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ScienceDirect
General and Comparative Endocrinology 155 (2008) 552–557
GENERAL AND COMPARATIVE ENDOCRINOLOGY
www.elsevier.com/locate/ygcen

Urinary androgens and cortisol metabolites in field-sampled bonobos (*Pan paniscus*)

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Received 5 December 2006; revised 30 July 2007; accepted 12 August 2007
Available online 25 August 2007

Abstract

Urinary metabolites of androgens and cortisol were measured in free-living male and female bonobos. Sex differences and correlations between adrenal and gonadal steroid excretion were investigated. The immunoreactive concentrations of androgens were measured with two different androgen assays. One assay used a testosterone (T) antibody raised with a 17β-hydroxy group, and the other employed an antibody raised against a reduced form, 5α-androstane-17α-ol-3-one-CM (17α) with cross reactivity for epitestosterone and 5α-androstanedione. Both assays have been used in bonobo and chimpanzee studies where non-invasive techniques were employed. The levels of 17α-androgen metabolites were 1.7- and 3-fold higher than those of T-metabolites in males and females. The two androgen assay results correlated in males but not females. There was a sex difference in the T-metabolites measured. Male levels were significantly higher. Levels of 17α in the two sexes were similar. Cortisol metabolite levels (CORT) were similar between the sexes. The T-metabolites were significantly correlated with CORT in males but not in females. In females, the 17α-androgen metabolites correlated with CORT. This suggests that either androgen secretion or metabolism differs between the sexes. A parsimonious interpretation of the androgen assay cortisol/androgen correlation differences would be that larger components of dehydroepiandrosterone (DHEA), androstenedione or epitestosterone from the adrenal androgens were being excreted and measured in the females. The CORT/T metabolite interactions in males may reflect male-specific social or metabolic endocrine conditions.

Keywords: Urinary testosterone/epitestosterone; Cortisol; Wild male and female bonobos

1. Introduction

The measurements of urinary and fecal androgens and corticosteroids in great apes have been used to study a wide range of interactions between physiology and behavior associated with reproductive cycles, nutrition and metabolism, dominance relationships and coping styles (e.g. Whitten et al. 1998; Möhle et al., 2000; Möhle 2001; Deschner et al. 2004; Muller and Wrangham 2004a, 2004b; Hagey and Czekala, 2003; Shimizu et al., 2003). Noninvasive monitoring techniques have been the only feasible way to get serial physiological data on individuals. Nonetheless field studies are plagued by methodological problems related to sampling techniques and the uncertainty about how metabolite concentrations reflect biologically relevant endocrine profiles (Palme 2005). Variation in systemic and environmental steroid metabolism along with the cross reactivity of the metabolites in immunological assays (Bahr et al. 2000; Möhle et al. 2002)

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doi:10.1016/j.ygcen.2007.08.009

often lead to erroneous estimates of endocrine secretion patterns and their functions. Another, perhaps less evident problem is that the development and validation of noninvasive techniques for field studies have usually been done with captive animals. The effects of captivity on gonadal and adrenal function are quite pronounced and difficult to assess in the field (e.g. Whitten et al. 1998). There is some consensus on the standardization of validation procedures for noninvasive endocrine techniques (Möhle et al. 1998; Palme 2005). Studies have demonstrated that there are predictable differences in steroid metabolism and excretion on both the species level and the individual level within a species, according to sex and age, and even temporal sampling patterns (e.g. Bahr et al. 2000; Möhle et al. 2002; Anestis and Bribiescas 2004).

The most common use of these techniques in primate research is associated with studies on behavioral endocrinology. Investigations have concentrated on androgen and cortisol metabolites, and the relationships among excretion patterns and behaviors associated with mate competition and reproductive (e.g. Heistermann et al. 1996; Deschner et al. 2004; Muller and Wrangham 2004a,b), social interactions (Strier et al. 1999; Möhle 2001; Goymann et al 2001), and environmental stressors (Steinmetz et al. 2006; Weingrill et al. 2004). The present study fits quite well into this line of primate research. The aim was to document the excretion of urinary androgen and cortisol metabolites in free-living bonobos, *Pan paniscus*. One key issue was the comparisons of the 17α-androgen metabolites with T-metabolites. Data on the former metabolites have been used as androgen markers for studies in behavioral endocrinology on captive bonobos (e.g. Sannen et al. 2003, 2004b). The two assay systems used show high cross reactivities with epitestosterone metabolites in one, and T-metabolites in the other. A second issue was the comparison of androgen excretion with cortisol excretion. A final point of study was the examination of sex differences in the datasets.

The results will aid the study of bonobo behavioral endocrinology in the field. Unlike chimpanzees that live in a strictly male dominated society, dominance relations between male and female bonobos have been described as being egalitarian (de Waal 1995) or female dominated (Parish 1996; Vervaeke et al. 2000). Animals are very gregarious even at times of food shortage (Hohmann and Fruth 2002). In conflict situations both sexes perform regularly dyadic interactions that contain components of sexual behavior (Hohmann and Fruth 2000). Accordingly, the species provides an opportunity to explore the adrenal and gonadal endocrine correlates of sexual and agonistic behavior and investigate the physiological connection between non-reproductive sexual behavior and social stress. This first set of androgen and cortisol metabolite data should pave the way for future field studies.

2. Methods

2.1. Study site, subjects, and urine sampling

Samples were collected by GH and JE in October and November 2000 from bonobos at Lui Kotal, located in the southern part of the Salonga National Park, Democratic Republic of Congo (Hohmann and Fruth 2003). Subjects ($N = 30$) were not habituated to the presence of humans. They were located acoustically and followed until they constructed sleeping nests at night. On the next morning, urine was collected from underneath the nests when subjects left the sleeping site (around 06.00). Following a protocol used in a previous study (Marshall and Hohmann 2005), urine was removed with a micropipette from the ground vegetation. Samples were only taken when no contamination with fecal material was present and when the spatial position of urine and feces for sex analyses were clearly related to a single nest. Samples were not collected when another nest was in close proximity. In some cases, urine was collected before the individual left the nest, but usually sampling took place after departure. Fresh urine was applied to filter paper (2×2 cm). Filter paper was stored in sealed containers with silica gel, a method that has been employed in a number of other primate species (e.g. Knott 1997; Marshall and Hohmann 2005). Using the criteria described above, following individuals that had been identified as adults, unambiguous sample allocation to a nest, and the fact that the nest was separated from other nests we can conclude that the samples were collected from adult individuals. Juveniles and adolescents nests are in close proximity to adults. Samples were collected in one run from an individual nest group. The nest group, in turn was associated with different social communities of Bonobos consisting of related and some unrelated females and a few males with little to no intergroup associations. Repeated individual sampling was thus highly improbable.

2.2. Sex determination

Identification of the sex of the urine donor was based on information from fecal samples collected at the same spot. After wakening, bonobos defecate from the rim of the nest, a habit that allows allocation of droppings to a given nest. From this and the spatial criteria described above, we assumed that feces and urine on the same patch were from the same individual. Sexing of fecal samples followed the procedure established in the MPI for Evolutionary Anthropology, Leipzig (Bradley et al., 2001; Eriksson et al., 2004, 2006). Assignments of sex were made when the same result was obtained in three independent extractions of nuclear material. Following this protocol, 14 samples were positively identified as male and 15 samples were identified as female.

2.3. Analyses of cortisol and androgen metabolites

2.3.1. The following antibodies were used in EIAs

A testosterone antibody raised against 17β-OH testosterone (the T assay, Palme and Möstl 1993), an androgen assay with an antibody raised against 5α-androstane-17α-ol-3-one-CMO-BSA (the 17α assay, Möhle 2001; Möhle et al. 2002) and a corticosteroid assay using an antibody against cortisol-3-CMO (the CORT assay, Palme and Möstl 1997).

The major cross-reactivities of the rabbit anti-testosterone used in the T assay, were 100% for testosterone, 23.7% for 5α-DHT, 12.3% for 5β-DHT, 7.6% for 4-Androsten-3α, 17β-diol. This was combined with a 5α-androstene-3β, 17β-diol3-HS-DADOO-biotin label and a starting standard of 20 pg testosterone/well.

Cross-reactivities of the 17α-androgen assay were 100% for epitestosterone, 3.7% for androstenedione, 1.8% for testosterone and 1.3% for 5α-androstanedione. This was combined with a epitestosterone-3CMO:DAADOO-biotin label and a starting standard of 200 pg epitestosterone/well.

For CORT, the metabolites were measured with EIA using DADOO-biotinylated cortisol-3-CMO labels (dilution: 1:250) and bovine serum albumin-coupled antibodies (dilution 1:20) against cortisol-3-CMO. The antibody was raised in rabbits. Cross-reactions of relevant steroids have been

described in detail elsewhere (Palme and Möstl, 1997). Briefly, the highest cross-reaction was found for 4-pregnene-11β, 17-α, 21-Triol-3, 20-Dione (100%) and 5α-pregnane-11β, 17α, 21-Triol-3, 20-Dione (4.6%). The assay has been shown to accurately reflect cortisol metabolites in feces and urine in a number of primate species including the chimpanzee (Bahr et al. 2000).

2.4. Assay procedures

Before analysis, the urine was re-suspended from the filter paper in 10 ml of methanol. Creatinine was measured using the standard Jaffe method (Slot 1965). After drying, the samples were re-suspended in acidic buffer and deconjugated. Ten microliters of a 1:10 dilution with assay buffer were used in the T assay, 20 μl of a 1:10 dilution were used in the CORT assay and 40 μl of a 1:1 dilution were used in the 17α assay. An incubation with a 1 μl glucuronidase/sulphatase followed to oxidize or deconjugate the steroids as has been described in Möstl and Palme (1993). In the assays, dilutions produced 20–85% binding values. Further information on assay sensitivities and procedures can be found in Palme and Möstl (1993, 1997), Möhle (2001), Bahr et al. (2000) and Möhle et al. (2002). The amount of excreted creatinine was used as a basis for concentration analyses. Intra- and inter-assay coefficients of variation of high- and low-value quality controls were 8%/12.3% for the 17α assay, 13.7%/15.2% for 17α and 9.4%/10.1 for CORT.

2.5. Statistics

Inspection of the field data showed that all three variables, for both sexes, were not normally distributed (One-sample Bootstrap Kolmogorov–Smirnov Goodness-of-fit test (KS) p-values < 0.05). Sex differences were documented with the Bootstrap Yuen-Welch (YW) test for mean differences and 95% Bootstrap YW confidence intervals (CI) as described in Pesarin (2001). In addition Two-sample Bootstrap Kolmogorov–Smirnov Goodness-of-Fit Tests were applied to each dataset pair, e.g. male and female CORT, to document sex differences in the distribution of the data. The resulting curves were obtained by smoothing the frequency distribution histograms with the method of Kernel Density Estimation (Venables and Ripley 2002). These bootstrap analyses differ from the more classical asymptotical approaches in a few ways. To begin with values of tests statistics are classically defined from asymptotic distributions. Using bootstrap analysis with 999 runs, an equal number of test statistics were produced. These test statistics themselves produce bootstrap distributions. These distributions are then used as a measure of significance. The combination of the YW and KS tests is important because differences in means or distributions can be attributed to different biological causes. For correlations between androgen and cortisol metabolites, a Bootstrap test was used again but in this case for association between Paired Samples using Spearman correlation coefficients (Good 2005). As above, but this time for the Spearman test, the values of test statistics are not relevant in the analyses here because an equal number of runs are done in each case that produce a statistical significance.

3. Results

To begin with, the levels of urinary 17α-androgen metabolites were higher than those of T-metabolites in both males and females (Fig. 1 bottom). The difference

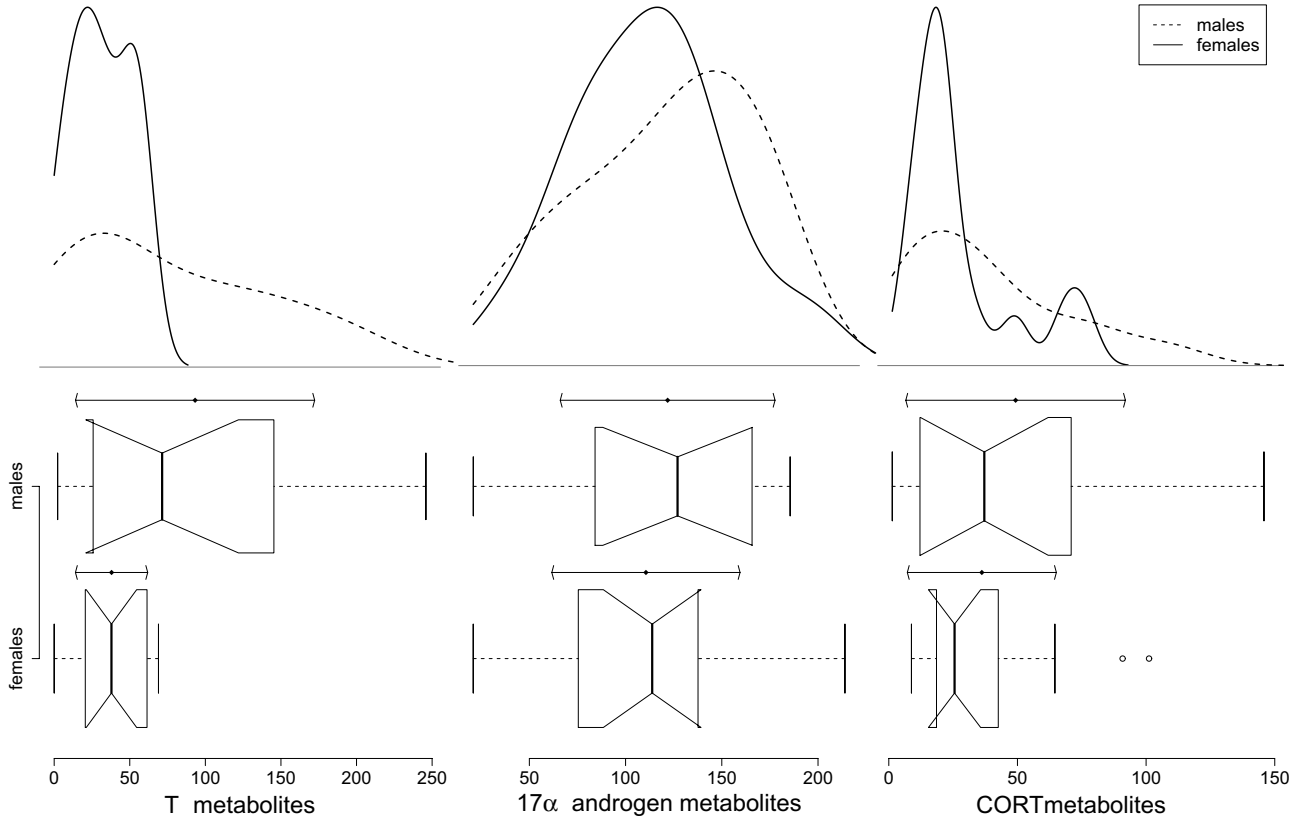

Fig. 1. Upper graph: The distributions (probability density functions) of T metabolites (left), 17α-androgen metabolites (center) and CORT (right) metabolites in the urine of male (dashed) and female (solid line) Bonobos. The curves result from smoothing of frequency distribution histograms using the method of Kernel Density Estimation (Venables and Ripley 2002). Two-sample Bootstrap Kolmogorov–Smirnov Goodness-of-Fit Tests were applied to each dataset pair. In all cases the KS analyses showed that the distributions differed between males and female. For T-metabolites the difference was based on location and amplitude of distribution. For 17α-metabolites there was a slight difference in the location and for CORT the amplitude differed between males and females. Lower graph: Box-and-whisker plots (with medians and quartiles) of individual hormone metabolite concentrations in urine (pg/mg creatinine). Means and standard deviations of the data are shown above the plots.

between 17α- and T-androgen titers in individual samples was larger in females than in males. In males, 17α-androgens were on average 1.7-fold higher and in females, 3.0-fold. These patterns are evident in the distribution patterns derived from Kernel Density Estimation and are plotted in Fig. 1 (upper graph). Two-sample, Bootstrap Kolmogorov–Smirnov Goodness-of-Fit Tests demonstrated a significant sex difference in the distributions for each of the endocrine parameters measured. For T metabolites, the KS test statistic was 0.433 and the p-value = 0.001, for 17α-androgen metabolites a KS of 0.188 and a p-value = 0.001 and for CORT, a KS value of 0.286 and a p-value = 0.001. Nonetheless, when one examines the density distributions it is evident that the T metabolite difference resulted from the predominance of lower T levels in females. For the 17α-androgen metabolites, a wide distribution of metabolite levels was found in both sexes, in spite of the significant differences in the overall distribution. Finally, the CORT distribution patterns in females had a predominance of low values with clusters of higher levels. This was in contrast to the male pattern that was much smoother over the concentration gradient.

The next point to consider was sex differences in the individual metabolite concentrations. There was a large inter individual variation in males for both androgen assays and in females with the 17α assay. None of the data sets were normally distributed (see Section 2). For this reason, bootstrap tests were employed to compare the means. This alleviated the issue of non random and perhaps even bimodal distribution of the ranks. The tests demonstrated that values of T-metabolites were significantly different between males and females (see Table 1). There were no sex differences in 17α-androgen metabolites and no sex differences in CORT. Again, is should be reiterated that although the distributions of the datasets differed significantly between the sexes for all three metabolites, the only significance in the mean values was found for T metabolites.

In a final analysis we opted to examine whether the datasets co-varied among individuals. To do this, we employed Bootstrap tests for the association between paired samples using Spearman correlation coefficients (see Section 2 and Good 2005). The analyses showed that 17α-androgen and T-metabolites were positively correlated in males ($p = 0.005$) and not in females ($p = 0.18$). The relationships between androgen metabolites and cortisol metabolites also differed between the sexes. T metabolites were positively related to CORT in males ($p = 0.005$) and not in females ($p = 0.2$). The correlation between 17α-androgen and CORT metabolites on the other hand were only close to significance in males ($p = 0.06$) and significant in females ($p = 0.03$).

In summary, males have higher T excretion levels than females in the field. The relative amount of 17α-androgen metabolites vs. T-metabolites excreted in urine was higher in females. Distribution pattern of the metabolites differed between the sexes. The two androgen assays correlated well with one another in males but not in females. Finally, the T-metabolite levels correlated with CORT in males and the 17α-androgen metabolites correlated with CORT in females.

Table 1
Urinary Androgen and corticosteroid differences between the sexes

	95% Confidence intervals	YW statistic	p-value
T-metabolites	(10.19, 100.31)	2.2579	0.02
17α-androgen metabolites	(−30.42, 52.98)	0.4825	0.61
Cort metabolites	(−16.00, 42.24)	0.8566	0.38

Bootstrap 95% confidence intervals (left and right boundaries) are shown for the male-female differences in means as are the YW test statistics and p-values of significance. The only significant sex differences found were in the analysis of means for T-metabolites.

4. Discussion

The data presented provide information about adrenal and gonadal steroid excretion of male and female bonobos in the field. The results contribute to the existent bonobo data because both cortisol and androgen metabolites were examined in the same samples, and two androgen assays were compared. The two androgen assays produced different results when compared to sex specificity and their relationships with cortisol excretion.

To date, most endocrine data from bonobos had been restricted to subjects living in captivity (Dahl et al 1991; Heistermann et al. 1996; Reichert et al. 2002; Sannen et al 2004a,2004b). The only exception was a short-term study on individually recognized males from a habituated community at Lomako/DRC (Marshall and Hohmann 2005). From studies on primates and other social mammals it is known that the captive situation is likely to affect social relations, competition for resources and the expression and execution of dominance. Accordingly, hormonal profiles may also be affected (Goymann et al. 2001), even in bonobos (Marshall and Hohmann 2005). Unfortunately, there are no published data on urinary cortisol concentrations in captive bonobos. However, using the same androgen assay system, which had been implemented and validated to an extent on captive bonobos (e.g. Bahr et al. 2000; Möhle et al. 2002), the data from wild animals are relevant to assess the impact of constraints imposed by the captive situation on these androgens. The levels of both types of androgen metabolites found in the captive studies were similar to those reported here from the field. In captivity, a marginal difference was found in urinary 17α-androgen metabolites; male levels were higher than female. The argument followed that male and female bonobo androgen secretion levels were comparable, and that they represented a form of demasculinisation in males that had not been found in chimpanzees (Sannen et al. 2003). This argument has been weakened by the addition of T metabolite data.

Sex differences *were* found with the T assay with antibodies raised against 17β-OH androgens.

One must then address the issue of what is being measured in the 17α-androgen assay, and whether the metabolites are related to biologically active androgens in circulation. Here, it is important, first, to note that the two androgen assays correlated well in males but not at all in females. The latter sex excreted proportionally larger amounts of epitestosterone metabolites. This means that there is either a common source or at linked sources of the two androgen metabolites in males. The different sources would naturally be gonadal and adrenal.

The CORT/T/17α-metabolite correlations are of interest here. In males, T- and 17α-androgens were significantly correlated. In females, only 17α-androgen metabolites were related to CORT. The differences in these correlations suggests that the female 17α-androgen metabolites may be produced by the adrenals and not be of gonadal origin. The sources would be epitestosterone, DHEA and androstenedione (Möhle 2001). Without further intracellular metabolism in target tissues, these androgens would be of little biological relevance. Hence, one is forced to question the relevance of this type of data in behavioral endocrinology. One last problem in the assumption that the female 17α-androgen metabolites may stem from the adrenal is the differences one sees in the distribution patterns of CORT and 17α-metabolites. The begs the issue that ACTH driven reticularis secretion is the only source of the metabolites. Other sources like the follicles have to be considered (e.g. Starka 2003).

The data presented in this paper also demonstrate the need for more detailed chemical analyses of urine before one can apply immunological assays to monitor gonadal and adrenal function. So-called immuno screening after HPLC separation is an easy approach. This could be complemented by GC analyses and tracer studies to document the chemical composition of the steroids and their metabolic pathways. These studies are in planning.

In conclusion, one should mention the implications of these preliminary results for the understanding of bonobo biology:

First, wild males have higher and more variable testosterone levels than females. Elevated adrenal activity, related to social and/or physical stress, can suppress testicular function but it does not apparently did not do so in the males. There were positive correlations between adrenal and gonadal steroids as if the males could up-regulate both testes and adrenal without any negative interactions. This would be characteristic for early phases of agonistic encounters but not for prolonged fights or subordination. It is also known in conflict situations with no apparent winner or socially instable societies that the two endocrine systems can be up-regulated in parallel. The fission fusion nature of bonobo societies and the relatively peripheral social role of males would allow one to predict this positive correlation. Sexual competition is not associated with monopolization. Males can and do engage in agonistic interactions, but they can easily withdraw participation. They then perhaps have a degree of resistance to stress-related gonadal down-regulation. This has not been directly investigated in captivity.

Female production of adrenal androgens like epitestosterone, androstenedione or DHEA is a topic that has puzzled physiologist for years. Although these androgens have little inherent biological activity they are important as precursors or prohormones of testosterone, DHT or estrogens. They may then have roles in reproduction, immune function or mineral and fat balance. More work is needed here to characterize the metabolites better and follow their excretion patterns associated with reproductive cycles and conditional changes in the females.

Acknowledgments

We thank Ibraham Elmadfa for his advice and help in creatinine questions, Carol Deimel for her laboratory help and constructive discussions, Anna Schöbitz for her exact and conscientious laboratory work and last but not least Dagmar Rotter for her unfailing psychological, administrative and academic support.

Statistical analysis was supported by MRTN-CT-2005-019564 (EVAN) and VEGA 1/3023/06. Thanks are due to the Institut Congolaise pour la Conservation de la Nature and to the Dept. de l'Enseignement Superieur et Universitaire et de la Recherche Scientifique for granting permission to conduct fieldwork and for giving the permit to export urine samples, and to the people of Lompole for giving permission to work in their forest. GH and JE received funding from the Max-Planck-Society, The Leakey Foundation for Anthropological Research, and from the Volkswagen Foundation.

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