Adrenarche in bonobos (*Pan paniscus*): evidence from ontogenetic changes in urinary dehydroepiandrosterone-sulfate levels

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Abstract

Adrenarche is characterized by the onset of adrenal secretions of increasing amounts of dehydroepiandrosterone-sulfate (DHEA-S). While the function of adrenarche remains a matter of speculation, evidence suggests that the morphological and physiological changes related to it are restricted to humans and closely related primates. Within the primate order, adrenarche has been described only in humans and chimpanzees, but bonobos, the sister species of chimpanzees, have not yet been studied regarding the early ontogenetic changes such as adrenarche. While bonobos and chimpanzees share many morphological and behavioral characteristics, they differ in a number of behavioral traits, and there is a growing interest in terms of the physiological differences that can be linked to species-specific patterns of social behavior. In this study, we measured urinary DHEA-S levels to determine whether bonobos experience physiological changes that are indicative of adrenarche. We measured DHEA-S in urine using ELISA and analyzed its levels in the samples from 53 bonobos aged 1–18 years. Our results show that bonobos experience an increase in DHEA-S levels after 5 years of age, which is comparable with the patterns observed in humans and chimpanzees. This indicates that bonobos do undergo adrenarche and that the timing of onset is similar to that of the two *Pan* species. The extraction procedures described in this report demonstrate the use of urine for monitoring ontogenetic changes in DHEA-S excretion. If applicable to other species, the technique would facilitate more research on the evolutionary origin of adrenarche and other developmental processes.

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Introduction

Adrenarche is a distinct developmental process in the ontogeny of humans and has also been reported to occur in chimpanzees, *Pan troglodytes* (reviewed in Campbell (2006)). Various criteria have been used to define adrenarche including morphological, biochemical, and hormonal parameters (Conley *et al.* 2012). In this study, we used hormonal profiles to identify age-related changes in dehydroepiandrosterone-sulfate (DHEA-S) secretions and to investigate whether adrenarche in bonobos followed the pattern found in studies on humans and chimpanzees (Ducharme *et al.* 1976, Cutler *et al.* 1978, Collins *et al.* 1981, Ibáñez *et al.* 2000), as hormonal studies have shown to be sufficient for detecting the onset of adrenal secretions of DHEA and DHEA-S and this onset is related to the maturation of the adrenal cortex. Although temporarily related to gonadarche, the two processes are considered to be physiologically independent (Sklar *et al.* 1980). In humans, adrenarche precedes the activation of gonads at the end of the prepubertal phase of development. Specific changes in androgen secretions have been related to the development of the *zona reticularis* (ZR) and its major androgen products, DHEA and DHEA-S (Dhom 1973, Conley *et al.* 2004). After birth, the prominent fetal zone of the adrenal gland undergoes involution but is restored in early childhood when the neocortex develops into the adult adrenal with a ZR (Bech *et al.* 1969). This process of morphological transformation correlates with an increase in the production of DHEA and DHEA-S (e.g. Ducharme *et al.* 1976, Ibáñez *et al.* 2000, Havelock *et al.* 2004). In humans, adrenarche is a gradual process that starts early in life (Palmert *et al.* 2001, Martin *et al.* 2004) and continues until the age of 8 years in females and 9 years in males (Wiermann *et al.* 1986, Ibáñez *et al.* 2000). Levels of DHEA-S and DHEA, both in human serum and urine, showed a decrease after birth followed by an increase during childhood and leading to a peak in adulthood (Ducharme *et al.* 1976, de Peretti & Forest 1978, Kelnar & Brook 1983, Parker 1999, Remer *et al.* 2005).

A number of studies have reported ontogenetic changes that are indicative of adrenarche in chimpanzees (Conley *et al.* 2004, Oberfield & White 2009), but information from other hominoid primates such as gorillas and orangutans is limited (Cutler *et al.* 1978, Collins *et al.* 1981, Bernstein *et al.* 2012) and nothing is known about adrenarche in bonobos (*Pan paniscus*), the sister species of chimpanzees. Unlike in hominoids, studies on macaques and baboons indicate

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that DHEA-S levels decline steadily throughout the lifespan (Castracane *et al*. 1981, Meusy-Dessolle & Dang 1985, Crawford *et al*. 1997, Lane *et al*. 1997). Based on this information, adrenarche is considered to be a derived trait that has evolved relatively recently (Havelock *et al*. 2004) and, by inference, has been related to neural developments characteristic of humans and hominoid primates (reviewed in Campbell (2006)).

Given the paucity of data on ontogenetic developments in hominoid primates, exploration of the ontogenetic developments of other species is of general interest. In addition, studies on the two *Pan* species, bonobos and chimpanzees, which are our closest living relatives, may be particularly relevant. Due to their close phylogenetic ties, it is not surprising that these two species share a number of behavioral and morphological traits. At the same time, there is growing evidence that the two species differ consistently in terms of patterns of social behavior as well as cognitive skills, and recent studies have highlighted the divergent development of the physiological mechanisms that are functionally linked to certain behavioral patterns (Furuichi 2011, Hare *et al*. 2012).

Given the growing interest in the evolution of social and cognitive skills in the two *Pan* species, more information on ontogenetic development is required to detect events that may lead to the observed differences in behavior. Bonobos and chimpanzees are equally closely related to humans, and both species are often used as referential models to reconstruct evolutionary developments in human evolution (Zihlman 1996). Based on their close relationship to humans and chimpanzees, it is reasonable to assume that bonobos undergo adrenarche. However, based on the patterns of cranial anatomy, it has been suggested that bonobos are pedomorphic relative to chimpanzees (e.g. Shea 1983, Lieberman *et al*. 2007), and recent behavioral studies have reported species differences that are also indicative of pedomorphism (Hare *et al*. 2012, reviewed in Wobber *et al*. (2010)). Two general possibilities that would be in line with the presumed heterochrony are that adrenarche in bonobos is either delayed or occurs earlier than in chimpanzees.

The aims of the study are to i) validate a commercial ELISA that was originally developed to measure DHEA-S in human serum, for use in urine; ii) compare DHEA-S in samples from bonobos of both sexes and of different ages to investigate whether bonobos show age-related changes in DHEA-S secretion that are indicative of the adrenal activity associated with adrenarche as reported in chimpanzees and humans, provided that the first two aims were successfully achieved; and iii) compare the timing of adrenarche in the two *Pan* species.

Materials and Methods

Animals and sample collection

To detect age-related changes in bonobos, we measured 77 urine samples from 23 males and 30 females from ten zoos (Table 1). Urine samples were collected between 0900 and 1600 h. Samples were either placed directly into plastic cups or taken from the ground with plastic pipettes and then transferred into plastic tubes. Samples were collected only when the individual could be identified and when contamination with feces could be excluded. After collection, the samples were stored at −20 °C and transported on dry ice to the Max Planck Institute for Evolutionary Anthropology in Leipzig, Germany, for processing. The individuals sampled included six males and six females aged 1–5 years, six males and seven females aged 6–9 years, and 11 males and 20 females aged between 10 and 18 years, inclusive. In this study, we measured urinary DHEA-S levels in bonobos aged 1–18 years. Individual samples were chosen to match the age distribution used in the corresponding studies on chimpanzees. To our knowledge, the oldest chimpanzee involved in a study assessing DHEA-S levels in this species was 22 years (Copeland *et al*. 1985), which is comparable with the age of the oldest bonobo used in our study.

Age classes were chosen to facilitate comparison with ontogenetic changes in DHEA-S levels reported in chimpanzees (Smail *et al*. 1982) and to account for the finding that the development of the ZR is considered to be complete in humans at 10 years of age (Dhom 1973). The urine collection procedure was in accordance with NIH published standards. For validation purposes, urine samples from two women (28 and 31 years of age) and one man (30 years of age) were collected at Max Planck Institute for Evolutionary Anthropology in Leipzig, Germany. Samples were handled as described for bonobo urine.

While previous work on DHEA-S relied mainly on serum to assess adrenal activity, new endocrinological techniques permit the measurement of changes in adrenal activity in other matrices such as urine. Unlike invasive serum collection, extraction of androgens from urine allows for the exploration of patterns of adrenal activity in a noninvasive way; thus, this method expands the applicability of such studies to subjects living in natural environments (reviewed in Lasley & Kirkpatrick (1991)). As only one study to date has investigated variations in DHEA-S levels in the context of

Table 1 Zoo name, location, and number of individuals (separated by sex)

Zoo	Country	Males	Females
Apenheul	The Netherlands	0	4
Zoo Frankfurt	Germany	5	6
Kölner Zoo	Germany	1	4
Zoo Leipzig	Germany	2	3
Milwaukee	USA	5	4
Planckendael	Belgium	3	1
San Diego WAP	USA	2	1
San Diego Zoo	USA	1	2
Twycross Zoo	Great Britain	2	3
Zoo Wuppertal	Germany	2	2

WAP, wild animal park.

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adrenarche in urine from captive chimpanzees (Anestis *et al.* 2009), the potential for such studies on hominoid primates has gone largely unexplored.

Sample preparation

Samples were prepared in two different ways to facilitate i) measurements of DHEA-S by an liquid chromatography–tandem mass spectrometry (LC–MS/MS) and ii) by an ELISA. LC–MS/MS measurements were used for the validation of the ELISA results.

LC–MS/MS sample preparation

For the LC–MS/MS measurement, 21 urine samples were purified by solid-phase extraction (SPE) followed by a liquid–liquid extraction (LLE, see both below), resulting in a tenfold concentration of the original sample while simultaneously reducing matrix effects. Nine of the samples were used to validate the extraction method, eight additional samples were prepared for comparison with the ELISA measurement, and one pool was prepared out of four samples from four individuals and spiked for a recovery experiment (see below).

Solid-phase extraction

Samples were spiked before purification with the same mixture of internal standards used for the calibration curve, at 50 ng/ml final extract (for detailed information, see Hauser *et al.* (2008)). A mixture of 500 μl urine and 500 μl distilled water (Aqua B. Braun, Melsungen, Germany) was loaded onto a polymer-based reversed phase cartridge (Chromabond HR-X, 30 mg, Macherey-Nagel, Duren, Germany). Before sample application, cartridges were conditioned with 1 ml methanol (supplied by Roth, Karlsruhe, Germany), followed by 1 ml water (Aqua B. Braun) and 1 ml phosphate buffer (pH 6·9). Cartridges were washed three times with 1 ml water followed by 1 ml of 40% methanol. Steroids were eluted with 1 ml methanol followed by 1 ml ethyl acetate (Roth) and collected in a single fraction (for details, see Weltring *et al.* (2012)). Samples were evaporated until completely dry and reconstituted in 1 ml water.

Liquid–liquid extraction

Free steroids were separated from conjugated ones by LLE with *tert*-butyl methyl ether (TBME: VWR, Darmstadt, Germany). The reconstituted samples after SPE were added to 5 ml TBME in a reaction tube. The reaction tubes were vortexed for 10 min (VX-2500 Multi-Tube Vortexer) and then centrifuged for 5 min at 2000 ***g***/min (Multifuge 3 S-R Heraeus) before being frozen overnight. In the next step, the organic layer was decanted and the aquatic layer was evaporated with compressed air at 45 °C. For nine samples (three from humans and six from bonobos, all between 2 and 31 years of age), we evaporated both the layers to test the extent to which DHEA-S was dissolved in the aquatic layer. The evaporated samples were reconstituted in 50 μl of 30% acetonitrile (ACN) in water. ACN was supplied by Roth.

LC–MS/MS measurement

LC separation was performed on a reverse phase C-18 column (Gemini C18; 150×2 mm, 3 μm, Phenomenex, Torrance, CA, USA) protected by a guard column of the same material (Security Guard; 4×2 mm, 5 μm, Phenomenex). We used a Waters Alliance 2695 separation module equipped with a quaternary pump and column oven (Waters, Milford, MA, USA). The composition of eluent A and eluent B was water/ACN 95+5 (v/v) and water/ACN 5+95 (v/v), respectively, and both contained 0·1% formic acid. The gradient was 30% B (0 min), linear increase to 70% B (0–20 min), linear increase to 90% B (20–21 min), 100% B (21–30 min), 30% B (30–39 min). Twenty microliters of the extract were injected. For further information, see Hauser *et al.* (2008). The retention times of DHEA-S and DHEA were 16·1 and 19·2 min respectively.

The MS analyses were carried out on a Quattro Premier XE tandem mass spectrometer with Z spray ESI interface (Micromass, Manchester, UK) and the settings were the same as those described in Hauser *et al.* (2008). We examined LC–MS/MS data with MassLynx (Version 4.1; QuanLynx-Software). We excluded samples in which the recovered value of the internal standard deviated more than ±50% from the expected value.

ELISA sample preparation

To investigate ontogenetic changes in DHEA-S excretion, we measured 53 urine samples from 53 different individuals by a commercial DHEA-S ELISA (DHEA-S ELISA, Ref. RE52181, IBL International GmbH, Hamburg, Germany). In addition, to monitor within-individual longitudinal changes, we measured four samples, each taken at a different age, from five individuals, for a total of 20 samples.

Liquid–liquid extraction

A mixture of 500 μl urine and 500 μl distilled water (Aqua B. Braun) was added to 5 ml TBME in a reaction tube and treated as described in the LLE section for LC–MS/MS measurements earlier. After evaporation, samples were reconstituted in 50 μl Standard A (a buffer provided by IBL International consisting of human serum and stabilizers), which resulted in a tenfold concentration of the original sample. The use of this buffer was necessary because initial testing with the assay revealed unrealistically high levels of DHEA-S when pure urine was applied to the assay or when water or phosphate buffered solution was used for the dilution or reconstitution of samples after LLE.

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ELISA measurement

The prepared samples were analyzed by a DHEA-S ELISA, a commercial assay from IBL International for the quantitative determination of DHEA-S in human serum or plasma. The assay was performed following the instructions from the supplier. Briefly, 25 μl of each sample extract and 200 μl of enzyme conjugate were added to each well on the plate. After incubation, the plates were washed and tetramethylbenzidine substrate solution was added. The reaction was stopped with the provided stop solution after incubation and measurement was carried out photometrically at 450 nm. All standards and controls were measured in duplicate. Because the measurement of the first 38 sample extracts resulted in a deviation of duplicate levels of <10% in each single measurement, the remaining samples were measured only once. Interassay variation of five separate runs for high- and low-quality controls were 2·96 and 5·84% respectively. Intra-assay variation was 8·47 and 7·45% respectively.

To compensate for variation in volume and concentration of the urine, we measured creatinine (cr) concentrations in each urine sample (Bahr *et al*. 2000) and expressed all DHEA-S levels in nanogram per milligram cr. As very low urinary creatinine concentrations can lead to overestimated DHEA-S levels, we excluded all urine measurements with creatinine concentrations below 0·1 mg/ml from the analyses (15·4% of all samples measured).

Cross-reactivities, as listed in the assay description, were 100% for DHEA-S, 5·67% for androsterone sulfate, 0·13% for 17-α-hydroxyprogesterone sulfate, 2·62% estrone, 2·13% testosterone, and 0·93% for progesterone. Because no cross-reactivity was observed for DHEA, we tested it with a DHEA standard (supplied by Steraloids CAS 53430, Batch H233, New Port, CT, USA). DHEA standard was diluted with Standard A to 1 μg/ml and applied five times to the DHEA-S ELISA plate. We found 347% (S.D. 12·7) cross-reactivity.

For assessment of parallelism, two studies were conducted to investigate whether DHEA-S levels were comparable across different stages of dilution and concentration or whether unspecific matrix effects interfered with the DHEA-S binding reaction. In the first experiment, six sample extracts (one human, five bonobo) were measured undiluted and in 1:2 and 1:3 dilutions. As DHEA-S levels of all diluted sample extracts were below the linear range of the assay, we conducted a second experiment in which six sample extracts (two human, four bonobo) were measured undiluted and five and ten times concentrated. Two bonobo urine samples without increased concentration were out of range and could therefore not be used to predict the levels of the concentrated samples (data not presented). Results of other samples are shown in Table 2. The average recovery of urine was 96% (S.D. 5·24) in human and 92% (S.D. 10·14) in bonobo.

Accuracy

To assess the accuracy of DHEA-S measurements by LC–MS/MS, a pool of the following bonobo samples was prepared: 2 ml from a 4-year-old male, 1 ml from an 11-year-old female, 1 ml from an 8-year-old male, and 2 ml from a 10-year-old female. For the LC–MS/MS measurements, the standard DHEA-potassium sulfate (supplied by Steraloids) was diluted to 0·1, 0·3, 0·9, and 2·7 μg/ml calculated as free DHEA-S, comparable with the Standard B, C, D, and E of the assay kit, and an additional dilution of 0·03 μg/ml was introduced. We added 50 μl of each diluted Standard to 50 μl of our internal standard mix with 900 μl Braun water. These samples were then extracted in the same way as the other samples were for LC–MS/MS measurement (see earlier).

To assess the accuracy of the DHEA-S measurement by DHEA-S ELISA, the same sample as described earlier was used. Subsamples of the pooled samples were spiked with Standard B (0·1 μg/ml), C (0·3 μg/ml), D (0·9 μg/ml), E (2·7 μg/ml), and F (10 μg/ml) from the assay kit respectively. We added 250 μl of the pool sample to 25 μl of each Standard with 725 μl Braun water. Afterward, the samples were prepared with LLE and reconstituted in Standard A (see above). The recovery was calculated as:

$$\mathrm{REC}\ (\%) = \frac{(C_{\mathrm{SS}} - 0{\cdot}5 \times C_{\mathrm{OS}}) \times 100}{0{\cdot}5 \times C_{\mathrm{S}}}$$

with C_{SS} being the measured concentration of the spiked sample, C_{OS} being the measured concentration of the pooled sample, and C_{S} being the concentration of the added standard.

Table 2 Recovery of DHEA-S (μg/ml) in concentrated bonobo and human urine samples

Species	Sex	Age	Untreated (μg/ml)	Concentration level	Expected (μg/ml)	Observed (μg/ml)	Recovery (%)
Human	Female	31	0·395	10:1	3·95	3·95	100·0
				5:1	1·98	1·93	97·5
Human	Female	2	0·137	10:1	1·37	1·35	98·5
				5:1	0·69	0·61	88·4
Bonobo	Male	28	0·089	10:1	0·89	0·79	88·8
				5:1	0·45	0·46	102·2
Bonobo	Female	13	0·148	10:1	1·48	1·46	98·6
				5:1	0·74	0·59	79·7

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Stability experiment

To test the stability of DHEA-S in urine, we conducted two experiments with five samples each. In the first experiment, we thawed and froze portions of the five samples up to four times. In the second experiment, we left portions of the same samples at room temperature for 0, 6, 9, 33, and 57 h.

Circadian influence

While plasma DHEA-S levels show only minor circadian fluctuations, those of DHEA seem to follow a circadian pattern similar to that of cortisol, with higher levels in the early morning and lower levels toward the afternoon (Rosenfeld *et al*. 1971). As we are not aware of any published data on circadian fluctuations in DHEA-S levels in urine, we used a subset of 34 urine samples collected between 0900 and 1600 h from adult bonobos between 10 and 18 years of age to investigate diurnal fluctuations in urinary DHEA-S excretion.

Statistical analysis

To investigate the differences in urinary DHEA-S levels across different age categories, we used a subset of 53 samples, in which each individual was represented only once. DHEA-S levels were square root (sqrt) transformed to achieve normal distribution (assessed by visual inspection of residuals plotted against fitted values) and tested for homogeneity of variances using Levene's test ($P=0{\cdot}116$). To explore age differences, we used a one-way ANOVA applied to sqrt-transformed averages of each of the four age categories. For subsequent *post hoc* comparison, we used a Tukey test. We used Mann–Whitney *U* tests to explore between-sex differences in DHEA-S levels (in bonobos older and younger than 10 years). Spearman's rank correlation was used to investigate the relationship between age (in months) and DHEA-S levels in urine, time of day of urine excretion and DHEA-S concentrations in bonobos older than 10 years, and to compare DHEA-S levels of the same samples measured by LC–MS/MS vs ELISA. A Wilcoxon matched-pair signed-rank test was used to compare urinary DHEA-S levels measured by LC–MS/MS with measures obtained by ELISA. All tests were performed with SPSS 20 (IBM, Germany).

Results

Assay and extraction validation

Testing the separation efficiency of DHEA and DHEA-S after LLE showed that, in all eight tested samples, no DHEA-S could be detected in the organic phase while no DHEA was found in the aquatic phase. Accuracy was tested for ELISA and LC–MS/MS. Average recovery of ELISA was 77% (range: 64–90%, S.D. 5·96; Table 3). Average recovery of LC–MS/MS measurements was 99% (range 89–111%, S.D. 8·15; Table 4). DHEA-S levels measured by LC–MS/MS correlated significantly with DHEA-S levels of the same samples measured by ELISA (Spearman's correlation: rS$=0{\cdot}833$; $n=8$; $P=0{\cdot}01$). When measured by LC–MS/MS, average DHEA-S concentrations did not differ significantly from measurements by ELISA (5·89 pg/µl, S.D. 4·09 vs 7·18 pg/µl, S.D. 7·46, Wilcoxon matched-pair signed-rank test $V=22$; $P=0{\cdot}575$).

Urinary DHEA-S stability

Repeatedly thawing the bonobo urine samples (up to four times) led to a consecutive decrease in DHEA-S levels (Fig. 1). After the first thawing cycle, an average of 78% of the

Table 3 Recovery of DHEA-S in spiked bonobo urine pool samples measured by ELISA

		DHEA-S (µg/ml)		
Sample	**Standard**	expected	measured	**Recovery** (%)
Pool A				
	B	0·41	0·31	74
	C	0·51	0·39	77
	D	0·77	0·66	86
	E	1·7	1·34	79
	F	5·37	3·97	74
Pool B				
	B	0·34	0·27	80
	C	0·54	0·41	75
	D	0·8	0·66	82
	E	1·73	1·32	76
	F	5·36	3·97	74
Pool C				
	B	0·24	0·19	78
	C	0·34	0·31	90
	D	0·77	0·58	75
	E	1·67	1·25	75
	F	5·39	3·45	64

Standard concentrations: B–F.

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Table 4 Recovery of DHEA-S in spiked bonobo urine pool samples measured by LC–MS/MS

		DHEA-S (μg/ml)		
Sample	**Standard**	expected	measured	**Recovery** (%)
Pool				
	0·03	116·72	113·21	97
	0·1	121·48	134·84	111
	0·3	180·92	183·62	102
	0·9	202·23	194·14	96
	2·7	379·97	338·17	89

original DHEA-S levels was still detectable but samples differed widely (S.D. 36). A second thawing cycle reduced the DHEA-S levels to about 50% of the original concentration and DHEA-S levels remained stable for two additional thawing cycles.

DHEA-S levels in bonobo urine samples stored at room temperature remained stable for the first 12 h. After 33 h, average DHEA-S levels dropped to ∼85% (S.D. 8·46) from their original concentration. No further degradation was observed after 57 h (Fig. 2).

Age-related changes in urinary DHEA-S levels

In both sexes, a significant positive correlation between age (in months) and urinary DHEA-S levels was found (Spearman's: $r_{males}=0{\cdot}628$, $n=23$, $P<0{\cdot}001$; $r_{females}=0{\cdot}608$, $n=30$, $P<0{\cdot}001$). Urinary DHEA-S levels remained low until the age of 5 years. Thereafter, an increase in DHEA-S levels accompanied by an increasing variance in DHEA-S levels was detected (Fig. 3) and high DHEA-S levels were detected at the age of 11 years.

As shown in Fig. 4, bonobos younger than 5 years had average urinary DHEA-S levels of 246·13 ng/mg cr (S.D. 131·91), while 5–9-year olds had average levels of 737·84 ng/mg cr (S.D. 292·06) and individuals 10 years and older had average levels of 897·19 ng/mg cr (S.D. 359·48).

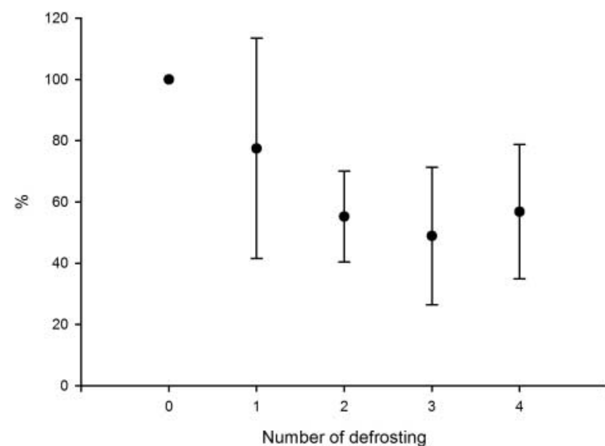

Figure 1 Mean DHEA-S recovery in five bonobo urine samples during four freezing cycles (bars indicate the S.D.).

The differences in DHEA-S levels between the three age categories were significant ($F(2,51)=30{\cdot}31$; $P<0{\cdot}001$), and a pairwise comparison of age categories revealed that DHEA-S levels in bonobos younger than 5 years were significantly lower than in individuals between the ages of 5 and 9 years (Tukey test, $P<0{\cdot}001$) and in individuals older than 9 years (Tukey test, $P<0{\cdot}001$). No significant difference was found for bonobos older than 5 years but younger than 10 years and individuals older than 10 years (Tukey test, $P=0{\cdot}331$).

Measurements of DHEA-S concentrations from five individuals from the same facility (Frankfurt Zoo) that were sampled repeatedly at different ages are shown in Fig. 5. All individuals showed an increase in DHEA-S related with age. Note that the two older individuals (Zomi and Kutu) exhibit a three- and fourfold increase in DHEA-S levels between the ages of 7 and 13 years.

Sex-related changes in urinary DHEA-S levels

Urinary DHEA-S levels did not differ between males and females older than 10 years, which is when adrenarche is expected to have subsided (Mann–Whitney U test: $Z=-0{\cdot}105$, $n_{males}=14$, $n_{females}=17$, $P=0{\cdot}916$), nor in individuals younger than 10 years ($Z=-1{\cdot}436$, $n_{males}=9$, $n_{females}=13$, $P=0{\cdot}151$). No clear diurnal pattern in urinary DHEA-S levels was detected, as the time of urine excretion did not correlate with urinary DHEA-S levels for individuals older than 10 years (rS$=-0{\cdot}21$, $n=31$, $P=0{\cdot}234$).

Discussion

This study shows age-related changes in urinary DHEA-S levels in bonobos that are similar to developmental changes in the adrenal activity of humans and chimpanzees and, by inference, are indicative of adrenarche in bonobos. Based on the data from this study, there is no evidence for a species-specific pattern that would support speculations about heterochrony in early adrenal activity of the two *Pan* species.

The aim of this study was to validate a commercial ELISA kit designed for the quantification of DHEA-S in human serum for measurements of DHEA-S in bonobo urine. In addition to DHEA-S, the urine samples also contained DHEA, which cross-reacted heavily with the antibody of the assay, rendering the measurement of DHEA-S levels in pure

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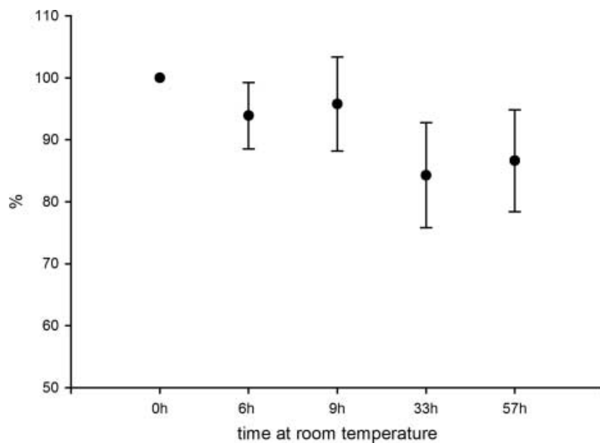

Figure 2 Mean DHEA-S recovery in five bonobo urine samples left for 57 h at room temperature (bars indicate the S.D.).

urine invalid. Therefore, an LLE had to be performed as described in the Materials and Methods section to reliably measure DHEA-S in bonobo urine. LC–MS/MS measurements showed that this step successfully separated DHEA from DHEA-S, resulting in high and consistent recoveries of DHEA-S in the aquatic phase, rendering the high cross-reactivity of the ELISA to DHEA unproblematic. Sample extracts then had to be reconstituted in Standard A of the assay kit (a standard without any hormones used as buffer) and measured in a ten-times concentrated form. After having executed this extraction, assay performance was acceptable with respect to specificity, precision, accuracy, and parallelism. Furthermore, the assay results are comparable with those of LC–MS/MS. The results from two additional experiments indicate that repeatedly thawing the urine samples was seriously detrimental to DHEA-S levels while exposure of the samples to room temperature resulted in advanced degradation of the DHEA-S only after 9 h. Finally, in contrast to Zhao *et al.* (2003), who found a peak of DHEA-S level at 1600 h in serum samples from humans, we did not find a diurnal excretion pattern of DHEA-S in the urine of bonobos. This might be due to DHEA-S having a slow metabolic clearance rate (Haning *et al.* 1989), which may have blurred diurnal patterns of DHEA-S levels in urine.

Until now, most studies on ontogenetic changes in DHEA-S secretion associated with adrenarche have used serum samples from humans (e.g. Rosenfield & Eberlein 1969, de Peretti & Forest 1978, Parker 1999) and nonhuman primates (e.g. baboons, Castracane *et al.* (1981); rhesus macaques, Kemnitz *et al.* (2000); and chimpanzees, Winter *et al.* (1980), Copeland *et al.* (1985) and Nadler *et al.* (1987)). Studies on DHEA-S secretion in urine have almost exclusively been carried out on humans (e.g. Tanner & Gupta 1968, Kelnar & Brook 1983, Remer *et al.* 1994). One study on DHEA-S levels in chimpanzee feces did not detect the age-related changes that were found in serum samples (Seraphin *et al.* 2008), but it should be noted that individuals of age categories that are most likely to show the sudden upward shift in DHEA-S were under-represented. There are other preliminary reports on DHEA-S levels in chimpanzees that have used fecal or urine samples (Fontenote *et al.* 2001, Anestis *et al.* 2009), but between-species comparisons are still difficult.

Measuring steroid levels in urine has become increasingly important for the noninvasive monitoring of endocrine correlates of behavior in captive and wild animals (Sheriff *et al.* 2011). Taking blood samples from study subjects is stressful and, in the case of wild animals, is a complicated procedure. Similarly, studies on human infants might also benefit from the use of urine as a medium for endocrinological studies. As urine can be easily and noninvasively collected from a wide range of species, our study offers an alternative to invasive sampling techniques. The technique described here facilitates the exploration of species-specific patterns of adrenal development and steroid secretion as well as comparative studies across a wider range of species.

Adrenarche in primates

Our results show that age-related DHEA-S excretion in bonobos closely resembles the changes detected in serum samples from chimpanzees (Cutler *et al.* 1978, Copeland *et al.* 1985) and humans (de Peretti & Forest 1978), indicating that humans and both the *Pan* species are similar in terms of early changes in adrenal activity. This finding supports the scenario that assumes that adrenarche is a relatively recent development in primate evolution (Havelock *et al.* 2004). The described technique can be applied to urine samples from other primate species and would facilitate comparisons across a larger number of primates in terms of the development of the ZR or DHEA-S excretion patterns. In some New World monkeys (e.g. *Callithrix jacchus*), development of the ZR in females seems to depend on their social status and their gonadal development, whereas males seem to lack a functional ZR (Pattison *et al.* 2005, 2009). As a consequence, adrenarche

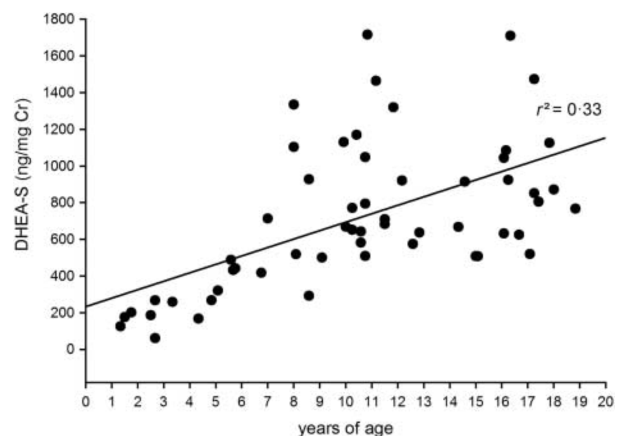

Figure 3 Urinary DHEA-S concentration in bonobo urine sample in relation to age (each dot represents one individual).

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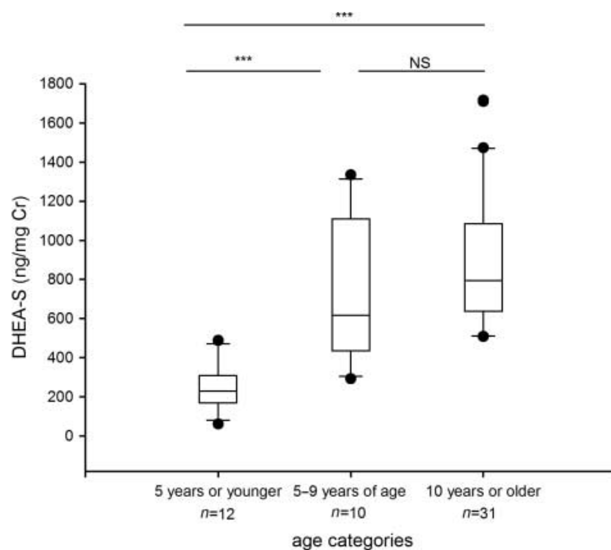

Figure 4 Boxplot of average DHEA-S concentrations in bonobo urine samples across three different age classes. The boxes indicate the 25th and 75th percentiles, the whiskers indicate the 10th and 90th percentiles, the bars indicate the range, and the circles indicate outliers. NS, non-significant. *** is $P<0{\cdot}01$.

does not occur as a single, temporarily well-defined event (Pattison *et al.* 2009). All Old World monkeys thus far investigated, including macaques and baboons, do not exhibit a distinct rise in DHEA-S before puberty (Meusy-Dessolle & Dang 1985, Crawford *et al.* 1997, Kemnitz *et al.* 2000), and it has been suggested that, in these taxa, fetal zone regression and adrenarche might overlap (Nguyen & Conley 2008, Conley *et al.* 2011). The information on DHEA-S levels in plasma from two other hominoids, gorilla and orangutan, is limited, making any conclusions about the existence of adrenarche speculative (Cutler *et al.* 1978, Collins *et al.* 1981). Further studies on other primate species and the extension to parameters other than hormones will shed light on age-related dynamics of adrenal activity and may revise current concepts of adrenarche (Conley *et al.* 2012).

Adrenarche in bonobos

Individual differences in DHEA-S excretion In the subset of longitudinally collected samples, we observed a pronounced increase in urinary DHEA-S levels over time. This increase corresponds well with the findings of studies on plasma androgens during adrenarche in humans (Korth-Schutz *et al.* 1976, Sizonenko *et al.* 1976, Ilondo *et al.* 1982). For bonobos older than 10 years, a wide range of DHEA-S levels was found, and for the five individuals for whom longitudinal measurements were available, one individual exhibited very high levels at the age of 8 years, while another showed very low levels at the age of 11 years. Similar ranges of variation were found for urinary DHEA-S levels in human children (Tanner & Gupta 1968) and remain to be explained. The wide range of DHEA-S levels during early adulthood may be due to sex differences in excretion patterns during ontogeny. While women reach a peak in DHEA-S level at 20 years of age, men only reach a peak at 25 years of age (Orentreich *et al.* 1984). Bonobos have a slow life history and there are reports of individuals living for 35 years or more (Furuichi *et al.* 2012). The oldest bonobo in our study was still younger than 19 years and it is possible that some or all the individuals included in this study had not yet reached their peak levels of DHEA-S excretion. In humans, peak levels of DHEA-S are reached during adulthood around 30 years of age (Šulcová *et al.* 1997) and thereafter show a modest but steady decline (Orentreich *et al.* 1984). Adult levels are considerably low at the age of 50 years (females) and 40 years (males), but a wide range of individual differences in DHEA-S levels may still prevail (Orentreich *et al.* 1984, Barrett-Connor *et al.* 1986, Salvini *et al.* 1992, Parker 1999). An investigation of changes in the adrenal activity of older bonobos would be instructive, but is unfortunately limited by the small number of old bonobos living in captive facilities. Currently, there are less than ten individuals worldwide of 40 years or older (Bonobo – International Studbook update 2011), making such studies a challenging task.

The variation in urinary DHEA-S levels in young individuals (<10 years) may reflect individual differences in the development of the ZR (Dhom 1973). Given that DHEA-S levels are elevated in obese children (Pintor *et al.* 1984, Reinehr *et al.* 2005), it is possible that individual differences in body mass index in bonobos contribute to differences in DHEA-S levels.

Onset of adrenarche Bonobos younger than 5 years had significantly lower DHEA-S levels than older individuals. In chimpanzees, urinary DHEA-S levels indicate an onset of adrenarche between the ages of 5 and 7 years; however,

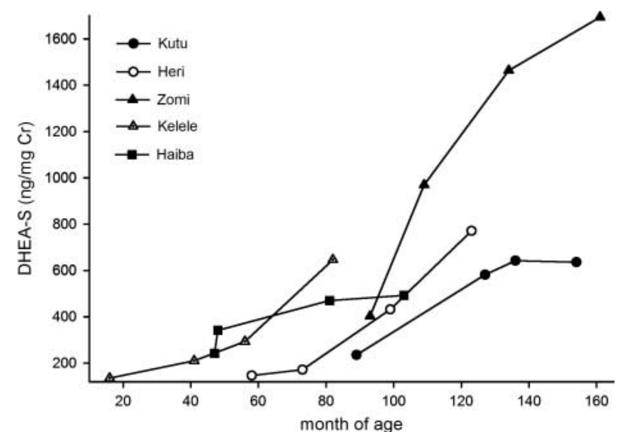

Figure 5 Individual DHEA-S profiles for five bonobos from Frankfurt Zoo (females, closed symbols; males, open symbols) during prepubertal and pubertal phases.

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the age of onset was highly variable across individuals (Anestis *et al.* 2009).

In most prepubertal children, individual DHEA-S excretion rates of urine are low until the age of 6 years and increase thereafter (Remer & Manz 1999).

Studies on DHEA-S levels in serum have produced conflicting results in terms of the onset of increased DHEA-S secretion. In chimpanzees, Cutler *et al.* (1978) found the earliest increase in DHEA and DHEA-S at about 2 years of age while Smail *et al.* (1982) and Copeland *et al.* (1985) found an increase in both sexes at around 6 years of age. In humans, Palmert *et al.* (2001) found an increase in DHEA-S levels in serum at the age of 3 years, but other studies (e.g. Rosenfield & Eberlein 1969, de Peretti & Forest 1978, Sklar *et al.* 1980) considered the onset of adrenarche to be at 6–8 years of age. In the current study, we did not find a significant difference in urinary DHEA-S levels between individuals aged 5–9 years and those older than 9 years. This might indicate that adrenarche had already occurred in most of the individuals between 5 and 9 years. Therefore, in our study, the onset of adrenarche, defined by increases in urinary DHEA-S levels in bonobos, is comparable with the results from studies on DHEA-S levels in human and chimpanzee urine. As DHEA-S levels begin to increase at a similar time in humans, chimpanzees, and bonobos, at least for this aspect of their physiology, there is no indication of a bonobo-specific developmental pattern, as has been suggested.

Effect of sex

We did not find any sex-specific differences in urinary DHEA-S levels in individuals of any age class. The different age classes were tested separately because the sex effect occurs around puberty in humans (Remer *et al.* 1994). In line with our findings in bonobos, no sex differences in urinary levels were found in human children (Gupta 1970), but sex differences have been reported in adult humans, with men having higher DHEA-S levels than women (e.g. Dhom 1973, Orentreich *et al.* 1984, Remer & Manz 1999). As in bonobos, several studies on chimpanzees did not detect sex differences in DHEA-S levels (Cutler *et al.* 1978, Fontenote *et al.* 2001, Anestis *et al.* 2009). Copeland *et al.* (1985) found higher levels of DHEA-S in serum samples from female vs male chimpanzees aged 2–6 years, and Smail *et al.* (1982) observed the opposite in 4- to 7-year-old individuals. This inconsistency in younger individuals could be due to individual differences in the timing of the onset of adrenarche, as shown in our results and in Anestis *et al.* (2009). Therefore, it is possible that a sex difference in DHEA-S levels in individuals older than 20 years exists but that this was not detected in our study because the oldest bonobos included in this study were only 18 years of age. Future studies that include samples from individuals older than 20 years could shed more light on this.

In conclusion, we validated an extraction method and a commercially available ELISA kit designed for the quantification of DHEA-S in human serum for measurements of DHEA-S in bonobo urine. With this method, we found pronounced age-related changes in urinary DHEA-S levels in bonobos. This indicates that, like their closest relatives, chimpanzees and humans, bonobos undergo the developmental process of adrenarche and onset occurs at a comparable time in all the three species. Therefore, morphological and behavioral indications of heterochrony, as have been suggested for bonobos, do not seem to be manifested in the physiological correlates of adrenarche.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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