Adrenal Androgen Production in Catarrhine Primates and the Evolution of Adrenarche

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ABSTRACT Adrenarche is a developmental event involving differentiation of the adrenal gland and production of adrenal androgens, and has been hypothesized to play a role in the extension of the preadolescent phase of human ontogeny. It remains unclear whether any nonhuman primate species shows a similar suite of endocrine, biochemical, and morphological changes as are encompassed by human adrenarche. Here, we report serum concentrations of the adrenal androgens dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) measured in 698 cross-sectional and mixed longitudinal serum samples from catarrhine primates ranging from 0.6 to 47 years of age. DHEAS in *Pan* is most similar to that of humans in both age-related pattern and absolute levels, and a transient early increase appears to be present in *Gorilla*. DHEA levels are highest in *Cercocebus*, *Cercopithecus*, and *Macaca*. We also tested for evidence of adaptive evolution in six genes that code for proteins involved in DHEA/S synthesis. Our genetic analyses demonstrate the protein-coding regions of these genes are highly conserved among sampled primates. We describe a tandem gene duplication event probably mediated by a retrotransposon that resulted in two 3-β-hydroxysteroid dehydrogenase/Delta 5-Delta 4 genes (*HSD3B1* and *HSD3B2*) with tissue specific functions in catarrhines. In humans, *HSD3B*2 is expressed primarily in the adrenals, ovary, and testis, while *HSD3B1* is expressed in the placenta. Taken together, our findings suggest that while adrenarche has been suggested to be unique to hominoids, the evolutionary roots for this developmental stage are more ancient. Am J Phys Anthropol 147:389–400, 2012.

Humans grow up slowly. In particular, the period of time between birth and adolescence is extended relative to other nonhuman primates (Leigh, 2001). The childhood stage of development, spanning from approximately 3–6 years of age, has been proposed as uniquely inserted into the human life history trajectory (Bogin, 1997), possibly as early as *Homo ergaster* (Aiello and Wells, 2002). During childhood, there is a continued dependence on older individuals for assistance with provisioning and protection, extending the period of parental or alloparental investment and potentially linking to a number of other human traits, including extended postreproductive life span (e.g., Hawkes et al., 1998).

One developmental event proposed to be of critical import for the extension of early human ontogeny is adrenarche, broadly defined as the differentiation of the zona reticularis (ZR) of the adrenal cortex and associated increases in adrenal androgen secretion (Nguyen and Conley, 2008). Specifically, it has been proposed that by delaying the onset of adrenarche, human ontogenetic, cognitive, and social factors are affected which facilitate plasticity in both somatic and neurological development (Bogin, 1997; Campbell, 2011). This has important consequences for reconstructing the evolution of human life history, and carries implications for understanding associated adaptations (e.g., timing of puberty, aging, and senescence). By investigating hormonal correlates of adrenarche, and the evolution of genes involved in the enzymatic regulation of adrenal androgen production, in a comparative context, this study aims to provide a broad comparative perspective regarding the evolutionary history of the physiological regulation of this developmental stage.

ADRENAL GLAND, ADRENARCHE, AND HORMONE SYNTHESIS

The adrenal gland is situated atop the kidney, and is composed of two parts: the outer medulla and the inner cortex. The adrenal medulla is responsible for the production of catecholamines. The adrenal cortex consists of dif-

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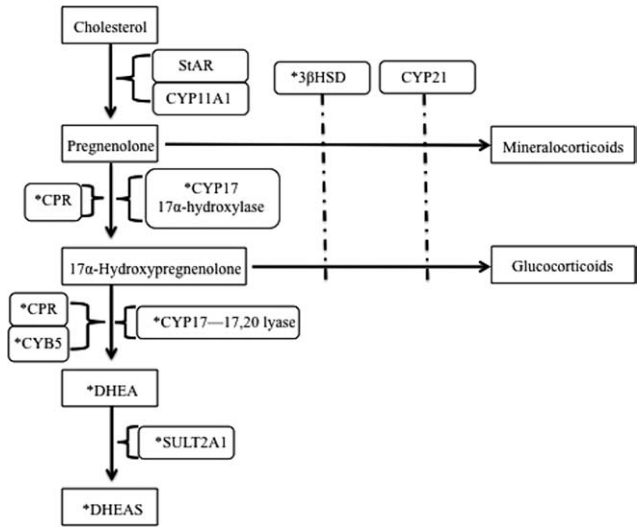

Fig. 1. Enzymes involved in the DHEA/DHEAS conversion pathway; asterisks indicate hormones and enzymes included in endocrine and evolutionary analysis in the current paper (modified from Nakamura et al., 2009). Abbreviations explained in text.

ferentiated zones, which are principally responsible for the production of different types of steroids. In adult humans, the zona glomerulosa (ZG) produces mineralocorticoids, the zona fasciculata (ZF) produces glucocorticoids, and the ZR is the major source of adrenal androgens (e.g., dehydroepiandrosterone (DHEA), and its sulfate ester, dehydroepiandrosterone sulfate (DHEAS)). The fetal zone (FZ) is the main site of adrenal androgen production during late gestation in humans and nonhuman primates (e.g., Axelson et al., 1984), and regresses shortly before birth or during postnatal life. The other portion of the fetal adrenal cortex, the neocortex, develops into the ZG, ZF, and ZR (Mesiano and Jaffe, 1997). The differentiation of these zones, and the short- and long-term regulation of steroidogenesis within the zones, is regulated by hormones and enzymes within each zone (Hornsby, 1995). In humans, the functional ZR develops in postnatal life, while in some nonhuman primates it appears to develop during the first few months after birth (Rhesus macaques—Conley et al., 2011), and in others it appears to not develop altogether (male marmosets—Pattison et al., 2007). The variation in the presence of a functional ZR, and the timing of its appearance, has raised the question of whether human adrenarche is a unique derived trait, or one shared with other primates.

Adrenarche can be defined by morphological, biochemical, and hormonal criteria. In humans, associated phenotypic changes include the appearance of axillary and pubic hair, and evidence of a "midgrowth spurt" in stature around 7 years of age in some populations, although adrenarche is likely not the mechanism driving this transient increase in growth rate (e.g., Remer and Manz, 2001). Adrenarche has been proposed to begin as early as ~3 years, or as late as 6–8 years, in humans based on evidence from different lines of investigation (Palmert et al., 2001).

Several enzymes are involved in the production of adrenal androgens (Fig. 1). Briefly, steroidogenic acute regulatory factor (StAR) and cytochrome P450 cholesterol side chain cleavage (CYP11A1) assist cholesterol transport to the mitochondria and also convert cholesterol to pregnenolone, respectively (Miller, 2002). CYP17 (P450 17α-hydroxylase/17,20-lyase) catalyzes the 17α-hydroxylation of pregnenolone and the 17,20-lyase reaction on 17α-hydroxypregnenolone. In both of these reactions, it works in a complex with its obligate redox partner CPR (NADPH-cytochrome-P450 oxidoreductase) (Conley et al., 2004). 3βHSD (3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase) effectively decreases the production of DHEAS by competing with CYP17 for substrate and removing precursors from the DHEA conversion pathway (Hornsby, 1995). CYP21 (21-hydroxylase cytochrome P450) assists in the production of mineralocorticoids and glucocorticoids by rerouting products of 3βHSD. CYB5 (cytochrome b5) plays a key role in regulating the 17,20-lyase function of CYP17 during the 17α-hydroxypregnenolone to DHEA conversion (Havelock et al., 2004). Finally, SULT2A1 (steroid sulfotransferase) is required for conversion of DHEA into DHEAS (Nakamura et al., 2009). It has been suggested that due to their temporal- and zone-specific expression, CYB5, 3βHSD type 2, and SULT2A1 are particularly important for understanding ZR adrenal androgen production at adrenarche (Rainey and Nakamura, 2008). The regulation of the expression of these enzymes during adrenarche is not fully understood, although several transcription factors have been identified (see below).

Adrenarche: a comparative perspective

Hormonal evidence of adrenarche is gathered by measuring adrenal androgen concentrations and many studies focus on DHEAS because in postnatal life, it is present in higher concentrations in circulation than DHEA. Studies of adrenal gland development, in a comparative primate context, have not examined the period of ZR development extensively, until recently (discussed below). Therefore, much of the comparative evidence for or against the presence of adrenarche in nonhuman primates has come from studies focusing on adrenal androgens in circulation.

In humans, evidence from blood serum documents a steep decline in concentrations of both DHEA and DHEAS after birth associated with the regression of the FZ, followed by elevations beginning around 6–8 years of age, peak concentrations reached in the second decade of life, and rounded out by decreasing the concentrations in later life (Šulcova et al., 1997). Chimpanzees approximate humans both in concentrations of these hormones as well as their pattern of postnatal secretion, and a chimpanzee-like pattern of an increase in postnatal DHEA and DHEAS secretion has been described in gorillas (although no values were reported); investigations of hormonal evidence for adrenarche in orangutans has not yielded any evidence to support its presence, but sample sizes have been very small (Cutler et al., 1978; Collins et al., 1981; Copeland et al., 1985; but see Nadler et al., 1987 and Seraphin et al., 2008).

While evidence for a postnatal increase in chimpanzee adrenal androgens is best supported amongst all the nonhuman apes, there remains a large gap between the oldest chimpanzee samples analyzed and published to date (22 years of age; Copeland et al., 1985) and older age-related changes remain unknown. Postnatal concentrations of adrenal androgens have been quantified in

TABLE 1. Study sample sex, age composition, and average hormone levels (± SEM), in (ng/ml) by genus

Genus (species)	*N* (m,f)	Age range (years)	Mean DHEA (± SEM)	Mean DHEAS (± SEM)
Cercocebus (atys,[a] *chrysogaster)*	231 (113,118)	0.8–19	36.15 (± 7.39)	119.98 (± 14.52)
Cercopithecus (petaurista, lhoesti, neglectus, ascanius, wolfi)	46 (23,23)	2.6–31	32.69 (± 6.36)	134.87 (± 26.01)
Colobus (guerezaangolensis)	14 (7,7)	3.5–22.9	10.53 (± 2.17)	35.54 (± 6.53)
Macaca (mulatta, fuscata, silenus)	38 (29,9)	5–30.8	28.16 (± 5.85)	186.03 (± 24.7)
Homo (sapiens)[b]	462 (159,303)	0.08–100	5.4 (na)	1,206.7 (na)
Mandrillus[c] *(sphinx, leucophaeus)*	46 (27,19)	2.5–34	–	174.6 (± 32.4)
Papio[c] *(papio, anubis*[a]*)*	175 (128,147)	0.6–22	–	256.21 (± 11.37)
Symphalangus (syndactylus)	11 (6,5)	4–28.7	20.29 (± 5.63)	116.19 (± 22.44)
Gorilla (gorilla gorilla)	49 (25,24)	2–42	8.96 (± 1.28)	227.55 (± 23.67)
Pan (troglodytes, paniscus)	49 (21,28)	1.5–45	5.09 (± 0.75)	669.46 (± 61.62)
Pongo (abelii, pygmaeus)	39 (17,22)	4–47.6	5.41 (± 0.68)	109.06 (± 19.76)

[a] Data included from previous study (Bernstein et al., 2008).
[b] Human data summarized from Sulcova et al. (1997).
[c] Not included in analysis of DHEA.
"na"—no SEM available.

other nonhuman primates such as marmosets, macaques, and baboons (Smail et al., 1982; Muehlenbein et al., 2001; Pattison et al., 2005), and while circulating concentrations are higher than those in other mammals, there is no evidence of similarity to humans or chimpanzees with respect to absolute levels of adrenal androgens or the postnatal pattern of secretion. Because of this, adrenarche had previously been posited to be a shared, derived characteristic in humans and chimpanzees (Cutler et al., 1978).

However, recent work examining functional changes in the adrenal gland in the early postnatal life of rhesus macaques suggests that a re-examination of various nonhuman primate taxa is warranted (Nguyen et al., 2009). Specifically, one recent study suggests that adrenarche occurs in rhesus macaques in a restricted time window in very early postnatal life (Conley et al., 2011). This intriguing finding points to the possibility that adrenarche is not a uniquely derived aspect of development in *Pan* and *Homo*, but rather that it has deeper evolutionary roots and may instead be delayed in onset and prolonged in these taxa.

Given the gaps in current knowledge regarding the comparative evidence for adrenarche, including substantive evidence of postnatal adrenal androgen patterns in catarrhine primates, the first objective of this study was to describe comparative data on DHEA and DHEAS levels from a large sample ($N = 698$, from ten primate genera). These data will help develop a framework within which hypotheses can be constructed regarding the biochemical and morphological bases of these patterns, as well as the evolutionary implications of differences among species.

From our brief review of the enzymatic regulation of adrenal androgen synthesis, it should be clear that these enzymes provide potentially potent mediators of evolutionary change in the timing of adrenarche, and for patterns of adrenal androgen secretion throughout life. As a result, the second objective of this study was to examine the genes that code for proteins involved in the synthesis of DHEA and DHEAS (*CYB5A, CYP17A1, HSD3B1, HSD3B2, POR,* and *SULT2A1*) at the sequence level. Using publically available genome sequence data, we examined the protein-coding sequences of these genes for evidence of adaptive evolution and the promoters of *CYP17A1* and *SULT2A1* in order to assess the conservation among functionally important regulatory motifs present in humans. Additionally, we examined the aspects of gene family evolution for these genes.

MATERIALS AND METHODS

Sample collection

Serum samples were collected from 2001 to 2011, some of which include repeat samples from single individuals; data were also included from previously published studies (Table 1). It should be emphasized that the collection of samples for this study was opportunistic, and dependent on sample availability from banked collections at zoological institutions. This method of sample acquisition did not permit for a number of variables to be controlled, including time of day or season of sample collection. Adrenal androgens are secreted within circadian and ultradian rhythms, so it is possible that some variation attributable to time of day or season may affect our results (e.g., Muehlenbein et al., 2003). In addition, previous research has suggested that adrenal androgen periodicities vary among human populations (Zhao et al., 2003), and that adrenal androgen rhythms are dampened in both humans and nonhuman primates at older ages (Liu et al., 1990; Downs et al., 2008). Another limitation to using opportunistically collected samples is the relative paucity of samples from very young individuals, who are usually not included in anesthetic knockdowns unless necessary, and when they are, smaller quantities of blood are drawn. For future, more detailed comparative studies of adrenal androgens in nonhuman primates, sampling strategies could focus on the first two years of life.

All samples were obtained during the course of veterinary or routine medical examinations, where animals were sedated and whole blood was collected via venipuncture. Collection of samples was minimally invasive, as part of regularly scheduled examinations in all cases, and fully compliant with guidelines for animal care at the relevant institutions. Serum was separated and stored at −80°C in veterinary pathology archives in zoological institutions, and shipped on dry ice. Ages were known for most individuals and estimated based on various developmental parameters for others (i.e., aged individuals who were wild-trapped). None of the animals sampled were obese or unwell at the time of examination. Reproductive

status was mostly unknown, although none of the females sampled were pregnant at the time of sampling.

Hormone analysis

Assays. Two hundred ninety-two serum samples were analyzed for DHEA and DHEAS concentrations (Table 1). Data from previous studies were also included in the analysis (see below). Hormones were measured using commercially available enzyme immunoassay kits (ALPCO Diagnostics, Salem NH), validated by parallelism and recovery tests for each species. The ranges of detection and sensitivities of the assays were as follows: DHEAS 0.005–10 μg/ml, 5 pg/ml; DHEA 0.37–30 ng/ml, 0.108 ng/ml. Initial tests indicated that while the majority of samples fell within the range of standard curves for both assays, some exceeded the highest value of the curve. These were subsequently diluted 1:10 and reanalyzed. The dilution factor was incorporated into final calculation of results. The cross-reactivity of DHEAS and DHEA were less than 1% for their respective assays. Plates were read on a Dynex MRX absorbance reader (Dynex Technologies, Chantilly VA).

Analysis of hormone data. As a consequence of the mixed longitudinal nature of the sample, age ranges of samples from different species varied (Table 1). Due to the small sample sizes associated with some of the species collected, species within the same genus were pooled for the purposes of our analysis. While prior research suggests that species-level differences in DHEAS levels exist (e.g., Muehlenbein et al., 2002), our aim was to relate concentrations of adrenal androgens in catarrhines to the general pattern of evolution of enzymes important in the production of DHEA/DHEAS. Therefore, we undertook our analysis at the same level as the evolutionary analyses (see below). In total, including samples analyzed previously (*Papio* and *Cercocebus*—Bernstein et al., 2008), 698 samples were utilized for analysis of DHEAS, and 477 were utilized for analysis of DHEA. DHEAS concentrations were converted to ng/ml for illustration. First, LOESS regression was used for description of ontogenetic variation in the hormones analyzed (Efron and Tibshirani, 1991). Data were log-transformed (base-10) for subsequent analysis. Analysis of covariance (ANCOVA) was used to assess variation attributable to taxonomic category and sex by regressing hormone levels against age, with sex and genus as covariates. In order to circumvent complications associated with estimating degrees of freedom in analyses of mixed longitudinal samples, we set a restrictive significance level ($P < 0.01$) and Bonferroni correction was applied to multiple pairwise comparisons. To further explore differences in hormone levels among apes and monkeys included in our analysis, we employed two-sample *t*-tests of pooled and separate variance with Bonferroni correction. Since initial tests found no significant differences in DHEA or DHEAS, concentrations among sexes (male and female samples) were pooled for all analyses. In order to examine age-related variation in adrenal androgens, we created age categories (Table 2) that best encompassed the variation in age present in our sample while permitting enough samples in each category to test for differences among them. Similar groupings have been used for cross-sectional analyses of human DHEAS concentrations (e.g., Orentreich et al., 1984). We used Mann–Whitney *U* tests to assess whether significant differences existed among adjacent age categories, separately for each genus.

TABLE 2. Age categories

Age category	Years included
1	0–3
2	4–6
3	7–9
4	10–12
5	13–15
6	16–20
7	21–25
8	26–30
9	30–40
10	40–50

Evolutionary analyses

Sequence data and alignments. Putatively orthologous protein-coding sequences for *CYB5A* (cytochrome b5 type A, microsomal), *CYP17A1* (cytochrome P450, family 17, subfamily A, polypeptide 1), *HSD3B1* (3-beta-hydroxysteroid dehydrogenase/Delta 5->4 type 1), *HSD3B2* (3-beta-hydroxysteroid dehydrogenase/Delta 5->4 type 2), *POR* (P450 (cytochrome) oxidoreductase), and *SULT2A1* (sulfotransferase family, cytosolic, 2A, DHEA-preferring, member 1) were obtained primarily from Ensembl (Release 58, May 2010; Vilella et al., 2009). *Gorilla gorilla CYP17A1* and *Macaca mulatta HSD3B1* and *HSD3B2* sequences were obtained from the UCSC Genome Browser using the BLAT search tool (Kent, 2002; Fujita et al., 2011). The longest available transcript was used. Sequences were aligned using Clustal W2 (Larkin et al., 2007) and adjusted to preserve reading frame with MacClade 4.08 (Maddison and Maddison, 2005). Only sequences without missing or ambiguous bases were included in the alignments, with the exception of *SULT2A1* where available sequence data were more limited. Supporting Information Table S1 depicts the taxa sampled for all sequence-based evolutionary analyses.

HSD3B1 and HSD3B2 gene trees. In order to examine the evolutionary relationships of primate *HSD3B1* and *HSD3B2* sequences we used maximum likelihood methods to infer the gene tree for the *HSD3B1*/*HSD3B2* dataset. Phylogenetic trees were inferred for the DNA alignment using RAxML v7.04 (Stamatakis, 2006). A majority rule consensus tree was generated from trees derived from 1,000 bootstrap replicates using default (−f d) options.

Tests for evidence of adaptive evolution. The ratio of nonsynonymous to synonymous nucleotide substitution rates ($\omega \approx dN/dS$) is commonly used to detect evidence of adaptive evolution in protein-coding sequences. We estimated ω using the codeml program in PAML v4.1 (Yang, 2007). Purifying selection is assumed when $\omega < 1$. Positive selection is assumed when $\omega > 1$. Neutral evolution is indicated when $\omega \approx 1$. In order to determine whether ω varied among lineages, a fixed ratio model (M0; ω is fixed across all branches of the phylogenetic tree) was compared to a free ratio model (M1; ω is allowed to vary across branches of the phylogenetic tree). In order to test whether these genes have experienced different selective pressures in species with previously described evidence of adrenarche, we implemented a model that allowed one ω for the *Homo*/*Pan* clade and one ω for all remaining branches. The dN/dS ratios were estimated on the well-

established primate species tree (Goodman et al., 2005), and all analyses were repeated three times with different initial ω values (0.1, 1.0, and 3.0).

Transcription factor binding sites important for the regulation of *CYP17A1* and *SULT2A1* expression in human adrenal cells have been described (Lin et al., 2001; Fluck and Miller, 2004; Saner et al., 2005, respectively). In order to examine the evolution of these binding sites we aligned approximately 400 base pairs of sequence directly upstream of the transcription start site. For *CYP17A1*, we examined human, chimpanzee, orangutan, rhesus macaque, and marmoset. For *SULT2A1*, we examined human, chimpanzee, gorilla, rhesus macaque, marmoset, mouse lemur, and bushbaby. All sequences were obtained from Ensembl (v. 58) and were examined using PROMO (version 3.0.2; maximum matrix dissimilarity rate = 15; Messeguer et al., 2002; Farre et al., 2003).

RESULTS

Hormones

Age-related patterns. Patterns and levels of DHEA and DHEAS varied among taxa (Supporting Information Fig. S1). Generally, DHEAS levels exceeded DHEA levels at all ages (Table 1). The small sample sizes for some of the genera (e.g., *Colobus, Symphalangus*) preclude a definitive description of age-related changes in levels of these hormones. While LOESS regression can provide a description of a pattern for existing data, in some cases there is substantial spread in hormone values. In most taxa, no significant differences were found among consecutive age categories (Table 3). However, a few returned significant differences among consecutive categories, lending strength to patterns observed from the LOESS plots. Specifically, *Gorilla* shows a significant difference between age category two and three, and three and four for DHEAS (spanning 4–12 years), and between age categories two and three for DHEA (spanning 4–6 years). Significant differences in DHEAS concentrations were shown between age categories four and five, and five and six (spanning 10–20 years) in *Pan*, with no significant differences in DHEA among any age category. *Papio* DHEAS concentrations decline from higher levels at earlier ages, supported by the significant difference between age categories one and two, and three and four. Similarly, *Cercocebus* DHEAS concentrations differ significantly from age category two to age category three. Declining DHEA concentrations in *Cercopithecus* contribute to significant differences between age category two and three. *Macaca* returns perhaps the most surprising results, with significant differences in DHEAS concentrations consistently from age category two through seven. These results show some similarity to those of an older study which showed increase in DHEAS concentrations in individuals aged six to nine years (Smail et al., 1982); however, since this rise occurs well after the age of sexual maturation, it is likely that these patterns reflect the influence on gonadal function on adrenal androgen secretion (Conley et al., 2004).

Differences among primates. ANCOVA confirms that *Pan* has higher DHEAS concentrations than other taxa at all ages ($P < 0.001$). *T*-tests show that nonhuman apes (gorillas, chimpanzees, orangutans, and gibbons) have significantly higher concentrations of DHEAS than monkeys ($P < 0.0001$), and further that monkeys have significantly higher DHEA concentrations than apes ($P < 0.0001$). Pairwise comparisons reveal that in addition

TABLE 3. Significant differences across adjacent age categories indicated by P-value (Mann–Whitney U). Pongo and Colobus not shown, all comparisons were not significant

	Cercocebus		*Cercopithecus*		*Gorilla*		*Macaca*		*Mandrillus*		*Papio*		*Pan*	
Age categories	DHEA	DS	DHEA	DS	DHEA	DS	DHEA	DS	DHEA	DS	DHEA	DS	DHEA	DS
1–2	ns	ns	ns	ns	ns	ns	ns	ns	–	ns	–	<0.001	ns	ns
2–3	ns	0.039	ns	ns	0.045	0.034	ns	<0.001	–	ns	–	ns	ns	ns
3–4	ns	ns	0.045	ns	ns	0.040	ns	<0.001	–	ns	–	0.026	ns	ns
4–5	ns	ns	ns	ns	ns	ns	ns	0.028	–	ns	–	ns	ns	0.004
5–6	ns	ns	ns	ns	ns	ns	<0.001	<0.001	–	ns	–	ns	ns	0.006
6–7	–	–	ns	ns	ns	ns	ns	<0.001	–	ns	–	ns	ns	ns
7–8	–	–	–	–	ns	ns	ns	ns	–	ns	–	–	ns	ns
8–9	–	–	–	–	ns	ns	ns	ns	–	ns	–	–	ns	ns
9–10	–	–	–	–	ns	ns	–	–	–	–	–	–	–	–

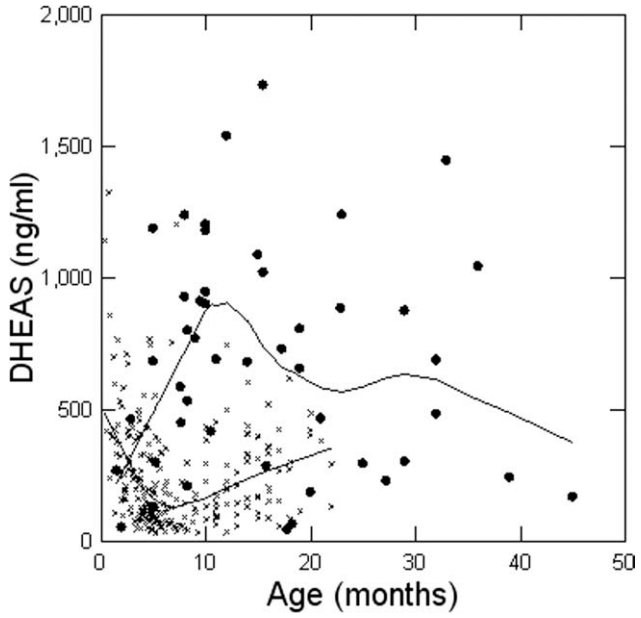

Fig. 2. DHEAS with age in *Pan* (filled circles) and *Papio* (crosses).

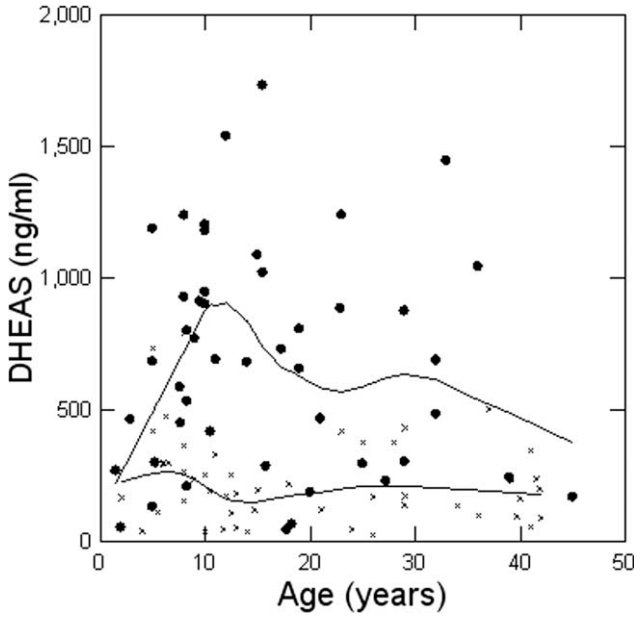

Fig. 3. DHEAS with age in *Pan* (filled circles) and *Gorilla* (crosses).

to *Pan*, *Gorilla* and *Papio* have significantly higher DHEAS concentrations than *Cercocebus*, *Cercopithecus*, and *Colobus* ($P < 0.001$). *Gorilla* and *Papio* do not have significantly different DHEAS concentrations from one another. In addition, *Pongo*, *Macaca*, and *Mandrillus* all have significantly higher DHEAS concentrations than *Colobus* ($P < 0.001$). *Cercocebus*, *Cercopithecus*, *Symphalangus*, *Macaca*, *Mandrillus*, and *Pongo* DHEAS concentrations are not significantly different. Significant differences in DHEA concentrations were also noted among taxa. *Cercocebus* DHEA concentrations are significantly higher than *Gorilla*, *Pan*, or *Pongo* ($P < 0.001$). Both *Cercopithecus* and *Macaca* have higher DHEA concentrations than *Pan* and *Pongo* ($P < 0.001$). *Symphalangus*, *Cercocebus*, *Macaca*, and *Cercopithecus* DHEA concentrations do not differ significantly from one another.

In order to further examine the nature of the results suggesting *Gorilla* and *Papio* have higher DHEAS concentrations than *Cercocebus, Cercopithecus,* and *Colobus*, LOESS-smoothed scatterplots of raw DHEAS values against age were constructed to compare the age-related patterns of DHEAS in these taxa to that in *Pan*. The results showing significantly higher concentrations of DHEAS in *Pan* and *Papio* compared to several other taxa are not reflective of underlying similarity in age-related patterns of secretion (Fig. 2). Interestingly, a smaller, moderate peak in *Gorilla* precedes a larger, steeper peak in *Pan* (Fig. 3).

Evolutionary analyses

HSD3B1 and HSD3B2: Gene duplication predates Old World monkey/ape divergence. Two isomerase types have been described for 3βHSD in humans (Luu et al., 1989; Rheaume et al., 1991; McBride et al., 1999). *HSD3B1* (Type 1) is expressed in the placenta, skin, and mammary gland tissue (Rheaume et al., 1991). *HSD3B2* (Type 2) is expressed in the adrenals, ovary, and testis (Rheaume et al., 1991). We interrogated primate genomes for orthologous *HSD3B1* and *HSD3B2* sequences in order to determine whether both genes were present in nonhuman primate species and to place this gene duplication event in an evolutionary context. Phylogenetic analyses demonstrated both genes are present in the human, chimpanzee, orangutan, and macaque genomes (Fig. 4 and Supporting Information Fig. S2) suggesting the duplication event occurred on the stem catarrhine lineage or earlier. The marmoset *HSD3B* gene fell outside of the *HSD3B1* and *B2* clade, which provides evidence that the duplication event occurred on the stem catarrhine lineage after platyrrhines and catarrhines diverged from each other.

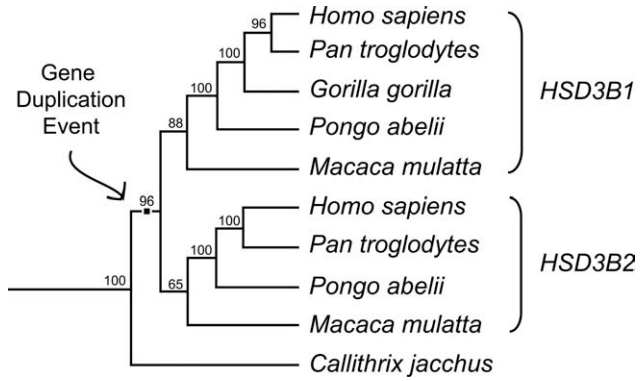

Fig. 4. Gene duplication event in the HSD3B gene family in primates. Maximum likelihood *HSD3B1* and *HSD3B2* gene tree with percentage bootstrap support (1000 replicates) given for each node (full tree given in Supporting Information Fig. S2).

Evidence of conservation in the protein-coding sequence of adrenarche-related proteins. The free ratio model (M1) is a significantly better fit to *CYB5A, POR, HSD3B1/HSD3B2*, and *CYP17A1* than the one ratio model (M0) (Table 4). For each gene, the data fit better to a model in which ω varies across lineages

TABLE 4. Parameter estimates and likelihood values under branch models

Gene	Model	P	ln$L$[a]	ω	LRT	$2(l_1 - l_0)$	P value
CYB5A	M0	1	−2854.42	0.1329	M0 vs. M1	73.25	0.001
	M1	40	−2817.79	see Figure S8			
	2ω	1	−2854.29	ω_1: 0.13227, ω_2: 0.21231	M0 vs. 2ω	0.25	0.62
POR	M0	1	−8341.63	0.0462	M0 vs. M1	42.69	0.01
	M1	26	−8320.29	see Figure S9			
	2ω	1	−8341.14	ω_1: 0.04654, ω_2: 0.02342	M0 vs. 2ω	0.97	0.32
HSD3B1& HSD3B2	M0	1	−13564.01	0.2838	M0 vs. M1	145.17	<0.001 (≈0)
	M1	64	−13491.42	see Figure S10			
	2ω[b]	1	−13563.95	ω_1: 0.28494, ω_2: 0.15523	M0 vs. 2ω	0.11	0.74
CYP17A1	M0	1	−13960.75	0.3328	M0 vs. M1	163.53	<0.001 (≈0)
	M1	44	−13878.98	see Figure S11			
	2ω	1	−13959.22	ω_1: 0.33432, ω_2: 0.09307	M0 vs. 2ω	3.05	0.08
SULT2A1	M0	1	−3046.13	0.58328	M0 vs. M1	18.28	0.69
	M1	22	−3036.99	N/A (test not significant)			
	2ω	1	−3046.13	ω_1: 0.58296, ω_2: 0.63216	M0 vs. 2ω	0	0.95

[a] Average of 3 runs with different initial ω.
[b] *HSD3B2* gene only.
P = parameters.
ω_1: all branches except the *Homo*/*Pan* clade.
ω_2: only branches included in the *Homo*/*Pan* clade.

(Supporting Information Figs. S2–S6). The coding regions of these genes are largely conserved across primates and show evidence of suggestive of purifying selection. Within primates, two branches (stem anthropoid and terminal *Pan troglodytes*) in the *CYB5A* dataset and two branches (stem African apes and terminal *Papio ursinus*) in the *CYP17A1* dataset show evidence suggestive of positive selection (Fig. 5 and Table 5). The free ratio model (M1) is not a significantly better fit than the one ratio model (M0) for *SULT2A1* (Table 4). In addition, the likelihood score in the two ω model was not significantly different from the score in the one ω model (Table 4) for all genes examined suggesting the *Homo* and *Pan* sequences have evolved under similar selection pressure as the other examined species.

Evidence of conservation in promoter sequence binding sites of CYP17A1 and SULT2A1. Expression of *CYP17A1* mRNA in adrenal cells is regulated by several DNA-binding transcription factors including steroidogenesis factor 1 (SF-1), GATA binding proteins (GATA-4 and GATA-6), nuclear factor 1-c (NF-1C), specificity proteins (Sp1 and Sp3), and sterol regulatory element binding protein (SREBP) (Sewer and Jagarlapudi, 2009). These transcription factors recruit co-regulators that together form a complex assembly of proteins on the promoter of *CYP17A1* (Sewer and Jagarlapudi, 2009). As a result, transcription of this gene is also regulated by co-regulatory proteins that post-translationally (e.g., phosphorylation and histone modification) modify this complex. Direct binding of SF-1 and Sp1 to the *CYP17A1* promoter in response to adrenocorticotropin (ACTH) is important for increased *CYP17A1* expression in human adrenal cells (Rodriguez et al., 1997; Sewer et al., 2002; Fluck and Miller, 2004). Both binding sites characterized in these studies are present and identical (with one exception) in all primates examined (human, chimpanzee, orangutan, rhesus macaque, and marmoset; Fig 6). The orangutan Sp1/3 binding site has one mismatch (CTCTTCCTC).

Expression of *SULT2A1* mRNA in adrenal cells is regulated in part by the binding of SF-1 and GATA-6 to the *SULT2A1* promoter (Saner et al., 2005). Highest expression is achieved when both factors are present and their respective *cis*-elements are intact; however SF-1 and GATA-6 are able to increase expression above basal levels independently of one another (Saner et al., 2005). Both SF-1 binding sites and the GATA-6 binding site identified by Saner et al. (2005) are conserved and identical among human, chimpanzee, and gorilla (Fig. 6). The SF-1 sites were also identified in the rhesus macaque and marmoset promoter. The GATA-6 binding site is conserved in the rhesus macaque, although this binding site is not present in marmoset, mouse lemur, or bushbaby (Fig. 6). The marmoset promoter sequence included a potential GATA-6 binding site (AGATAACC) 5′ of this region.

DISCUSSION

Adrenarche: A conserved developmental event

Our results support previous research suggesting that *Pan* most closely approximates the human postnatal adrenal androgen levels and patterns of secretion (Cutler et al., 1978; Smail et al., 1982), and perhaps this is not surprising given the close phylogenetic affinity of humans, chimpanzees, and bonobos (Goodman et al., 1998). A novel finding presented here suggests that gorillas may experience a transient increase in DHEA/DHEAS levels prior to sexual maturation. Comparison of *Gorilla* and *Pan* patterns makes clear that the *Gorilla* DHEAS increase is modest compared to what is seen in *Pan*, as peak levels in *Pan* are two times as high as peak levels in *Gorilla*. These differences in the timing of the DHEAS peak, if substantiated, align with what is known regarding general patterns of growth and development in these taxa. Specifically, *Gorilla* grows faster, and reaches sexual maturation earlier, than *Pan* (Leigh and Shea, 1996). Since adrenarche is an endocrine event that normally occurs prior to the onset of gonadarche, it should be seen earlier in *Gorilla* than in *Pan*.

Although the specific mechanisms underlying initiation of adrenal androgen production during adrenarche are unclear, changes in human adrenal tissue morphology and expression levels associated with an increase in adrenal androgen (specifically DHEA and DHEAS) production have been described (Nakamura et al., 2009).

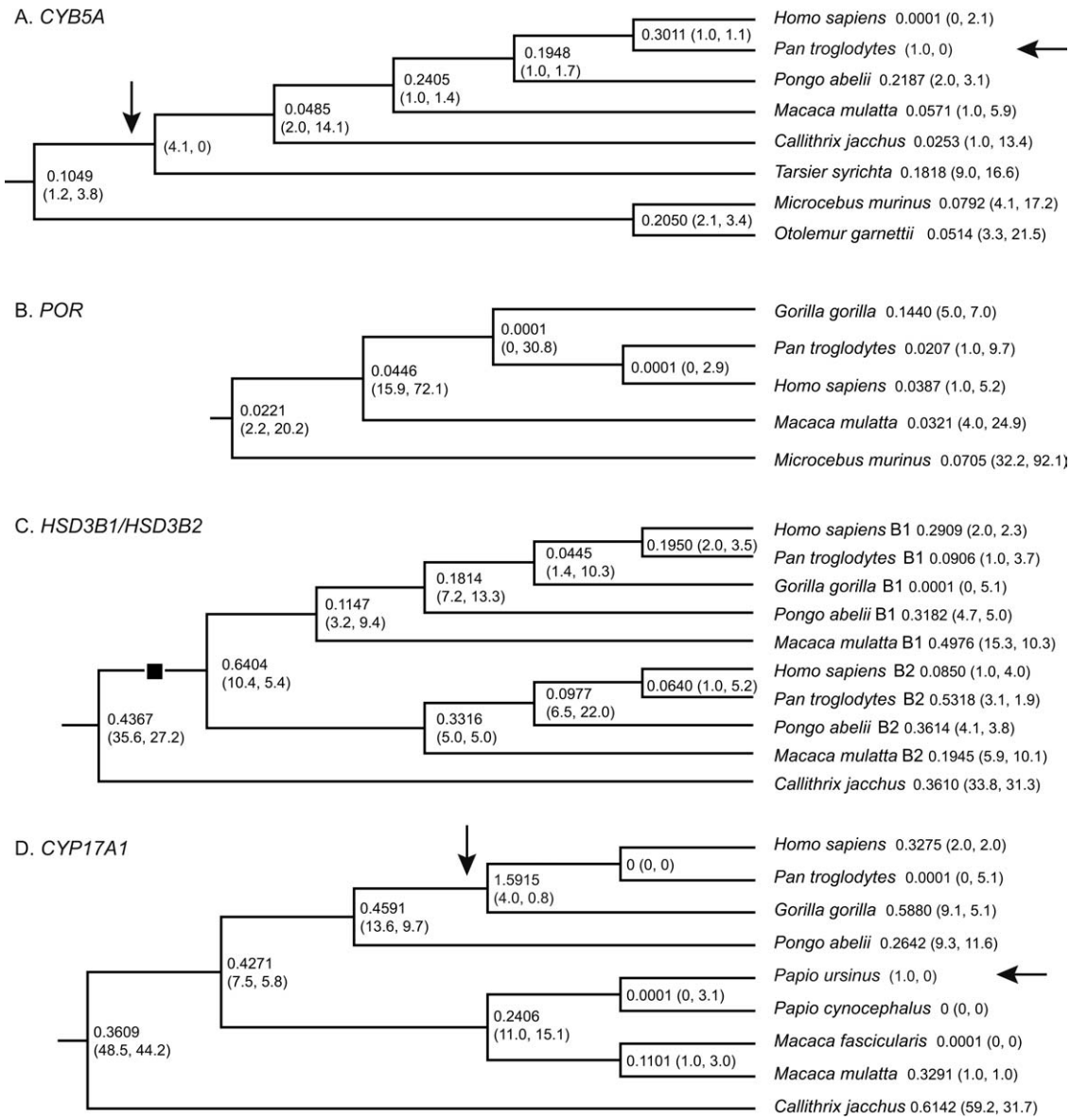

Fig. 5. *CYB5A, POR,* HSD3B gene family and *CYP17A1* M1 trees. The free ratio model (M1) is a significantly better fit to the data than the one ratio model (M0) for all four loci. ω Values are given for primate branches (full dataset shown in Supporting Information Fig. S3–S6). Arrows denote branches with evidence suggesting positive selection. The square denotes a gene duplication event.

TABLE 5. Evidence of positive selection

Locus/Branch	ω (N^* dN, S^*dS)	Replacement[a]	Change in physicochemical property[b]
CYB5A/Stem Anthropoid	(4.1, 0)	Lys7Glu	change in charge and hydrophobicity
		Thr93Asn	change in hydrophobicity
		Leu121Val	change in size
		Val122Ala	change in aromaticity
CYP17A1/Stem Ape	1.6 (4, 1)	Val325Leu	change in size
		Ile373Leu	no change
		His403Gln	change in charge and hydrophobicity

[a] *CYB5A* positions are based on the human sequence UniProt Accession P00167; *CYP17A1* positions are based on the human sequence UniProt Accession P05093.
[b] Common physicochemical properties examined included hydrophobicity, aromaticity, size, and charge as defined by Exarchos et al. (2009), BMC Bioinformatics 10:113.

Human or primate specific changes in adrenal androgen output may suggest enzymes involved in the biosynthesis of these hormones function differently and/or have evolved adaptively in certain taxa. Investigating the second of these possibilities, we found that although the ratio of nonsynonymous to synonymous substitution rates

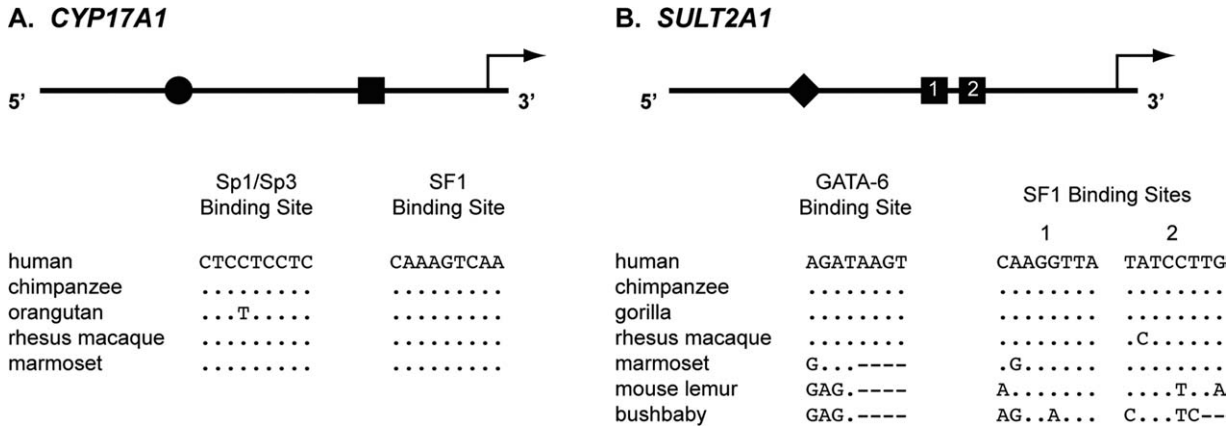

Fig. 6. Functionally active binding sites in the promoter region of *CYP17A1* (**A**) and *SULT2A1* (**B**). For each gene, (1) a schematic of binding site positions upstream of the transcription start site and (2) alignments of binding site regions showing matched sequences (denoted by '.') to the human sequence are shown. Binding site regions are based on the human sequence as reported by Lin et al. (2001), Fluck and Miller (2004), Saner et al. (2005). Functionally active transcription factor binding site regions are aligned for all currently available primate sequences (downloaded from Ensembl). Flanking sequences are not shown. Symbol key: circle = Sp1/Sp3 binding site; diamond = GATA-6 binding site; and squares = SF1 binding sites.

vary across lineages sampled, *CYB5A, POR*, *HSD3B1*/*HSD3B2*, and *CYP17A1* protein-coding sequences are conserved across primates and show evidence of purifying selection. The observed amino acid replacements are mostly conservative and thus probably do not greatly affect protein structure. We found no evidence that humans and chimpanzees share exceptionally divergent protein-coding sequences.

The observation that DHEAS levels in *Papio* are higher than seen in other species results from high levels of this adrenal androgen at young ages followed by low levels in older individuals. Other Old World monkeys follow this pattern, including *Cercocebus* and *Macaca*. Recent investigation by Conley et al. suggests that these early, high levels (in *Macaca*) are actually products of a much-earlier timed adrenarche, resulting from a near-coincident development of the ZR and regression of the FZ (Conley et al., 2011). Arlt et al. (2002) examined sequence differences in CYP17 cDNA and genomic DNA and enzymatic activity in primates that are believed to undergo adrenarche (human and chimpanzee) and two species that were, at that time, believed to not experience adrenarche (rhesus macaque and baboon). While enzyme activities were similar between the human and chimpanzee CYP17, enzyme activities (17α-hydroxylase and 16α-hydroxylase) differed significantly between macaque and baboon CYP17. They found only one amino acid difference between the rhesus macaque (His255) and baboon (Arg255) CYP17 sequence but determined that while this amino acid was important for enzymatic activity, this substitution cannot explain differences observed in the activity of the rhesus macaque and baboon enzyme. Using a larger dataset, we sought to determine whether Arg255 in human, chimpanzee, and baboon represents a derived or ancestral state. The analyses presented here demonstrate that Arg255 (found in human, chimpanzee, gorilla, orangutan, baboon) represents the ancestral state for anthropoid primates (P = 0.983). Although mouse lemur sequence for this gene was incomplete and not included in these analyses, mouse lemur CYP17 also has an arginine at position 255. Therefore, His255 is a homoplasy shared between macaques (*M. mulatta* and *M. fascicularis*) and marmosets. This substitution is conservative (i.e., both amino acids are polar and have positively charged side chains) which is consistent with observations pointing to similarities observed in 17α-hydroxylase activity of wild-type (Arg255) and mutant (Arg255His) CYP17 (Arlt et al., 2002). Arlt et al. (2002) also identified 25 amino acids shared between human and chimpanzee and suggested these amino acids might contribute to differences in enzyme activity observed between the apes (human and chimpanzee) and monkeys (rhesus macaque and baboon) in their study. In order to characterize these 25 amino acids as either derived in human/chimpanzee (species with documented evidence of adrenarche) or conserved across apes, we examined these sites in our expanded dataset (Supporting Information Fig. S7). We found that none of these amino acids were uniquely shared by these two species when more primates were considered. Of these 25 amino acids, 5 are actually shared among human, chimpanzee and gorilla, 19 are shared among all apes examined, and 1 is shared between human, chimpanzee, and orangutan. Because the majority of these sites are shared among all apes examined, it would be interesting to test whether the enzymatic activity of gorilla and orangutan CYP17 is similar to human and chimpanzee CYP17. Overall, however, our results lend evolutionary support to the recent description of the presence of what appears to be adrenarche in macaques (Conley et al., 2011); in other words, this developmental process is not unique to humans and chimpanzees. What might be unique, or at least uniquely shared among humans, *Pan*, and perhaps *Gorilla*, is the extended delay in adrenarche relative to the regression of the FZ.

Transforming ontogeny: Regulation and modification

Adrenarche, and the extended period of low adrenal androgen levels preceding it in human development, has been suggested as a mechanism that has allowed the "childhood" stage of development to evolve, and is implicated in mediating the "5–7 year shift" in cognitive function (Bogin, 1997). In this way, adrenal androgens might be implicated in the evolutionary modification of growth periods and therefore in mediating heterochronic trans-

formations. However, it has been broadly accepted that only humans have extended the prepubertal phase of growth long enough to permit the introduction of the childhood stage. The present study did not address whether there is physiological evidence for uniqueness of this developmental stage in humans; however, the evidence presented does imply that delayed adrenarche, and therefore a potential endocrine mechanism for extending growth and modifying cognitive function, may be present in gorillas as well as chimpanzees, bonobos, and humans. Our results, taken together with the recent findings that adrenarche is present in a compressed period in very early neonatal life in macaques (Conley et al., 2011), leave open the possibility that instead of modification through the insertion of evolutionarily novel phases, early growth periods can be truncated or extended through alterations in the timing of adrenal gland zonation and maturation. The notion that hormones play key roles as modifying prereproductive phases of development is certainly not novel, as investigations into the regulation of development in other organisms demonstrate (e.g., Brown, 1997). However, our results offer a starting point for further examination into how similar evolutionary modifications may occur in long-lived, long-developing primates.

Expression of genes required for steroidogenesis is tissue- and species-specific, developmentally programmed, and hormonally regulated (Martinez-Arguelles and Papadopoulos, 2011). Conservation in protein-coding sequence among the genes we studied, as well as evidence suggesting the expression of these enzymes is adrenal zone-specific and appear to be developmentally programmed, suggest changes in the transcriptional and post-translational regulation of these genes may be key factors in the evolution of adrenarche, or more specifically, the timing of adrenarche. We examined nonhuman primate promoter sequences of two key steroidogenesis genes (*CYP17A1* and *SULT2A1*) for transcription factor binding sites that are known to affect expression of these genes in human adrenal cells in response to ACTH, a hormone important for the steroidogenesis (Parker, 1999). None of the transcription factor binding sites previously described as important for *CYP17A1* and *SULT2A1* expression were found to be unique to the human and chimpanzee promoters. In addition to changes at the sequence level, differences observed between primate species in adrenal androgen production may also be explained by post-translational modifications to these proteins or their co-regulators. For example, phosphorylation of human P450c17 (on serine and threonine residues) is required for 17,20-lyase activity and may increase P450c17 affinity for P450 oxidoreductase (Zhang et al., 1995). This post-translational regulation of P450c17 may be developmentally regulated in species with adrenarche (Zhang et al., 1995). There are a number of potentially phosphorylated serine (Ser) and threonine (Thr) residues present in humans that are conserved across most placental mammals examined (Supporting Information Fig. S7). None of these are shared uniquely among humans and chimpanzees; however, Ser117, Ser210, Ser234, Thr265, and Thr314 are only found in apes.

Future directions

The results discussed here suggest that it would be reasonable to predict that the human, chimpanzee, and bonobo adrenal gland follow similar courses of maturation, and that the similarity in DHEAS levels is due to shared process within the adrenal cortex. Following this, one would expect that the tissue-specific expression of enzymes such as CYP17, CPR, CYB5, and 3βHSD would localize similarly in these species (Conley and Bird, 1997). The composition of the cross-sectional great ape data set used here is not detailed enough to permit a clear picture of initial elevation in DHEAS levels measured in *Pan* samples, but it is obvious that ranges of adult DHEAS levels overlap with those of humans. It could be proposed that orangutans and gorillas would show different patterns of expression, in locality or density, than humans, chimpanzees, or bonobos, relative to their differences in absolute concentrations and/or patterns of secretion.

The implications of these species-level pattern differences, if shown to result from the same physiological processes at the organ level, are far-reaching—this would indicate that the pattern of adrenal gland maturation seen in humans is at least as ancient as the last common ancestor of humans, chimpanzees, and bonobos ($\sim$5–6myr, Wildman et al., 2003). Indeed, it has been suggested that neuroprotective effects of DHEAS protect synaptic plasticity in metabolically active portions of the human neocortex (Campbell, 2011), and humans are known to show a different pattern of neocortical glucose utilization than is seen in rhesus macaques (Jacobs et al., 2005). There are no glucose utilization data available from apes, but the current study suggests that chimpanzees and bonobos may share the pattern of cerebral glucose utilization during the development that is seen in humans.

There remains considerable practical difficulty associated with obtaining whole adrenals representing the developmental span of these long-lived primates, for use in tissue analysis. However, other avenues for research remain open, including those presented here, which may provide some insight regarding whether chimpanzees and bonobos share specific aspects of human adrenarche and adrenal function. In particular, investigation of changes in the regulation of these steroidogenic enzyme genes and post-translational modifications to the proteins involved in the synthesis of DHEA and DHEAS offer key areas for future studies concerning the evolution of adrenarche.

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