies can lead to widely divergent results. For example, in a comparative study, significantly lower testosterone metabolite levels were found in bonobo males than in chimpanzee males when a group-specific 5α-androstane-17α-ol-3-one antibody was used (Sannen et al., 2003). The difference in mean testosterone metabolite levels between males and females was significant but much smaller in bonobos than in chimpanzees. In contrast, a study of free-living bonobos found a pronounced sex difference in urinary testosterone levels when a testosterone-specific EIA was used. This difference was not seen with the above mentioned 5α-androstane-17α-ol-3-one assay (Dittami et al., 2008). As stressed by the authors, an assay claimed to

* Corresponding author. Fax: +49 341 3550 299.
E-mail address: deschner@eva.mpg.de (T. Deschner).

0016-6480/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygcen.2010.09.012

measure testosterone levels should at least be able to clearly distinguish between males and females (Dittami et al., 2008).

In humans and a number of other primates, urinary testosterone concentrations parallel biologically active testosterone circulating in the blood (Dehennin and Matsumoto, 1993; Palonek et. al., 1995; Möhle et al., 2002). The metabolism of testosterone is characterized by a great number of structurally closely related metabolites. The relative proportion of androgen metabolites seems to vary considerably between the great ape species (Hagey and Czekala, 2003). A thorough validation of the analytical method is therefore indispensable before it is used on matrices such as urine and feces, especially when comparing between sexes and species (Möhle et al., 2002).

1.1. Synthesis and regulation of androgens

In male primates, androgens are mainly synthesized in the Leydig cells of the testes. In addition, androgens are synthesized in the adrenal cortex and the female ovaries. The enzymes involved in the synthesis of androgens are activated by luteinizing hormone. Androgen biosynthesis in the human testes preferentially proceeds via the Δ^5 pathway from pregnenolone to dehydroepiandrosterone (DHEA) and androst-5-ene-3β,17β-diol (A^5diol) before entering the Δ^4 pathway as androstenedione (A^4dione) and testosterone (Felig et al., 1995). Both DHEA and A^4dione do not bind to the androgen receptor (Arlt, 2004) and have only little androgenic activity. Therefore, if one is interested in monitoring gonadal activity, the co-measurement of these steroids should be avoided, especially since in humans DHEA is the most abundant urinary androgen metabolite. In humans, a pronounced increase in DHEA during adrenarche occurs between 8 and 9 years of age. In general, the decrease in DHEA and DHEAS production with age that is seen in adult humans is also common in a number of other species, including rhesus-monkeys, pig-tailed macaques, and baboons (Cutler et al., 1978; Conley et al., 2004). However, when comparing patterns of DHEA secretion in relation to age in chimpanzees, rhesus macaques (*Macaca mulatta*), and pig-tailed macaques (*Macaca nemestrina*) (Smail et al., 1982), and several rodents, domestic animals, and primates (Cutler et al., 1978), only chimpanzees show a profile that closely resembled that of humans (Conley et al., 2004).

1.2. Metabolism of testosterone in humans

The metabolism of androgens takes place in the liver, kidneys, and peripheral tissues. The molecules are reduced at the A^4-double bond. Subsequently, in humans, the 3-oxo-function is preferentially reduced to the 3α-hydroxyl-function (Michael, 1999). These compounds are then glucuronidated or sulphated before excretion. As only a small proportion of testosterone is excreted as native testosterone, it is unclear which metabolite or combination of metabolites is best used to assess gonadal function in non-human primates. Testosterone is converted to 5α-DHT, which has a 2.5 times greater androgenic potency than testosterone, by a 5α-reductase. 5α-DHT appears to be involved in the mediation of androgenic effects on beard growth and prostatic hypertrophy (determined in men who are deficient in 5α-reductase). Stimulatory effects on muscle and sexual potency do not appear to require 5α-reduction of testosterone (Felig et al., 1995).

Exogenous administration of testosterone to men has generally been found to increase urinary excretion of testosterone metabolites such as testosterone glucuronide, testosterone sulfate, glucuronides of androsterone, etiocholanolone, 5α- and 5β-androstane-3α,17β-diol, and to decrease excretions of conjugates of epitestosterone (ET) and its precursor androgen androst-5-en-3β,17α-diol (Dehennin and Matsumoto, 1993).

1.3. Metabolism of testosterone in chimpanzees

The metabolism of testosterone in chimpanzees has been investigated by a radiometabolism study within one male individual by Möhle and colleagues (2002). In this study three different enzyme immunoassays were employed to characterize testosterone metabolites. The first assay used a testosterone-antibody (for 17β-OH androgens), the second used an epiandrosterone-antibody (for 17-oxo-androgens), and the third used a 5α-androstan-17α-ol-3-one-antibody (for 17α-OH-androgens).

After ^{14}C-labelled testosterone was infused, peak radioactivity was detected in urine within 2 h. The results showed that 91% of recovered radioactivity was detected in urine, 90–99% of radioactive testosterone metabolites were conjugated, and 98% of these were found in the glucuronide fraction. Using HPLC and fraction collection, five distinct radioactive peaks were found in chimpanzee urine. The majority of radioactivity was found in fractions coeluting with 3α-hydroxy-5β-androstan-17-one (etiocholanolone) and 5α-androstane-3,17-dione (androstanedione). Other metabolites such as 3α-hydroxy-5α-androstan-17-one (androsterone) and 3β-hydroxy-5α-androstan-17-one (epiandrosterone) were detected as immunoreactive peaks with an epiandrosterone-assay.

The biological validation ($N = 5$) in chimpanzee urine revealed significantly higher levels of immunoreactive androgen metabolites in intact compared to castrated males with all three different assays. Androgen metabolite levels were clearly lower in the urine of female than of male chimpanzees, but differences were only significant using the 5α-androstan-17α-ol-3-one-assay. The 17-oxo-metabolites were generally found to be more abundant than 17-hydroxylated compounds. The 5α-androstan-3α-ol-17-one-assay produced the largest differences between the groups. The high levels of immunoreactive androgen metabolites measured in females and castrated males were probably due to DHEA metabolites. This was confirmed by the co-administration of ^{14}C-testosterone and ^{3}H-DHEA to one male long-tailed macaque, which resulted in a similar metabolic pattern in urine and feces (Möhle et al., 2002). To our knowledge, this study is the most detailed work published on the metabolism of testosterone in chimpanzees. As a mass-spectrometric characterization of isolated radioactive fractions has not been carried out, a clear identification and quantification of metabolites is still lacking. Therefore, in this study, we compare the relative proportion of a large number of androgens and their metabolites in intact and castrated male, and female chimpanzees. Castrated chimpanzees serve as control group to investigate, which androgen metabolites are of gonadal origin. We include serum as sample matrix, as serum testosterone is the biologically active androgen and therefore gives the most relevant information on gonadal activity. By comparing androgen metabolite levels in serum and urine between the different sex classes and investigating how specific metabolite levels correlate with each other, we aim to answer the following questions:

(1) Which urinary androgen metabolites represent testosterone levels in serum and are suitable markers of testicular activity in male chimpanzees?
(2) How do female, and intact and castrated male chimpanzees differ in their androgen metabolite levels?
(3) What is the relative level of androgen metabolites in urine?

2. Materials and methods

2.1. Samples and animals

The chimpanzees were members of two captive groups living in the Ngamba Island Sanctuary (Uganda), run by the Chimpanzee Sanctuary & Wildlife Conservation Trust. A total of 42 chimpanzees

Please cite this article in press as: Hauser, B., et al. LC–MS analysis of androgen metabolites in serum and urine from east African chimpanzees (*Pan troglodytes schweinfurthii*). Gen. Comp. Endocrinol. (2010), doi:10.1016/j.ygcen.2010.09.012

live on the island, including 19 males (3–23 years) and 23 females (6–24 years). Urine samples were collected from 15 females (10–24 years), 10 intact males (9–22 years), and 2 castrated males (21 and 23 years). Serum samples were collected from 17 females (10–24 years), 11 intact males (9–22 years), and 2 castrated males (21 and 23 years). We included only individuals over 9 years of age, when they become socially and sexually mature, and in captivity have been shown to reproduce (Carlsen, 2007). Usually serum and urine samples were obtained from the same individual, but sometimes only serum or only urine could be collected. Samples were collected during a yearly medical check-up in January and February 2007, between 8 and 12 a.m. Blood was obtained during anesthesia, for which a combination of ketamin (3 mg/kg) and meditomidin (0.03 mg/kg) was administered. Within minutes after collection, the blood was centrifuged and the serum was transferred to 2 ml cryotubes. Urine was collected from the ground of the cage with plastic pipettes after the individuals had woken up after anesthesia. Both, serum and urine samples were stored at −80° in liquid nitrogen until they were shipped on dry ice to the lab at the MPI EVAN in Leipzig where they were stored at −20 °C until analysis.

2.2. Extraction of steroids from serum

We added 400 µl of acetonitrile and 50 µl of internal standard mixture, which contained 10 pg/µl each of *d4*-cortisol, *d4*-estrone, d3-testosterone and *d9*-progesterone, to 200 µl of serum. Samples were vortexed for 1 min and then centrifuged for 10 min at 18.000*g*. The supernatant of the precipitated protein pellet was aspirated and diluted with 3 ml of de-ionized water. A solid phase extraction cartridge (C18 ec, 200 mg, Chromabond, Macherey & Nagel, Düren, Germany) was conditioned with 3 ml of methanol and 3 ml of de-ionized water. The serum extract was then applied, and the cartridge was washed with 5 ml of water and 3 ml of 20% methanol, and dried for 10 min. Elution of steroids was carried out with 3 ml of methanol. The eluate was evaporated and reconstituted with 300 µl of acetonitrile. After vortexing and centrifuging for 2 min, the extract was transferred to a 650 µl Eppendorf tube and evaporated in a Speedvac until it was dry. The residue was re-dissolved in 50 µl of 30% acetonitrile and transferred to a 150 µl insert of an HPLC vial. Quantitation limits were 0.025 ng/ml for testosterone and androstenedione, 0.125 ng/ml for 5α-DHT and androstenediol and 0.25 ng/ml for DHEA.

2.3. Analytical methods and extraction of steroids from urine

The extraction of steroid hormones from 200 µl of urine, as well as the materials and equipment used have previously been described in detail (Hauser et al., 2008a,b). In short, steroid glucuronides were hydrolyzed using β-glucuronidase from *Escherichia coli*. As this enzymatic preparation has no sulfatase activity, subsequently steroid sulfates were cleaved by solvolysis with ethyl acetate/sulfuric acid. Steroids were extracted with tert. butyl methyl ether, evaporated, and reconstituted in HPLC eluent. Extraction efficiencies ranged from 60.4% to 103% (Hauser et al., 2008a). The quantitative analysis was carried out by HPLC – tandem mass spectrometry in the range of 0.3–1000 ng/ml. The urinary hormone concentrations were indexed against creatinine, which was determined by the Jaffé method (Hauser et al., 2008a). The choice of analyzed androgens and androgen metabolites was based on their occurrence in human testosterone metabolism and on a radiometabolism study of testosterone in chimpanzees (Möhle et al., 2002). These androgens are listed in Table 1.

2.4. Statistics

Since we detected occasional outliers or skewed distributions in most of the datasets, parametric test assumptions were violated. Therefore, we used only non-parametric tests, since, in these cases, the corresponding non-parametric test is more reliable and may have more power (Hunter and May, 1993). We calculated Spearman's rank correlations using software written by R. Mundry, and Kruskall–Wallis *H*-tests and Mann–Whitney-*U*-tests using SPSS 11.0.1 or 15.0.0. Whenever possible, we calculated exact tests when small samples required their use (Siegel and Castellan, 1988; Mundry and Fischer, 1998), and indicated the two-tailed *P*-values throughout.

Testing the same null-hypothesis (no differences between the three groups) repeatedly for each metabolite required a correction for multiple testing. We achieved this by first combining *P*-values as for Fisher's omnibus test (Haccou and Meelis, 1994) into a single chi-square value. However, since *P*-values derived for different metabolites obtained from the same set of individuals are not independent, we could not use the standard chi-square distribution to obtain its significance (Potter and Griffiths, 2006). Thus, we derived the distribution of the chi-squares expected under a true null-hypothesis using a permutation procedure (Adams and Anthony, 1996; Manly, 1997). That is, we repeatedly randomized the subject's assignments to the three groups, and each time compared the groups using a Kruskal–Wallis test applied to each metabolite and summarized the *P*-values derived into a single chi-square. We ran a total of 1000 permutations (with the original data included as one permutation) and estimated the final *P*-value as the proportion of permutations revealing a chi-square at least as large as that of the original data.

3. Results

3.1. Description of absolute steroid concentrations in serum and urine

Androgen concentrations in serum (Table S1, supplementary material) were much lower than in urine (Table S2, supplementary

Table 1
Investigated steroids: systematic name, trivial name and abbreviation used.

Systematic name	Trivial name	Abbreviation
17β-Hydroxyandrost-4-en-3-one	Testosterone	–
17α-Hydroxyandrost-4-en-3-one	Epitestosterone	–
Androst-4-ene-3,17-dione	Androstenedione	A^4dione
Androst-5-ene-3β,17β-diol	Androstenediol	A^5diol
3β-Hydroxyandrost-5-en-17-one	Dehydroepiandrosterone	DHEA
17β-Hydroxy-5α-androstan-3-one	5α-Dihydrotestosterone	5α-DHT
5α-Androstane-3α,17β-diol	5α-Androstanediol	Androstanediol
3α-Hydroxy-5β-androstan-17-one	Etiocholanolone	–
3α-Hydroxy-5α-androstan-17-one	Androsterone	–
3β-Hydroxy-5β-androstan-17-one	Epietiocholanolone	–
3β-Hydroxy-5α-androstan-17-one	Epiandrosterone	–

Please cite this article in press as: Hauser, B., et al. LC–MS analysis of androgen metabolites in serum and urine from east African chimpanzees (*Pan troglodytes schweinfurthii*). Gen. Comp. Endocrinol. (2010), doi:10.1016/j.ygcen.2010.09.012

material). Therefore, the analysis of serum steroids often had to be carried out close to the detection limit of the LC–MS/MS-method. The most abundant adrenal steroid in chimpanzee serum was DHEA (1.4–16.3 ng/ml), while testosterone concentrations were up to 30 times lower than DHEA concentrations in females and castrated males. In general, serum steroids showed a high amount of inter-individual variance.

Quantitatively, testosterone represented only a minor part of all measured androgen metabolites in urine (Table S2).The same was true for the two testosterone metabolites, 5α-DHT and androstanediol, which were present in concentrations between 0.1 and 36 ng/mg creatinine. The most abundant urinary androgen was DHEA, which results from solvolytic cleavage of DHEAS. Other androgen metabolites that occurred in high concentrations in urine of up to 1000 ng/mg creatinine were androstenediol, androsterone and etiocholanolone.

The concentration range of androgen metabolites in urine (0.1–4000 ng/mg creatinine) was much wider than in serum (0.06–16 ng/ml) and extended over the whole calibration range of the analytical method. As in serum, we saw a high amount of inter-individual variance in urinary androgen metabolite levels.

3.2. Comparison of serum steroids of females, and intact and castrated males

Overall, intact males, castrated males and females differed significantly in their serum androgen metabolite levels (chi-square combining Kruskal–Wallis H-tests = 51.3, permutation test: $P = 0.001$). When comparing individual androgen metabolite levels in serum of females, and intact and castrated males, significant differences were seen in testosterone (Kruskall–Wallis H-test, $\chi^2 = 20.648$, df = 2, $P < 0.001$), DHEA ($\chi^2 = 8.669$, df = 2, $P = 0.013$), and A^4dione ($\chi^2 = 13.290$, df = 2, $P = 0.001$). Testosterone levels were significantly higher in intact males ($N = 11$) than in females ($N = 17$) (Mann–Whitney-U-test, $U = 0$, $P < 0.001$), and in intact males than in castrated males ($N = 2$) ($U = 0$, $P = 0.026$). There was no significant difference in testosterone levels between females and castrated males (Fig. 1). The serum concentration of 5α-DHT was significantly lower in females than in castrated males (Mann–Whitney-U-test, $U = 1$, $P = 0.012$). Furthermore, females showed a significantly higher level of DHEA (not DHEAS) than intact males ($U = 33$, $P = 0.003$), as well as a significantly higher level of A^4dione ($U = 21.5$, $P = 0.001$).

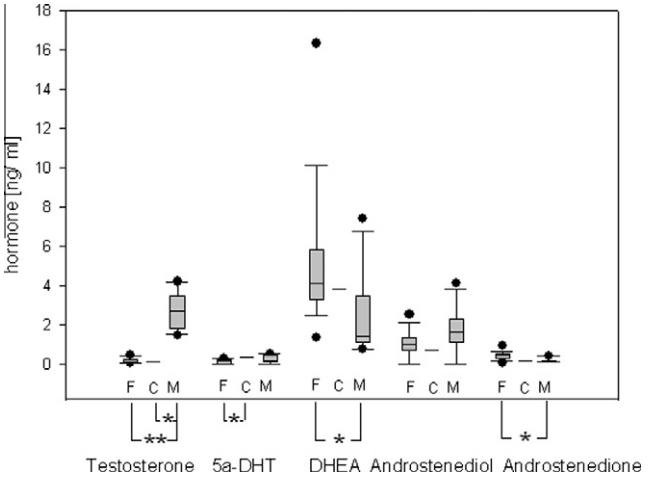

Fig. 1. Box plot chart of androgen metabolite concentrations in serum [ng/ml]. F = females, C = castrates, M = males. The top, middle, and bottom horizontal lines represent the 75th, 50th, and 25th percentiles, respectively. Dots represent outliers and were included in the analysis (see methods for further details). Significant differences are indicated by one ($P < 0.05$) or two asterisks ($P < 0.001$).

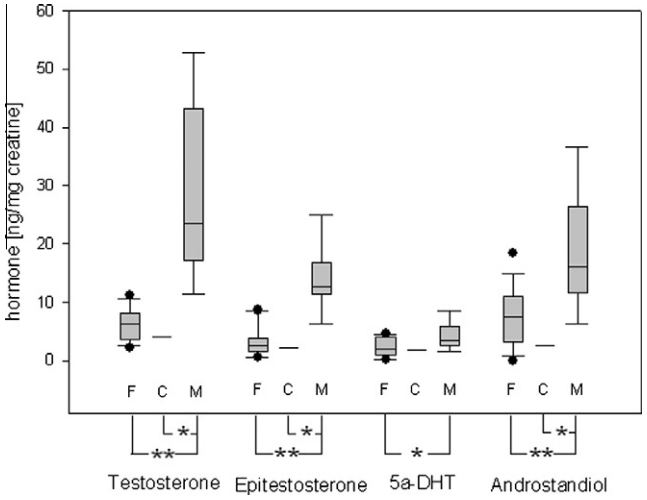

Fig. 2. Box plot chart of androgen metabolite concentrations in urine [ng/mg creatinine]. (For additional information see Fig. 1.).

3.3. Comparison of urinary steroids of females, and intact and castrated males

Overall intact males, castrated males and females differed significantly in their urinary androgen metabolite levels (chi-square combining Kruskal–Wallis H-tests = 73.8, permutation test: $P = 0.002$). The comparison of individual urinary androgen metabolite concentrations in females ($N = 15$), and intact ($N = 9$, age above 10 years) and castrated males ($N = 2$) revealed significant differences in testosterone (Kruskall–Wallis H-test, $\chi^2 = 17.349$, df = 2, $P < 0.001$), epitestosterone ($\chi^2 = 16.224$, df = 2, $P < 0.001$), androstanediol ($\chi^2 = 11.456$, df = 2, $P = 0.003$), etiocholanolone ($\chi^2 = 7.569$, df = 2, $P = 0.023$), and androsterone ($\chi^2 = 7.741$, df = 2, $P = 0.021$). When comparing intact males and females, males had significantly higher urinary concentrations of testosterone (Mann–Whitney-U-test, $U = 0$, $P < 0.001$), epitestosterone ($U = 2$, $P < 0.001$), 5α-DHT ($U = 34$, $P = 0.048$), androstanediol ($U = 1$, $P = 0.002$), etiocholanolone ($U = 30$, $P = 0.025$), and androsterone ($U = 25$, $P = 0.01$) than females (Figs. 2 and 3). Except for 5α-DHT,

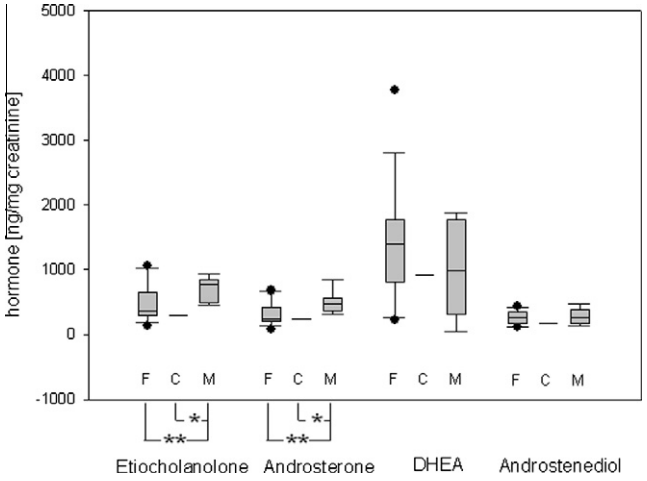

Fig. 3. Box plot chart of androgen metabolite concentrations in urine [ng/mg creatinine]. (For additional information see Fig. 1.).

Please cite this article in press as: Hauser, B., et al. LC–MS analysis of androgen metabolites in serum and urine from east African chimpanzees (*Pan troglodytes schweinfurthii*). Gen. Comp. Endocrinol. (2010), doi:10.1016/j.ygcen.2010.09.012

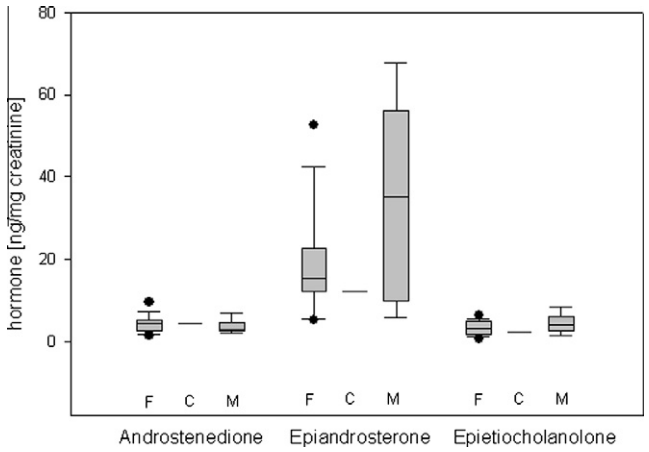

Fig. 4. Box plot chart of androgen metabolite concentrations in urine [ng/mg creatinine]. (For additional information see Fig. 1.).

the same pattern was seen when we compared the urinary androgen metabolite levels of intact and castrated males: intact males showed higher concentrations of testosterone, epitestosterone, androstanediol, etiocholanolone, and androsterone (Mann–Whitney-*U*-test, $U = 0$, $P = 0.036$ for each of the aforementioned androgen metabolite). By contrast, we saw no significant differences in these urinary androgen metabolite concentrations between females and castrated males (Figs. 2 and 3). The urinary levels of DHEA, androstenediol, A^4dione, epiandrosterone, and epietiocholanolone did not differ significantly between females, and intact and castrated males (Figs. 3 and 4).

For testosterone Mann–Whitney-*U*-tests were repeated separately for unconjugated testosterone plus testosterone glucuronide (T + TG, analyzed after hydrolysis of urine) and testosterone sulfate (TS, analyzed after solvolysis). Significant differences in urinary concentrations of T + TG were seen between intact males and females ($U = 0$, $P < 0.001$) and between intact and castrated males ($U = 0$, $P = 0.036$), but not between females and castrated males ($U = 10$, $P = 0.529$). When comparing urinary TS concentrations, these differed significantly between intact males and females ($U = 8$, $P < 0.001$), between intact and castrated males ($U = 0$, $P = 0.036$) and females and castrated males ($U = 1$, $P = 0.029$) with females having higher TS levels than castrated males.

3.4. Correlation of urinary testosterone concentrations with other urinary androgen metabolite levels

In order to test whether high levels of testosterone are associated with elevated urinary concentration of other androgen metabolites, we calculated Spearman's rank correlations for testosterone concentration versus the concentration of each androgen metabolite analyzed in the urine of 9 male individuals (10–22 years of age) under the assumption that if another androgen was a unique metabolite of testosterone, a significant correlation should be expected. These results are summarized in Table 2. The urinary concentration of testosterone was significantly correlated with the concentration of androstandiol. For all other androgen metabolites no significant correlation was found.

Table 2
Spearman's rank correlation between urinary testosterone concentration and the concentration of other urinary androgen metabolites in urine of 9 male individuals. Significant correlations are highlighted in bold.

Correlation between:	Males 10–22 years		
Testosterone_Androgen	Rho	*N*	*P*
Testosterone_Androstanediol	**0.783**	**9**	**0.012**
Testosterone_5aDHT	0.483	9	0.216
Testosterone_Epitestosterone	0.330	9	0.390
Testosterone_Etiocholanolone	0.067	9	0.868
Testosterone_Androsterone	0.333	9	0.397
Testosterone_DHEA	0.350	9	0.356
Testosterone_Epiandrosterone	0.217	9	0.563
Testosterone_Epietiocholanolone	0.483	9	0.185
Testosterone_Androstenediol	0.250	9	0.543
Testosterone_A^4dione	0.350	9	0.337

Table 3
Spearman's rank correlation of serum testosterone levels with urinary androgen metabolite levels of 10 adult male chimpanzees (9–22 years of age).

Serum testosterone_urinary androgen	Rho	*N*	*P*
Testosterone_testosterone (T + TG + TS)[a]	0.891	10	**0.001**
Testosterone_testosterone (T + TG)	0.903	10	**0.001**
Testosterone_testosterone (TS)[a]	0.539	10	0.113
Testosterone_5α-DHT	0.333	10	0.356
Testosterone_Androstanediol	0.624	10	0.068
Testosterone_Etiocholanolone	0.406	10	0.243
Testosterone_Androsterone	0.406	10	0.249
Testosterone_Sum_T-metabolites[b]	0.418	10	0.244

[a] T = testosterone, TG = testosterone glucuronide, TS = testosterone sulfate.
[b] Sum of 5α-DHT, androstanediol, etiocholanolone and androsterone.

3.5. Correlations between serum testosterone and urinary androgens and their metabolites

A highly significant correlation ($P = 0.001$) of serum testosterone concentration with urinary testosterone levels was found in intact males (Table 3). For androstanediol, the correlations approached significance ($P = 0.068$). For the other urinary androgen metabolites, we found no significant correlations with serum testosterone in male chimpanzees. When repeating the correlation separately for serum testosterone versus urinary testosterone plus testosterone glucuronide (T + TG) and serum testosterone versus testosterone sulfate (TS), only urinary T + TG correlated significantly with serum testosterone levels ($P = 0.001$).

4. Discussion

The aim of this study was to identify exclusive metabolites of testosterone in the urine of male chimpanzees in order to refine the analytical methodology for the assessment of gonadal function. First, we compared the levels of several androgens in serum from females, and intact and castrated males. Then we extended the analysis to urine by measuring a number of urinary androgen metabolites in these three sex-classes.

We found the absolute concentrations of androgens in chimpanzee serum to be in the low ng/ml range (0.1–5 ng/ml, except for DHEA). Intact males' serum testosterone levels were approximately 10 times higher than females and 10–40 times higher than castrated males. This difference in testosterone levels between intact and castrated males was considerably higher than the one found in an older study where a fivefold reduction of serum testosterone levels in chimpanzees as a result of castration was detected. (Albertson et al., 1984). Absolute serum concentrations of testosterone were in the same range as other published data on testosterone in chimpanzee serum determined by RIA (Smail et al., 1982: 0.2–1.3 ng/ml, Copeland et al., 1985: 7.2 ng/ml). While all serum DHEA levels in our study ranged between 0.8 ng/ml and 16.3 ng/ml, when measured with a RIA much higher concentrations of 20–200 ng/ml DHEA have been reported for chimpanzee serum (Smail et al., 1982). In humans, DHEAS occurs at up to 5000 times higher concentrations in serum than DHEA (Ceglarek et al., 2009). As DHEAS could not be analyzed with the LC method

Please cite this article in press as: Hauser, B., et al. LC–MS analysis of androgen metabolites in serum and urine from east African chimpanzees (*Pan troglodytes schweinfurthii*). Gen. Comp. Endocrinol. (2010), doi:10.1016/j.ygcen.2010.09.012

used in this study, we were not able to exhaustively analyze excretion patterns for these androgen metabolites.

For humans, a similar range of testosterone and androstenedione concentrations was reported in serum as we found in our study (Rauh et al., 2006). In 16–18 year-old adolescents, boys' testosterone levels were ten times higher than girls' (Rauh et al., 2006). A comparative study on different mass spectrometric steroid methods established reference values of 0.058–1.3 ng/ml for testosterone in serum of women and 2.91–9.0 ng/ml in serum of men (Thienpont et al., 2008). Therefore, similar relative testosterone ratios of males and females seem to exist in human and chimpanzee serum.

In serum, the level of native testosterone was the clearest difference between intact and castrated males, and intact males and females. The concentration of 5α-DHT would have been expected to show the same pattern, but a significant difference was only seen between females and castrated males. This is probably due to the low concentration of 5α-DHT in serum, which would require lower detection limits of the analytical system. This could be achieved by extracting larger amount of serum which unfortunately were not available in our study. As unconjugated androstanediol could not be detected in serum, perhaps the analysis of androstanediol glucuronide would provide us with better results, as this represents the predominant form of serum androstanediol in humans.

In urine, as in serum, testosterone concentrations showed the largest differences between intact and castrated males, and intact males and females, with intact males having the highest urinary testosterone levels. Epitestosterone levels revealed the same pattern. To our knowledge, the difference of epitestosterone concentration between male and female chimpanzees has not been previously investigated, however, epitestosterone is considered to have no androgenic, but, rather, an antiandrogenic, activity (Lapĉik et al., 1995; Stárka, 2003). The expected differences in urinary concentrations could also be seen for androstanediol and less clearly but still significantly for 5α-DHT, etiocholanolone, and androsterone.

The determination of 5α-DHT in urine is confounded by epietiocholanolone, as both compounds coelute and have the same transition (Hauser et al., 2008). Therefore, the analytical results for 5α-DHT are less reliable than for the other androgens and their metabolites.

Urinary testosterone concentrations correlated with urinary androstanediol, but not with any other urinary androgen metabolite, when 10–22 year old intact male chimpanzees were included in the test. This finding underlines the suitability of androstanediol as a marker of testosterone excretion in chimpanzees. To more conclusively verify this conclusion, repeated measurements of androgens and their metabolites within multiple samples of the same subject over a certain time period, in which some variance of testosterone excretion can be observed would be preferable. The expected differences in testicular androgen metabolite levels between intact and castrated males, and between intact males and females were also seen in urinary concentrations of etiocholanolone and androsterone, but, as these compounds can also result from adrenal DHEA and A^4dione (Wudy and Hartmann, 2004; Shackleton et al., 1997), it does not seem appropriate to use them as markers of gonadal activity. Therefore, only the measurement of testosterone and androstanediol in urine seem to accurately reflect gonadal activity in chimpanzees. As a consequence, when using immunoassays for steroid analysis, these should be specifically directed at these compounds without showing cross-reactivities to other androgens or their metabolites. Given the much higher urinary concentration (up to 50 times) of DHEA, androstenediol, androsterone, and etiocholanolone, even a minor cross-reactivity towards these compounds would bias the results. Regarding the different conjugates of testosterone, the sum of unconjugated testosterone, testosterone glucuronide and testosterone sulfate revealed the same results when comparing the three different sex classes as the sum of unconjugated testosterone and testosterone glucuronide. Serum testosterone concentrations did significantly correlate with urinary testosterone plus testosterone glucuronide levels but not with urinary testosterone sulfate concentrations. Therefore urinary testosterone sulfate alone does not seem to represent a valuable measure of circulating and biologically active testosterone levels.

We cannot exclude the presence of other relevant testosterone metabolites in chimpanzee urine (Hagey and Czekala, 2003). The LC chromatogram shows another isomer of androstanediol (presumably 5β-androstane-3α,17β-diol), which has not been identified by the injection of a standard until now. In the study of Möhle et al. (2002) the 5α-androstane-17α-ol-3-one-assay was the only one that could detect significant differences between male and female chimpanzees. Within our chromatographic method, 17α-hydroxy-5α-androstane-3-one coelutes with androsterone, but from the relation of the two fragments chosen, it can be concluded that it represents a minor part of the androsterone peak in chimpanzees.

Although the radiometabolism study revealed a distinct radioactive peak at the elution position of epiandrosterone (Möhle et al., 2002), we found no differences in epiandrosterone and epietiocholanolone levels between intact and castrated male and female chimpanzees. Therefore, we do not consider epiandrosterone to be an exclusive metabolite of testosterone. The absolute concentrations of urinary androgens and androgen metabolites in chimpanzees found in our study are problematic to compare with data from other studies, as the analytical methods we employed differed dramatically in measurement method as well as sample preparation. While Möhle and colleagues (2002) report ten times higher testosterone concentrations, Klinkova and colleagues (2004) describe 4 times and Muller and colleagues (2004) 4–10 times higher urinary testosterone levels. All three studies used an enzyme derived from *Helix pomatia* for hydrolysis of steroid conjugates and EIA for quantitative analysis. Since *H. pomatia* juice has additional enzymatic activities catalyzing the conversion of androst-5-en-3β,17β-diol into testosterone the detected testosterone levels in the aforementioned studies might not represent the actual levels of testosterone and its conjugates in the samples before the hydrolysis.

With respect to androgen metabolism, the metabolic pattern of chimpanzees and humans show great similarities. In both species, testosterone, androstanediol, and 5α-DHT occur in relatively low concentrations in urine, while DHEA is the most abundant adrenal androgen. However, in humans, the urinary concentrations of androstanediol and 5α-DHT exceed those of testosterone (Geyer et al., 1997), while in this and a preceding study (Hauser et al., 2008a), we found lower concentrations of both metabolites relative to testosterone in chimpanzee urine. While the concentrations of androsterone and etiocholanone are about 20–60 times higher than the concentrations of testosterone in chimpanzee urine in our study, they are 50–400 times higher than testosterone in human urine (Geyer et al., 1997). In contrast, the ratio of androsterone to etiocholanolone seems to be lower in chimpanzees than in humans and the ratio of testosterone to epitestosterone appears to be higher in chimpanzees than in humans (Dehennin and Matsumoto, 1993; Donike et al., 1995). These differences could indicate a lower 5α-reductase-activity in chimpanzees than in humans and would be worth further investigation.

In summary, urinary testosterone seems to be the most reliable marker of testicular activity in chimpanzees. The other two main testosterone metabolites that might reliably trace testicular activity are 5α-DHT and androstanediol, both of which are known testosterone metabolites in humans (Dehennin and Matsumoto, 1993).

Please cite this article in press as: Hauser, B., et al. LC–MS analysis of androgen metabolites in serum and urine from east African chimpanzees (*Pan troglodytes schweinfurthii*). Gen. Comp. Endocrinol. (2010), doi:10.1016/j.ygcen.2010.09.012

Acknowledgments

We thank all involved veterinarians and keepers from Ngamba Island Sanctuary (Uganda), run by the Chimpanzee Sanctuary & Wildlife Conservation Trust, for the collection of urine and blood samples. We thank Roger Mundry for help with the statistical analysis, Vera Schmeling for extracting urine samples, Linda Kortz for advice on extracting serum samples, Carolyn Rowney and two anonymous reviewers for comments on earlier versions of this manuscript. This research was supported by the Max Planck Society.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygcen.2010.09.012.

References

Albertson, B.D., Hobson, W.C., Burnett, B.S., Turner, P.T., Clark, R.V., Schiebinger, R.J., Loriaux, D.L., Cutler, G.B., 1984. Dissociation of cortisol and adrenal androgen secretion in the hypophysectomized, adrenocorticotropin-replaced chimpanzee. J. Clin. Endocrinol. Metab. 59, 13–18.

Adams, D.C., Anthony, C.D., 1996. Using randomisation techniques to analyse behavioural data. Anim. Behav. 51, 733–738.

Anestis, S.F., 2006. Testosterone in juvenile and adolescent male chimpanzees (*Pan troglodytes*): effects of dominance, rank, aggression, and behavioral style. Am. J. Phys. Anthropol. 130, 536–545.

Arlt, W., 2004. Dehydroepiandrosterone and ageing. Best Pract. Res. Clin. Endocrinol. Metab. 18, 363–380.

Carlsen, F., 2007. European studbook for the chimpanzee *Pan troglodytes*. Copenhagen Zoo, Roskildevej 38, PO Box 7, DK-2000 Frederiksberg, Denmark.

Ceglarek, U., Kortz, L., Leichtle, A., Fiedler, G.M., Kratzsch, J., Thiery, J., 2009. Rapid quantification of steroid patterns in human serum by on-line solid phase extraction combined with liquid chromatography–triple quadrupole linear ion trap mass spectrometry. Clin. Chim. Acta 401, 114–118.

Conley, A.J., Pattison, J.C., Bird, I.M., 2004. Variations in adrenal androgen production among (nonhuman) primates. Sem. Reproduc. Med. 22, 311–326.

Copeland, K.C., Eichberg, J.W., Parker, R., Bartke, A., 1985. Puberty in the chimpanzee: somatomedin-C and its relationship to somatic growth and steroid hormone concentrations. J. Clin. Endocrinol. Metabol. 60, 1154–1160.

Cutler, G.B., Glenn, M., Bush, M., Hodgen, G.D., Graham, C.E., Loriaux, D.L., 1978. Adrenarche: a survey of rodents, domestic animals, and primates. Endocrinology 103, 2112–2118.

Dehennin, L., Matsumoto, A.M., 1993. 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. Molec. Biol. 44, 179–189.

Dittami, J., Katina, S., Möstl, E., Eriksson, J., Machatschke, I.H., Hohmann, G., 2008. Urinary androgens and cortisol metabolites in field-samples bonobos (*Pan pansicus*). Gen. Comp. Endocrinol. 155, 552–557.

Dixson, A.F., 1998. Primate Sexuality. Oxford University Press, Oxford.

Donike, M., Ueki, M., Kuroda, Y., Geyer, H., Nolteernsting, E., Rauth, S., Schänzer, W., Schindler, U., Völker, E., Fujisake, M., 1995. Detection of dihydrotestosterone (DHT) doping: alterations in the steroid profile and reference ranges for DHT and its 5α-metabolites. J. Sports Med. Phys. Fitness 35, 235–250.

Felig, P., Baxter, J.D., Frohmann, L.A., 1995. Endocrinology and Metabolism, third ed. McGraw-Hill, Inc., New York.

Geyer, H., Mareck-Engelke, U., Schänzer, W., Donike, M., 1997. The Cologne protocol to follow up high testosterone/epitestosterone ratios. In: Schänzer, W., Geyer, H., Gotzmann, A., Mareck-Engelke, U. (Eds.), Recent Advances in Doping Analysis (4). Sport und Buch Strauß, Köln, pp. 107–125.

Haccou, P., Meelis, E., 1994. Statistical Analyses of Behavioural Data. Oxford University Press, Oxford.

Hagey, L.R., Czekala, N.M., 2003. Comparative urinary androstanes in the great apes. Gen. Comp. Endocrinol. 130, 64–69.

Hauser, B., Deschner, T., Boesch, C., 2008a. 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, 100–112.

Hauser, B., Schulz, D., Bosch, C., Deschner, T., 2008b. Measuring urinary testosterone values of the great apes–problems with enzymatic hydrolysis using *Helix pomatia* juice. Gen. Comp. Endocrinol. 158, 77–86.

Hunter, M.A., May, R.B., 1993. Some myths concerning parametric and nonparametric-tests. Can. Psychol. 34, 384–389.

Klinkova, E., Heistermann, M., Hodges, J.K., 2004. Social parameters and urinary testosterone level in male chimpanzees (*Pan troglodytes*). Horm. Behav. 46, 474–481.

Lapĉik, O., Hampl, R., Hill, M., Stárka, L., 1995. Plasma levels of epitestosterone from prepuberty to adult life. J. Steroid Biochem. Molec. Biol. 55, 405–408.

Manly, B.F.J., 1997. Randomization, Bootstrap and Monte Carlo Methods in Biology. Chapman & Hall, New York.

Michael, G., 1999. Biochemical Pathways. Spektrum Akadem. Heidelberg, Verlag.

Möhle, U., Heistermann, M., Palme, R., Hodges, J.K., 2002. Characterization of urinary and fecal metabolites of testosterone and their measurement for assessing gonadal endocrine function in male nonhuman primates. Gen. Comp. Endocrinol. 129, 135–145.

Muller, M.N., Wrangham, R.W., 2004. Dominance, aggression and testosterone in wild chimpanzees: a test of the 'challenge hypothesis'. Anim. Behav. 67, 113–123.

Mundry, R., Fischer, J., 1998. Use of statistical programs for nonparametric tests of small samples often leads to incorrect *P*-values: examples from Animal Behaviour. Anim. Behav. 56, 256–259.

Palonek, E., Gottlieb, C., Garle, M., Björkhelm, I., Carlström, K., 1995. Serum and urinary markers of exogenous testosterone administration. J. Steroid Biochem. Molec. Biol. 55, 121–127.

Potter, D.M., Griffiths, D.J., 2006. Omnibus permutation tests of the overall null hypothesis in datasets with many covariates. J. Biopharm. Stat. 16, 327–341.

Rauh, M., Gröschl, M., Rascher, W., Dörr, H.G., 2006. Automated, fast and sensitive quantification of 17α-hydroxy-progesterone, androstenedione and testosterone by tandem mass spectrometry with on-line extraction. Steroids 71, 450–458.

Shackleton, C.H.L., Roitman, E., Phillips, A., Chang, T., 1997. Androstanediol and 5-androstenediol profiling for detecting exogenously administered dihydrotestosterone, epitestosterone, and dehydroepiandrosterone: potential use in gas chromatography isotope ratio mass spectrometry. Steroids 62, 665.

Sannen, A., Heistermann, M., Van Elsacker, L., Möhle, U., Eens, M., 2003. Urinary testosterone metabolite levels in bonobos: a comparison with chimpanzees in relation to social system. Behaviour 140, 683–696.

Siegel, S., Castellan, N.J., 1988. Nonparametric statistics for the behavioural sciences. Second ed. McGraw-Hill, New York.

Smail, P.J., Faiman, C., Hobson, W.C., Fuller, G.B., Winter, J.S.D., 1982. Further studies on adrenarche in nonhuman primates. Endocrinology 111, 844–848.

Stárka, L., 2003. Epitestosterone. J. Steroid Biochem. Molec. Biol. 87, 27–34.

Thienpont, L.M., Van Uytfanghe, K., Blincko, S., Ramsay, C.S., Xie, H., Doss, R.C., 2008. State-of the-art of serum testosterone measurement by isotope dilution-liquid chromatography tandem mass spectrometry. Clin. Chem. 54, 1290–1297.

Wingfield, J.C., Hegner, R.E., Dufty, A.M., Ball, G.F., 1990. The 'challenge hypothesis': theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. Am. Nat. 136, 829–846.

Wudy, S.A., Hartmann, M.F., 2004. Gas chromatography–mass spectrometry profiling of steroids in times of molecular biology. Horm. Metab. Res. 36, 415–422.

Please cite this article in press as: Hauser, B., et al. LC–MS analysis of androgen metabolites in serum and urine from east African chimpanzees (*Pan troglodytes schweinfurthii*). Gen. Comp. Endocrinol. (2010), doi:10.1016/j.ygcen.2010.09.012