

RESEARCH ARTICLE

Androgens and Innate Immunity in Rehabilitated Semi-Captive Orangutans (*Pongo pygmaeus morio*) from Malaysian BorneoSEAN P. PRALL^{1**}, LAURENTIUS AMBU², SENTHILVEL NATHAN², SYLVIA ALSISTO², DIANA RAMIREZ², AND MICHAEL P. MUEHLENBEIN^{1*}¹Department of Anthropology, Indiana University, Bloomington, Indiana²Sabah Wildlife Department, Kota Kinabalu, Sabah, Malaysia

Despite the implications for the development of life-history traits, endocrine-immune trade-offs in apes are not well studied. This is due, in part, to difficulty in sampling wild primates, and lack of methods available for immune measures using samples collected noninvasively. Evidence for androgen-mediated immune trade-offs in orangutans is virtually absent, and very little is known regarding their pattern of adrenal development and production of adrenal androgens. To remedy both of these deficiencies, sera were collected from orangutans (*Pongo pygmaeus morio*) ($N = 38$) at the Sepilok Orangutan Rehabilitation Centre, Sabah, Malaysia, during routine health screenings. Testosterone, dehydroepiandrosterone (DHEA), and dehydroepiandrosterone-sulfate (DHEA-S) were assayed, along with two measures of functional innate immunity. DHEA-S concentrations, but not DHEA, increased with age in this sample of 1–18 year old animals. DHEA concentrations were higher in animals with higher levels of serum bacteria killing ability, while DHEA-S and testosterone concentrations were higher in animals with reduced complement protein activity. Patterns of DHEA-S concentration in this sample are consistent with patterns of adrenarche observed in other apes. Results from this study suggest that in addition to testosterone, DHEA and DHEA-S may have potent effects on immunological activity in this species. *Am. J. Primatol.* 9999:1–9, 2015. © 2015 Wiley Periodicals, Inc.

Key words: orangutan; dehydroepiandrosterone; testosterone; innate immunity; adrenarche; sepilok; bacteria killing assay; hemolytic complement assay

INTRODUCTION

The influence of androgens in immunological measures can provide insight into developmental patterns and physiological trade-offs in primates. Shifts in energy allocation toward immunocompetence may result in costs paid in reproduction and growth. Testosterone in particular has been investigated for its influence on mediating trade-offs between reproduction and immunity [Muehlenbein & Bribiescas, 2005]. However, other androgens have not been investigated well for their roles in this process. The adrenal androgens dehydroepiandrosterone (DHEA) and dehydroepiandrosterone-sulfate (DHEA-S) are known modulators of immune function, and act as precursors to other sex steroids. Inclusion of these androgens in a conceptual understanding of hormone-mediated trade-offs may further clarify how development and reproduction may influence immunological outcomes.

In vitro and rodent research into the effects of DHEA and DHEA-S on immune function implicates it in modulating multiple branches of immunity. DHEA inhibits production of inflammatory cytokines, including TNF α and IL-6 [Di Santo et al., 1996; Du

et al., 2001; Oberbeck et al., 2001], while stimulating IL-2 production [Daynes et al., 1990; Suzuki et al., 1991]. DHEA also modulates lymphocyte proliferation [Sakakura et al., 2006], stimulates the actions of T-cells, and natural killer cells [Casson et al., 1993; Khorram et al., 1997; Suzuki et al., 1991], and is generally characterized in the literature as supportive of immune function. Conversely, testosterone is

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*Correspondence to: Michael P. Muehlenbein, Department of Anthropology, Indiana University, 701 E Kirkwood Ave Student Building 130, Bloomington, IN 47405.
E-mail: mpm1@indiana.edu

**Current affiliation for Sean P. Prall: Department of Anthropology, New Mexico State University, Las Cruces, New Mexico

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generally characterized as immunosuppressive in action [Muehlenbein & Bribiescas, 2005] and acts to inhibit antibody production, suppress T-helper cells, modulate cytokine concentrations, and inhibit macrophage activity [Chao et al., 1994; Giltay et al., 2000; Grossman et al., 1991; Sakiani et al., 2013]. Testosterone is thought to be one of the primary drivers behind sexual dimorphism in humans [Abrams & Miller, 2011; Muehlenbein & Bribiescas, 2005]. Additionally, while results are mixed, there are several studies that indicate higher testosterone is related to worse disease outcomes [Muehlenbein et al., 2005, 2006].

Despite the importance of understanding the mechanisms of energy allocation between life history traits, there are relatively few studies that sufficiently examine associations between androgens and immune function in non-human primates, and none that examine DHEA or DHEA-S. Fecal testosterone concentrations are positively associated with intestinal parasite richness in Ngogo chimpanzees [Muehlenbein et al., 2004; Muehlenbein & Watts, 2010]. In macaques, baseline testosterone levels prior to infection are positively associated with viremia following infection with Venezuelan equine encephalitis virus, and infection causes significant decreases in testosterone concentrations [Muehlenbein et al., 2006]. Field studies in wild primates are restricted by the difficulty in collecting any samples other than feces and urine, and the relative lack of immunological measures capable using these samples. Many studies use fecal samples to measure egg counts from intestinal parasites, but such measures are poor proxies of immune function and are more dependent on parasite age and various host factors [Stear et al., 1995]. Additionally, factors like nutritional status, sex, dominance hierarchies, and sampling strategy all complicate understanding androgen-immune interactions in free-ranging primates [Prall & Muehlenbein, 2014].

In addition to its role in immune function, patterns of DHEA and DHEA-S secretion in apes are poorly understood, and additional data on orangutans in particular can yield insight into the evolution of developmental patterns. In humans, the development of the zona reticularis of the adrenal gland results in a dramatic increase in sex steroids in a developmental milestone known as adrenarche, which proceeds puberty by several years. This delayed adrenal development is thought to be a unique feature of great apes, and to play an important role in brain development [Campbell, 2006, 2010, 2011]. Studies of macaques, baboons, and others indicate a consistent decline in DHEA-S concentrations with age [Castracane et al., 1981; Crawford et al., 1997; Meusy-Dessolle & Dang, 1985; Muehlenbein et al., 2003; Perret & Aujard, 2005], but the human pattern of adrenal androgens surrounding adrenarche in adolescence is absent in these species. In a longitudinal examination of infant

macaques, Conley et al. [2011] finds that adrenarche does occur in this species in the first few months of life, prior to the typical pattern of age related decline. This suggests that adrenarche may be a feature of other anthropoid primates, but that significant heterochrony exists in this stage of development. In contrast, chimpanzees and bonobos exhibit adrenal development patterns very similar to humans [Behringer et al., 2012; Collins et al., 1981; Copeland et al., 1985; Cutler et al., 1978; Smail et al., 1982].

There are very few studies that investigate the relationship between age and DHEA and DHEA-S in orangutans, and those that are published find no evidence of delayed adrenal development [Bernstein et al., 2012; Cutler et al., 1978; Collins et al., 1981]. However, these studies have rather small sample sizes, particularly in infant and juvenile animals, where adrenarche could be detected via elevations in adrenal androgens. In order to investigate better the existence of delayed adrenarche in humans, a larger sample of younger apes is necessary.

Based on the relative absence of evidence regarding both the adrenal androgen secretion patterns of DHEA and DHEA-S, as well as the dearth of research regarding the role of androgens on innate immune function in orangutans, the goals of this study were to (1) document changes in adrenal androgens in a large sample of orangutans of varying age, and (2) document associations of adrenal androgens as well as testosterone with multiple measures of innate immune function in orangutans. We hypothesized that (a) orangutans would exhibit similar age-related changes in adrenal androgens as other apes, and (b) DHEA and DHEA-S would be positively and testosterone negatively related to functional innate immunity in this sample.

METHODS

Study Site

Sampling took place at Sepilok Orangutan Rehabilitation Centre (SORC), in Sabah Malaysia, in March, 2011. The primary goal of the SORC is to rehabilitate orangutans and other endangered species, typically those who have been previously kept as illegal domestic pets, were injured, or orphaned. Newly acquired animals are quarantined for several months, where an on-site veterinarian closely supervises their health. Following quarantine, animals are behaviorally rehabilitated and taught to forage before being released into the surrounding 5,529 ha forest reserve or otherwise relocated. The SORC also maintains a visitors center, where public feeding of rehabilitated and released orangutans occurs twice daily. A second sampling of four animals under similar conditions was also completed at an additional facility of the Sabah Wildlife Department, located at the Shangri-La Rasa Ria Resort. This

facility is adjacent to the Rasa Ria Nature reserve, and the orangutans sampled here originated from the SORC and were transferred for additional rehabilitation.

Sample Collection

Sampling took place as part of SORC's routine health assessment of all animals. Animal ages ranged from 1 to 18.6 years, and included 17 males (mean age = 7.9) and 21 females (mean age = 6.4). Blood collection took place between 9:00 AM and 4:00 PM, in a veterinary clinic under the supervision of a wildlife veterinarian. Orangutans were collected from either the clinic nursery or were free-ranging animals brought to a designated clinical exam room, where weight, skinfolds, arm circumference, and crown-rump length were obtained. Animal ages were ascertained from SORC records. With the exception of a single semi-flanged male who was tranquilized with approximately 2 mg/kg of Zoletil 100 (zolazepam-tiletamine; Virbac, South Africa), animals were manually restrained and blood samples collected from the forearm by a trained wildlife veterinarian using single-use vacutainer products (Becton-Dickinson). Serum was collected in duplicate, and stored at 0°C, before being shipped to the United States on dry ice, where samples were stored at -80°C until analysis.

Samples were imported to Indiana University, Bloomington using CITES permits 12US77006A/9 and 9062, and CDC import permits 2011-03-048 and 2013-06-111. Local research permission was granted by the Sabah Wildlife Department, and ethical permission through the Bloomington Institutional Animal Care and Use Committee at Indiana University. This research adhered to the American Society of Primatologists principles for the ethical treatment of primates.

Immunological and Endocrine Assays

Alpco enzyme immunoassay kits (#20-DHEHU-E01, 11-DHEHU-E01, 11-TESTU-E01, respectively) were used to assess concentrations of DHEA, DHEA-S, and testosterone in serum samples. These products have been used previously to assess androgen concentrations in primates of various species [Bernstein et al., 2012]. Intra-assay coefficients of variability, assessed by calculating the mean coefficient of variation between sample duplicates were 12.5%, 6.0%, and 6.4% for DHEA, DHEA-S, and testosterone, respectively.

A bacteria killing assay and hemolytic complement assay were used to determine immune function. Bacteria killing assays (BKA) measure the ability of integrative immunological components, particularly antibodies and opsonizing proteins, to lyse a quantity of *Escherichia coli* bacteria ex vivo [Demas et al., 2011]. Following methods modified

from Chester et al. [2010], sera were diluted 1:10 in CO₂-independent media (Gibco #18045) and supplemented with L-glutamine. *E. coli* lyophilized pellets (ATCC #8739, MicroBiologics EPower #0483E7) were reconstituted and diluted in sterile saline, and a volume was added to the diluted serum. After a 30 min incubation, samples were plated using sterile technique onto tryptic soy agar (BD BBL #211043) petri plates in triplicate and incubated overnight. The following day, colonies were tabulated and the percent bacteria killing calculated [average count for each sample subtracted from the positive control plate (bacteria and media only), divided by the positive control plate].

Hemolytic complement assays measure the results of the classical pathway of complement protein mediated lysis, and were run here to provide an additional measure of innate immunity [Demas et al., 2011]. Using modifications from a protocol developed by Sinclair & Lochmiller [2000] sera were diluted 1:2 and 1:4 in dextrose gelatin veronal buffer (Lonza BioWhittaker #10-539), and pipetted in duplicate into a 96-well round-bottom plate. Diluted anti-sheep red blood cell antibody (Sigma #S1389-1VL) was added to all sample wells and a 0.9% solution of diluted sheep red blood cells (MP Biomedicals #55876) was added to all sample wells and controls, and the plate vortexed and incubated for 1.5 hr. Following incubation, plates were centrifuged and supernatant transferred to a new plate, and absorbance measured in a spectrophotometer at 405 nm. Based on calculated percent lysis values (absorbance for dilution divided by absorbance for positive control) for each sample dilution, CH50 values (i.e. estimated dilution predicted to lyse 50% of cells) were calculated [Mayer, 1948].

Statistics

Age (as a continuous variable) and hormone concentrations (raw concentrations) were compared using Spearman's correlations. For ease of analysis, animals were also categorized into infant (0–4 years; including 3 males and 9 females), juvenile (4–8 years; 8 males and 2 females), and adolescent/adult (older than 8 years; 6 males and 10 females). Adults were not given a separate category, as there was only one individual older than 12 years, and exclusion of this individual in the analysis did not alter results. Data did not meet assumptions of normality, and included outliers in some age categories, so differences in hormone concentrations by age group were compared using the Kruskal–Wallis Test. Sex and immune category were compared using Mann–Whitney U Tests. When necessary, logistic regressions were performed to measure the influence of androgens and covariates on immunological outcomes. Additionally, in order to control the effects of covariates on androgen concentrations, linear regressions were

TABLE I. Correlations Between Androgens

	DHEA-S	Testosterone
DHEA	$\rho = 0.715$ $P < 0.0005$ $N = 28$	$\rho = 0.426$ $P = 0.021$ $N = 29$
DHEA-S		$\rho = 0.783$ $P < 0.0005$ $N = 34$

performed on transformed variables to calculate unstandardized residuals.

RESULTS

Endocrine Results

All androgens were significantly, positively correlated (Table I). Serum volume was limited, so not all samples were available for use in all endocrine and immune assays. Additionally, six samples had inappropriately high coefficients of variation in DHEA results, so these were excluded from all analyses. Median concentrations for androgens in each age group are reported in Table II. There were no significant differences in DHEA concentrations between age categories ($N = 32$, $\chi^2 = 2.963$, $df = 2$, $P = 0.227$; Fig. 1), but there were for DHEA-S ($N = 33$, $\chi^2 = 7.972$, $df = 2$, $P = 0.019$; Fig. 2), with significant differences between juvenile and adolescent/adult groups (adjusted $P = 0.051$). Similarly, testosterone was different between groups ($N = 35$, $\chi^2 = 8.581$, $df = 2$, $P = 0.014$; Fig. 3), with a significant difference between infant and adolescent/adult groups (adjusted $P = 0.023$). Results from Spearman's correlations with age yielded similar results, with testosterone and DHEA-S ($\rho = 0.533$, $P = 0.001$; $\rho = 0.412$, $P = 0.016$, respectively), but not DHEA ($\rho = 0.283$, $P = 0.319$) significantly and positively associated with age. Additionally, no sex differences were found for DHEA, DHEA-S, or testosterone ($U = 107$, $z = -0.722$, $P = 0.470$; $U = 93.5$, $z = -1.524$, $P = 0.129$; $U = 109$, $z = -1.28$, $P = 0.21$, respectively), even when infants were excluded from the analysis

Immune Results

Serum BKA results were highly bimodal, with clustering at 100% and 0% killing. Additionally,

several samples resulted in negative values for percent killing, indicating that bacterial growth occurred with those samples relative to the positive control. One animal grew what appeared to be *Staphylococcus* bacteria in all three plates, suggesting the possibility of a blood-borne bacterial infection in this individual. For these reasons, percent killing was converted into a dichotomous categorical variable of low killing ($N = 21$) and high killing ($N = 14$). In comparing androgen levels between BKA category, the high BKA had significantly higher DHEA concentrations than did the low BKA ($N = 29$, $U = 151$, $z = 2.061$, $P = 0.04$; Fig. 4). There were no other statistical differences in BKA category between other androgens, age, or weight. A binary logistic regression (with normalized androgens as predictive variables) yielded a statistically significant model ($\chi^2(3) = 9.608$, $P = 0.022$), and explained 38.8% of the variation in BKA category (Nagelkerke R^2), and correctly classified 64.3% of cases. Sensitivity was 61.5% and specificity was 66.7%. DHEA was the only predictive variable found to be statistically significant. Increased DHEA significantly increased the likelihood of being in the high BKA category ($B = 2.627$, $P = 0.018$).

Several of the serum samples had undergone hemolysis, so these results were excluded from the hemolytic complement analysis. Similar to serum BKA, hemolytic complement results were highly bimodal, with many percent lysis results above 100%. Elevated lysis results and a lack of variation between dilutions within samples resulted in CH50 calculations that were negative or inaccurate, indicating large variation in optimal sample dilution. Therefore, percent lysis values for the 1:4 dilution were dichotomized into high lysis ($N = 15$) and low lysis ($N = 18$) at 50%. There was no statistically significant difference in androgens between lysis categories. However, there was a significant difference in age between lysis categories ($U = 64$, $z = -2.581$, $P = 0.009$), where low lysis individuals tended to be older. Initially, a hierarchical binary logistic regression analysis was performed to ascertain the influence of age and androgens on complement lysis, with age as predictor in the first model, and age and normalized androgens in the second. However, none of the androgens were statistically significant in the second model, so linear regressions were subsequently performed with age as a predictor of androgen concentrations (transformed for

TABLE II. Median (and Standard Deviation) Concentration of Androgens by Age Group, With Sample Sizes Listed for Each

	DHEA (ng/ml)	DHEA-S (ug/ml)	Testosterone (ng/ml)
Infant	2.91 (3.28); $N = 11$	0.10 (0.42); $N = 10$	0.55 (2.68); $N = 11$
Juvenile	2.59 (2.87); $N = 6$	0.12 (0.19); $N = 8$	0.88 (1.87); $N = 8$
Adolescent/Adult	3.72 (3.18); $N = 15$	0.30 (1.58); $N = 16$	5.11 (5.23); $N = 16$

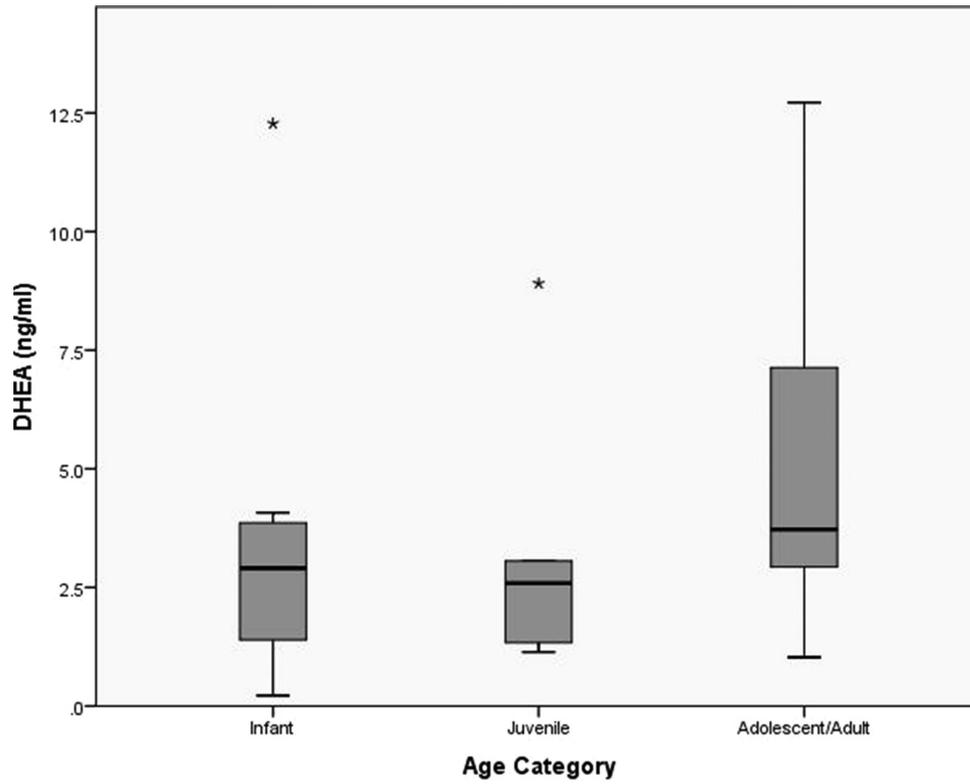


Fig. 1. Median DHEA between age categories, with 95% confidence intervals. Outliers indicated by asterisk.

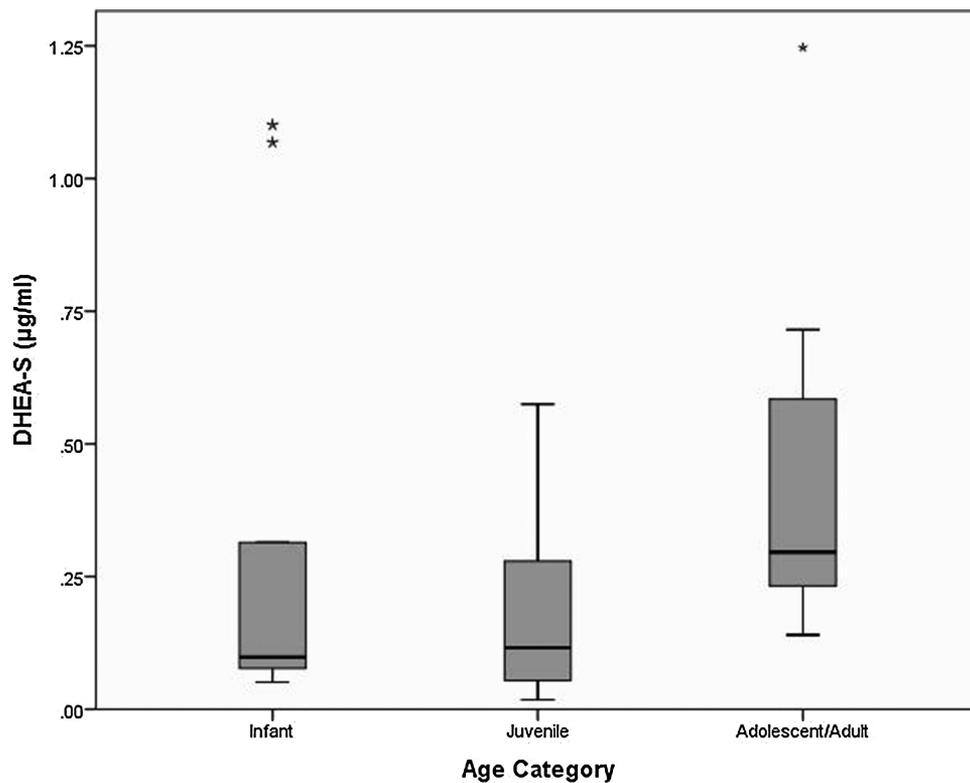


Fig. 2. Median DHEA-S between age categories with 95% confidence intervals. Outliers indicated by asterisks. For visual purposes, this graph excludes an outlier in the adolescent/adult category.

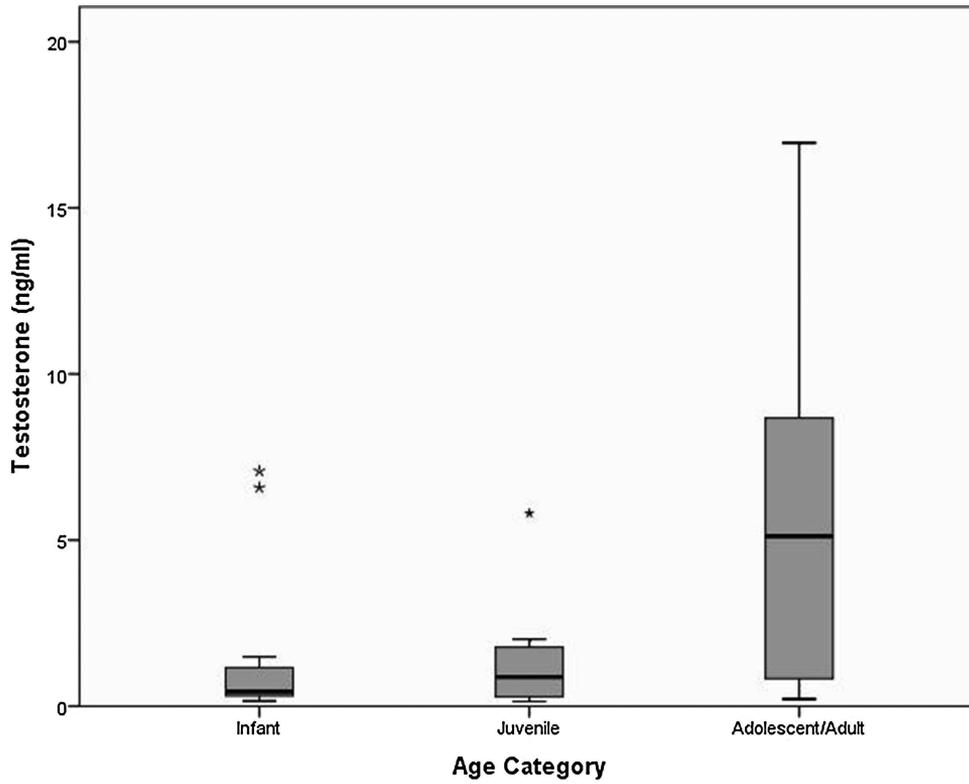


Fig. 3. Median testosterone concentrations between age categories, with 95% confidence intervals. Outliers indicated by asterisks.

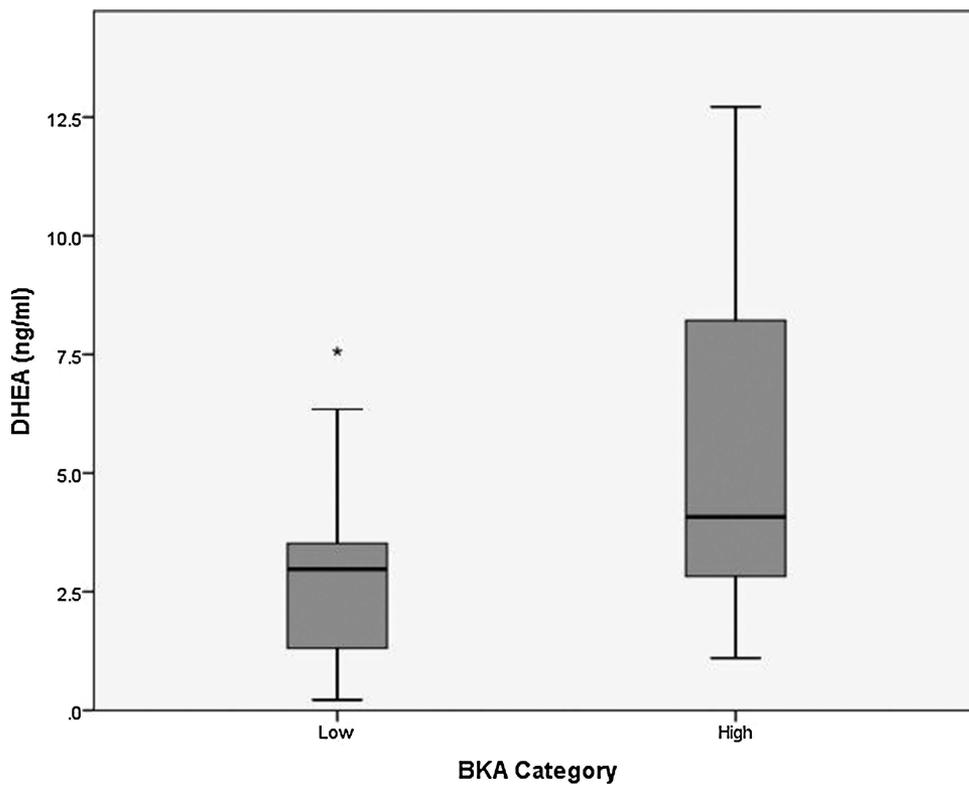


Fig. 4. Median DHEA concentrations between bacterial killing categories, with 95% confidence intervals. Outlier indicated by asterisk.

normality to generate unstandardized residuals). Logarithmic transformations resulted in normality for DHEA and DHEA-S (Shapiro–Wilk, $P > 0.05$), but transformation of testosterone did not yield a normalized variable (Shapiro–Wilk = 0.937, $P = 0.046$). Only DHEA-S and testosterone generated significant models as the result of a linear regression ($r^2 = 0.127$, $F = 4.654$, $P = 0.039$; $r^2 = 0.281$, $F = 12.891$, $P = 0.001$, respectively). Results indicate that age-corrected DHEA-S and testosterone were significantly higher in the low lysis category as compared to the high lysis category (both $N = 33$, $U = 64$, $z = -2.581$, $P = 0.009$).

DISCUSSION

Previous studies of patterns of adrenal androgen secretion in orangutans indicated few significant age-related patterns, and no evidence of a human-like pattern of delayed adrenarche [Bernstein et al., 2012; Cutler et al., 1978; Collins et al., 1981]. However, the present study finds significant differences in DHEA-S, but not DHEA, concentrations between age categories in orangutans. In particular, these changes occur between juvenile (4–8 years) and adolescent/adult (>8 years) categories, suggesting orangutan adrenal androgens experience a similar surge as seen in humans, bonobos, and chimpanzees.

There is considerable variation in adrenal androgen concentration within age categories in the current study, which may suggest variability in timing of development and subsequent androgen synthesis, or that unmeasured factors also influence adrenal androgen secretion. In particular, acute stress has been demonstrated to elevate DHEA and DHEA-S concentrations in humans [Lennartsson et al., 2012]. The animals in the current study were collected from a free-ranging habitat, or were being held in a nursery. Manual restraint and blood collection may have resulted in abnormal elevations in adrenal androgen secretion, although all of these animals were handled extensively as part of the rehabilitation process, and only a single animal exhibited behavioral signs of stress (the semi-flanged male that had to be tranquilized). All animals had received veterinary care at some point, and were receiving supplemental food as part of the public feedings that occur twice daily at the SORC. It is therefore possible that developmental patterns in these animals are not necessarily identical to wild orangutans without a supplemented diet. Longitudinal sampling of 5–10 year old orangutans would be of great benefit in further confirmation of a human-like pattern of adrenal development.

This study is the first examination of endocrine–immune interactions in orangutans. Routine health assessment at the SORC allowed for collection of serum for high quality functional measures of innate immune function that are far superior to methods

commonly used in wild primates. Results found here largely confirm predictions from general trends on the effects of these androgens. After controlling for age, testosterone and DHEA-S were associated with low hemolytic complement lysis, while DHEA was higher in the elevated bacteria killing category.

While testosterone and DHEA fit the predictions regarding relationship to immunocompetence, it is unclear why DHEA-S, the sulfated form of DHEA, was not similarly related to beneficial outcomes in innate immunity, particularly given that DHEA and DHEA-S were correlated in this sample. In humans, higher DHEA-S has been associated with a reduction in parasite infection and reinfection, even after adjusting for age on DHEA-S levels [Kurtis et al., 2006; Leenstra et al., 2003], suggesting a direct relationship between adrenal development and disease resistance. It is possible that elevated DHEA-S represents higher rates of conversion of DHEA to its inactive form, or even to increased testosterone synthesis. This relationship can only be explored with more sophisticated methods to measure production and activity of steroidogenic enzymes.

The need to dichotomize the immune results caused a significant loss of statistical power. Serum samples were frozen for a significant period of time, which is known to result in depreciation of immune activity in these measures [Liebl et al., 2009]. Additionally, due to limited volumes of serum, other dilutions could not be tested in each immune measure. Some samples from the BKA resulted in negative percent killing, which has been found in other studies [Brooks & Mateo, 2013; Rubenstein et al., 2008; Tieleman et al., 2005]. Negative percent killing could indicate overly diluted samples, bacterial growth in the presence of diluted serum, or possibly inhibition of growth in the positive control plates due to experimental error, although the latter is highly unlikely here. Hemolytic complement results were equally problematic, where many individuals in the high lysis category had little difference in percent lysis between the two dilutions, indicating an extremely active complement activity, but also making accurate CH50 calculations difficult.

Malaria infection in the study sample is high, with 57.9% of animals infected (including those with very low parasitemia), and 26.3% having multiple species present (Muehlenbein et al. 2015). Additionally, one individual exhibited what appeared to be *Staphylococcus* growth on all three bacterial killing plates, indicating a bacterial infection in this individual. Immune activation is known to result in reductions in testosterone concentrations [Boonekamp et al., 2008; Muehlenbein et al., 2005, 2010], and may impact DHEA and DHEA-S under some conditions [Arlt et al., 2006; Kolditz et al., 2010]. Finally, DHEA has moderate circadian rhythms, which may cause additional variation in these results [Hucklebridge et al., 2005].

In summary, this study is the first to find an age-related increase in DHEA-S, but not DHEA, in young orangutans, indicating that orangutans may exhibit delayed adrenarche in a similar manner to humans and other apes. We also find that DHEA is positively, and DHEA-S and testosterone negatively associated with functional measures of innate immunity in this sample of rehabilitated orangutans. These results further support the idea that, in addition to testosterone, adrenal androgens may contribute significantly to important developmental and immunological outcomes, demonstrating a probable role of adrenal androgens in primate life history trade-offs.

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