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Measuring urinary cortisol and testosterone levels in male Barbary macaques: A comparison of EIA and LC–MS

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ABSTRACT

The development of methods to quantify hormones from non-invasively collected samples such as urine or feces has facilitated endocrinology research on wild-living animals. To ensure that hormone measurements are biologically meaningful, method validations are strongly recommended for each new species or sample matrix. Our aim was to validate three commonly used enzyme immunoassays (EIA), one for analysis of cortisol and two for analysis of testosterone, to assess adrenocortical and gonadal endocrine activity, respectively, from the urine of male Barbary macaques. We compared EIA and liquid chromatography–mass spectrometry (LC–MS) results to determine if the EIA measurements truly reflect levels of the target hormone and to determine if antibody cross-reactivities with other steroids were potentially confounding results. Furthermore, we conducted a biological validation of testosterone to ensure that both EIA and LC–MS were able to capture physiologically meaningful differences in hormone levels. We found that cortisol measured by EIA correlated strongly with cortisol measured by LC–MS in both adult and immature males, without the need for deconjugation of steroids in the urine. Both testosterone EIAs correlated strongly with LC–MS in adult males, but only if steroids in the urine were deconjugated by enzymatic hydrolysis prior to analysis. However, in immature males, EIA and LC–MS results did not correlate significantly. Further correlation analyses suggest this is likely due to cross-reactivity of the testosterone antibodies with other adrenal steroids such as cortisol, DHEA, and likely others, which are present at much higher concentrations relative to testosterone in immature males. Testosterone levels were significantly higher in adult compared to immature males as measured by LC–MS but not as measured by EIA. Taken together, our results suggest that the testosterone EIAs are suitable to assess gonadal activity in adult but not immature males, and only if a hydrolysis of the urine is conducted prior to analysis.

1. Introduction

Steroid hormones facilitate a range of behaviors and developmental changes in animals. For example, glucocorticoids help to mobilize energy reserves and respond adaptively to environmental and social stressors (Sapolsky, 2002). Androgens, such as testosterone (T), the predominant male sex-hormone, promote the production of sperm, development of secondary sexual characteristics, and male reproductive competition (Wingfield et al., 1990). To study steroid hormones in wild-living animals, behavioral ecologists and wildlife endocrinologists are increasingly measuring hormone levels non-invasively, usually via the analysis of excreted hormone metabolites in urine or feces (e.g. Wasser et al., 2000, 1988; Brown et al., 1994; Robbins and Czekala, 1997; also see Higham, 2016 for a review, including more references and information on the history of non-invasive hormone measurements). This is in part due to the practical and ethical advantages these methods have compared to the traditional approach of measuring hormones invasively from blood. Notably, urine and fecal samples can be collected repeatedly over time without the need to capture or disturb the animal.

The most commonly used methods to quantify hormone concentrations in the various matrices are radio- (RIA) and enzyme- (EIA) immunoassays. These methods rely on the use of antibodies that bind to the hormone (or their metabolites) of interest, thus allowing for its

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concentration to be quantified in a sample (Grange et al., 2014). Despite their specificity, antibodies used in immunoassays may cross-react with other structurally similar metabolites. In blood, where native unconjugated steroid hormones circulate at much higher concentrations than their metabolites, the impact of such cross-reactivities is often negligible. However, steroids are extensively metabolized in the liver and/or by gut bacteria (Taylor, 1971), and as a result, the concentration of the native unconjugated hormone excreted in urine or feces is usually very low relative to its metabolites or conjugated forms (Bahr et al., 2000; Möhle et al., 2002; Palme and Möstl, 1997). Thus the impact of cross-reactivity is usually more pronounced in the analysis of urine or fecal samples than of blood samples. Immunoassays for the measurement of testosterone often lack the needed antibody specificity and thus may be of limited use for direct measurement of testosterone in urine (Venturelli et al., 1995). Nevertheless, if the cross-reacting metabolites originate from the parent hormone of interest, the signal detected by the assay may still be biologically meaningful. However, if the cross-reacting compounds measured by the antibody used originate from hormones with different biological functions, then results are confounded and may be uninterpretable. For example, some testosterone immunoassays co-measure androgen metabolites of non-gonadal origin (likely from dehydroepiandrosterone (DHEA), which is of adrenal origin) to such an extent, that they fail to find the predicted difference in testosterone levels between males and females (Goymann, 2005; Möhle et al., 2002). Similarly, two out of four glucocorticoid EIAs showed substantial cross-reactivity with testosterone metabolites in the urine and feces of male African elephants (*Loxodonta africana*), potentially leading to a confound when applied for the assessment of adreonocortical activity in this species (Ganswindt et al., 2003).

As steroids are primarily excreted in their conjugated form in urine (Bahr et al., 2000; Möhle et al., 2002; Ziegler et al., 2000), results from hormone-specific assays designed to measure the free (i.e. unconjugated) native hormone in blood may be improved by first deconjugating the steroids via hydrolysis and/or solvolysis (Hauser et al., 2008a; Venturelli et al., 1995), thus increasing the ratio of free native hormone in the sample. Failure to do so may produce inconsistent results. For example, two studies on wild chimpanzees (*Pan troglodytes*) tested for a relationship between dominance rank and urinary testosterone levels. One study found a significant positive correlation, where high ranking males had higher testosterone (Muller and Wrangham, 2004), whereas the other study did not find a significant relationship (Sobolewski et al., 2013). While this could be a true difference between populations, methodological differences in hormone analysis could also account for this discrepancy. The key difference is that in the former study, steroids in the urine were deconjugated via hydrolysis prior to analysis (Muller and Wrangham, 2004), but in the latter study, testosterone was analyzed from unprocessed urine samples (Sobolewski et al., 2013). Considering that the vast majority of testosterone is excreted as glucuronide conjugates in chimpanzee urine (Möhle et al., 2002), it is unclear whether using a testosterone EIA on non-hydrolyzed urine produces biologically meaningful results, particularly when cross-reactivity of the antibody with conjugated testosterone is not known or very low.

Given the discrepancies mentioned above, it is of paramount importance to validate hormone assay methods prior to their application in non-invasively collected samples (Heistermann et al., 2006; Möhle et al., 2002; Touma and Palme, 2005; Ziegler et al., 2000). Furthermore, due to variation in hormone metabolism, method validations are strongly recommended for each new species, sex, or sample matrix (Buchanan and Goldsmith, 2004; Goymann, 2005; Heistermann et al., 2006; Palme, 2019; Touma and Palme, 2005).

Traditionally, two methods have been used to validate measurements of immunoassays. First, radioinfusion studies work by injecting a small amount of radio-labelled hormone into the animal and collecting all subsequent excreta. Since the injected hormone is radio-labelled, researchers are able to deduce the time-lag to hormone excretion, the metabolism pathway, and whether an antibody really cross-reacts with the target hormone (Goymann, 2005; Palme, 2019; Wasser et al., 2000). Second, physiological validations of steroid hormone measurements may be conducted by pharmacologically inducing their release in the body, then checking if the immunoassay is able to capture the resulting change in hormone levels (Goymann, 2005; Heistermann et al., 2006; Kretzschmar et al., 2004; Palme, 2019; Wasser et al., 2000). While these methods provide invaluable information, one limitation is that they are invasive in nature and usually carried out in captivity, which may not always be practical.

One way to ensure that non-invasive methods can capture natural variations in hormone levels is to conduct biological validations. For example, a testosterone assay should be able to differentiate levels of adult males from those of immature males or females (Möhle et al., 2002; Pineda-Galindo et al., 2017), a glucocorticoid assay should be able to detect rises following putatively stressful events such as translocation, capture and restraint (Pineda-Galindo et al., 2017; Touma and Palme, 2005), and estrogen or progesterone assays should be able to detect changes in female reproductive condition (i.e. menstrual cycle, pregnancy: Pineda-Galindo et al., 2017; Fieß et al., 1999).

Liquid chromatography–mass spectrometry (LC–MS) can also be used as a tool to validate immunoassay measurements (Gesquiere et al., 2014; Habumuremyi et al., 2014; Preis et al., 2011). In contrast to immunoassays, LC–MS does not use antibodies for hormone detection, but allows for highly specific measurements of hormones in samples based on their molecular weight and charge (Cross and Hornshaw, 2016; Hauser et al., 2008a), thus avoiding any confounding effects of antibody cross-reactivity. Therefore, comparing immunoassay measurements to LC–MS is a useful way to deduce if measurements from an immunoassay indeed reflect the concentration of the target hormone and which cross-reacting metabolites may be potentially confounding results (Gesquiere et al., 2014; Habumuremyi et al., 2014; Preis et al., 2011). Both immunoassays and LC–MS may be applied to non-invasively collected samples and the comparison of their measurements offer a useful alternative when more invasive validation methods are not feasible or desirable. Once sufficiently validated, immunoassays have the advantage of being cheaper and having a higher throughput than LC–MS (Cross and Hornshaw, 2016). Therefore, as long as the focus of a study is on a single hormone, routine hormone measurements with immunoassays are more practical for end-users in terms of cost and resourcing especially in labs that may not have access to LC–MS.

In this study, we aimed to determine whether three commonly used EIAs, one cortisol and two testosterone, previously used in other non-human primates (Bahr et al., 2000; Möhle et al., 2002; Sobolewski et al., 2013) were suitable to assess adrenocortical and gonadal activity, respectively, in the urine of male Barbary macaques (*Macaca sylvanus*). In this species, validations have been conducted for assays measuring glucocorticoid (Heistermann et al., 2006; Young et al., 2014) and androgen (Rincon et al., 2017) metabolite levels in fecal samples. Only one study has measured cortisol levels in the urine of (female) Barbary macaques (Sonnweber et al., 2015), although, to our knowledge, no validation has yet been conducted for urinary cortisol and testosterone measurements in this species. First, we examined the pattern of conjugation of cortisol and testosterone in the urine of adult and immature male Barbary macaques. Then, we correlated the LC–MS cortisol and testosterone measurements to their respective EIA measurements from unprocessed urine to determine whether they would closely match, or whether deconjugation steps might be necessary prior to using EIA in order to improve results (Al-Dujaili, 2006). Thus, if these results did not correlate significantly, we then performed a deconjugation step (Venturelli et al., 1995; Ziegler et al., 2000) to see if the correlation improved and by this assessed the general suitability of the immunoassays used (c.f. Al-Dujaili, 2006). The deconjugation step performed (hydrolysis or solvolysis) was chosen based on the pattern of conjugation. To determine any potential influence of cross-reactivity and potential co-measurement of steroid metabolites of different origins

on our EIA measurements, we correlated measurements from the three EIAs to cortisol, testosterone and DHEA as measured by LC–MS. To complement the methodological testosterone validations, we additionally performed a biological validation of testosterone by comparing levels of adult males to those of immature males. We predicted that testosterone levels would be higher in adult males compared to immature males (Rincon et al., 2017).

2. Materials and methods

2.1. Ethical statement

All methods used in this study were non-invasive (i.e. animals were not handled at all) and followed the Animal Behaviour Society's guidelines for the ethical treatment of animals in behavioral research and teaching.

2.2. Study site and animals

Study subjects belonged to one out of three groups of Barbary macaques living in semi-free ranging conditions in 14.5 ha of enclosed forest at Affenberg Salem, Germany (de Turckheim and Merz, 1984). They are provisioned daily with fruits, vegetables, grains and have *ad libitum* access to water and monkey chow. The study group (group C) consisted of 13–14 adult males (one male died during the study period), 20 adult females, 2 large sub-adult males, 8 immature males, 10 immature females and 1 newborn infant male. All members of the group were individually recognizable by observers based on a combination of distinctive individual characteristics, such as facial spots and scars, canine size, body size, coat color and quality as well as unique tattoos on the inner right thigh.

2.3. Sample collection

A total of 62 urine samples were collected between April and October 2016 from a total of 21 males, including 30 samples from 13 adults (7–25 years old), and 32 samples from 8 immature individuals (1–4.5 years old). When monkeys were seen to urinate, the urine was caught with a plastic bag when possible or collected from leaves, branches, rocks or the ground by using a disposable pipette or salivette (Salivette Cortisol, Sarstedt, Nümbrecht, Germany; see Danish et al., 2015; Müller et al., 2017). Urine samples contaminated with feces were not collected. Urine samples collected by pipette were transferred to 2 ml cryotubes. Both samples stored in cryotubes and salivettes were kept in a thermos filled with ice while in the field. At the end of the day, urine was recovered from the salivettes by centrifugation using an electric centrifuge and also transferred to 2 ml cryotubes. All samples were then stored in a freezer at −20 °C. When data collection was complete, samples were transported in containers with dry ice to the endocrinology laboratory where they were once again kept frozen at −20 °C until hormone analysis.

2.4. Hormone analysis

2.4.1. Deconjugation and extraction of steroids

The deconjugation and extraction of steroids for LC–MS analysis followed a modified version of a protocol previously described (Hauser et al., 2008a; Preis et al., 2011). We used 20 µl urine for analysis. To each urine sample we added 50 µl internal standard mixture, to control for losses during extraction and purification, and matrix effects on ionisation of MS measurements. Internal standard mixtures contained 2 ng/ml each of testosterone-d3 and estrone-d4 from Sigma-Aldrich (St. Louis, MO, USA), progesterone-d9 from CDN Isotopes (Point-Claire, QC, Canada), and prednisolone from Steraloids (Newport, Rhode Island, USA). However, for this study, only values for testosterone-d3 were used for the correction of testosterone and DHEA and values for prednisolone were used for the correction of cortisol. Steroid glucuronides were hydrolyzed by mixing 20 µl urine of each sample with 10 µl β-Glucuronidase (K12 strain *Escherichia coli*, Prod. No. BGALS-RO, Sigma-Aldrich), 50 µl internal standard mixture (see above), and 250 µl 0.05 M phosphate buffer (pH 6.5) and incubating in a water bath at 55 °C for 30 min. Extracts were purified by solid phase extractions (Chromabond HR-X, 30 mg, 1 ml, Macherey-Nagel, Dueren, Germany) (Hauser et al., 2008a). Afterwards, steroid sulfates were cleaved by solvolysis: residues of the aqueous layer were resolved in 500 µl of methanol and 2.5 ml ethyl acetate/H_2SO_4 (250 ml ethyl acetate/200 mg H_2SO_4, 98%). This mix was then incubated for 15 min at 55 °C in a water bath. Following incubation, 150 µl 1 M KOH was added to the solvolyzed samples, vortexed and then centrifuged for 3 min at 870 g. The solution was evaporated to dryness at 45 °C under air. This solvolysis step was carried out only for the measurements of steroids by LC–MS (and not EIA). Extraction of steroids was carried out with 5 ml tert. butyl methyl ether (TBME), evaporated and reconstituted in 100 µl of 30% acetonitrile. Extraction efficiencies for the LC–MS measurements were 81.9% for cortisol, 77.4% for testosterone and 62.7% for DHEA (Hauser et al., 2008a). HPLC grade water and H_2SO_4 were purchased from J.T. Baker (Mallinckrodt Baker, Phillipsburg, NJ, USA). Potassium carbonate (K_2CO_3) was purchased from Merck (Kenilworth, NJ, USA). Ammonium acetate was purchased from Sigma-Aldrich. Methanol, ethyl acetate, monosodium phosphate, disodium phosphate, TBME, KOH, and UPLC grade acetonitrile were purchased from Roth (Karlsruhe, Germany).

To determine the pattern of conjugation, we first extracted the urine to obtain the unconjugated fraction, then performed a hydrolysis on the aqueous phase to obtain the glucuronide fraction and finally performed a solvolysis on the remaining aqueous phase to obtain the sulfate fraction. The concentration of each fraction was determined by LC–MS and summed to provide a measure of the total concentration of hormone (cortisol or testosterone). The pattern of conjugation is reported as the percentage of each fraction of the total sum.

To measure testosterone in the urine via EIA, we performed an enzymatic hydrolysis and extraction as described in Section 2.4.1, but without adding the internal LC–MS standard mixture to the samples. Furthermore, we decided not to perform a sovolysis on the samples. This was because we found that in both adult and immature individuals, consistently only a small proportion of testosterone was excreted as sulfates (see Results, Fig. 1). We are confident that disregarding the small amount of sulfated testosterone excreted does not influence the across individual/sample variation in testosterone concentrations that we were interested in. Therefore, for the routine measurement of samples with EIA we skipped the solvolysis step to save on time and costs. To assess the efficiency of the combined hydrolysis and extraction procedure, we prepared a stock solution of testosterone-glucuronide (Art. No. T-2000; Merck KGaA, Darmstadt, Germany) with a concentration of 50 pg/µl in MeOH/H_20 (50/50). Aliquots of 0.4 ml each of this stock solution were stored at −20 °C until used in the hydrolysis/extraction process as separate controls (i.e. using 20 µl of control solution instead of 20 µl of urine). Hydrolysis and extraction of controls were performed as described in Section 2.4.1 (see above). Testosterone levels were measured from hydrolyzed controls using T-EIA I as described in Section 2.4.3 (see below). To determine the combined hydrolysis/extraction efficiency, testosterone values measured in the controls were divided by the added amount and expressed as a percentage. The combined hydrolysis/extraction efficiencies ranged from 68% to 82% recovery (N = 11, mean ± SD: 76.6 ± 4.7%).

2.4.2. LC–MS analysis

LC measurements were carried out using a Waters Acquity UPLC separation module equipped with a binary solvent manager and a column oven (Waters, Milford, MA, USA) and separation was performed on a Waters Acquity BEH C18 column (2.1 × 100 mm, 1.7 µm particle

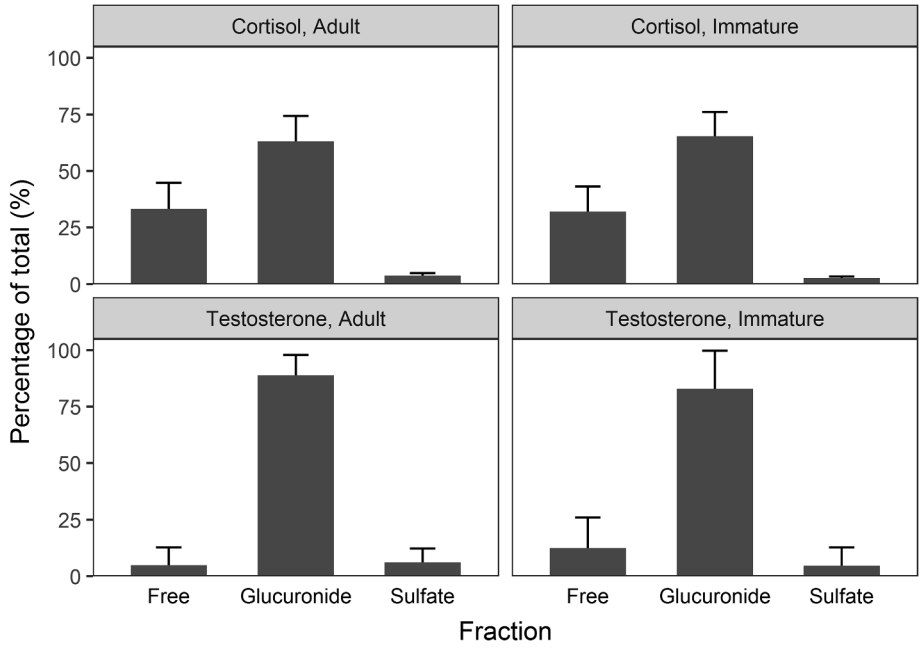

Fig. 1. Proportion of cortisol and testosterone excreted in conjugated (glucuronide or sulfate) and unconjugated (free) form in male Barbary macaque urine, as measured by LC–MS. N = 13 adult, N = 8 immature males. Error bars indicate standard deviation.

diameter). Eluent A was water with 0.1% formic acid and Eluent B was acetonitrile. MS analyses were carried out on a Waters XEVO TQ-S tandem quadrupole mass spectrometer (Micromass, Manchester, UK) with an electro spray interface (ESI) in positive mode (Wessling et al., 2018). The quantitative analysis by LC–MS was realized in the range of 0.03–100 ng/µl for cortisol and testosterone and 0.5–100 ng/µl for DHEA (Hauser et al., 2008a,b). One sample was excluded due to internal standard loss of > 80%. For all other samples (N = 61), internal standard loss was < 45%. For each sample analyzed, hormone concentrations were corrected for individual losses. We examined LC–MS data with MassLynx (Version 4.1; QuanLynx-Software).

2.4.3. EIA

Immunoreactive urinary cortisol ($iuCort_{EIA}$) concentrations were determined in unprocessed diluted urine by microtiter plate enzyme immunoassay using an antiserum against cortisol-3-CMO-BSA and biotinylated cortisol as enzyme conjugate (Palme and Möstl, 1997). Prior to analysis, samples were diluted 1:100 to 1:12,800 (to ensure concentrations fell in the linear range of the standard curve) in assay buffer and duplicate 50 µl aliquots of diluted samples and cortisol standard (50 µl, 0.6–40 pg/50 µl) were combined with labelled cortisol (50 µl) and antiserum (50 µl) and incubated overnight at 4 °C. After incubation, the plates were washed four times (plate washer model 405 TS, BioTek Instruments GmbH; Bad Friedrichshall, Germany), 150 µl (667 ng) of streptavidin-peroxidase (S5512; Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) in assay buffer was added to each well and the plates incubated at room temperature in the dark for 60 min and then washed again four times. TMB substrate solution (100 µl; 1-Step Ultra TMB, Thermo Fisher Scientific Inc., Rockford, USA) was subsequently added and the plates incubated at room temperature in the dark for another 45–60 min. The enzyme reaction was finally stopped by adding 50 µl of 2 M H_2SO_4 to each well and absorbance measured at 450 nm (reference 630 nm) in a plate spectrophotometer (EL 808, BioTek Instruments GmbH; Bad Friedrichshall, Germany). Cross-reactivity of the antibody is given in Palme and Möstl (1997). Serial dilutions of samples showed displacement curves that run parallel to the respective standard curve. Assay sensitivity at 90% binding was 0.6 pg. Intra-assay coefficients of variation (CV) of high and low value quality controls (pool samples of standard preparations) were 5.8% (high) and 7.7% (low) while respective figures for inter-assay CVs were 7.4% (high, N = 6 assays) and 6.2% (low, N = 6 assays).

For measurement of immunoreactive urinary testosterone (iuT_{EIA}) in all sample types (diluted unprocessed urine samples, and samples following extraction, and hydrolysis), we generally applied a testosterone EIA (T-EIA I) using an antiserum that was purchased from Rupert Palme (University of Veterinary Medicine, Vienna, Austria; Palme and Möstl, 1994). In addition, a subset of samples was also measured with a second testosterone EIA (T-EIA II) using an antiserum (R156/7) purchased from late Coralie Munro (Clinical Endocrinology Laboratory, UC Davis, USA). The latter was done in order to examine whether different EIAs designed for the measurement of testosterone would generally provide the same or different results when applied to the measurement of testosterone metabolites in the urine of Barbary macaques. Antibodies were both raised in rabbits against testosterone-3-CMO-BSA (T-EIA I) and testosterone-6-CMO-BSA (T-EIA II). For the assay, in brief, 50 µl aliquots of diluted samples (1:20–1:2500; to ensure concentrations fell in the linear range of the standard curve) and testosterone standard (50 µl, 0.31–20 pg/50 µl) were combined with HRP-labelled testosterone (50 µl) and antiserum (50 µl) and incubated overnight at 4 °C. After incubation, the plates were washed four times, after which TMB substrate solution (100 µl; 1-Step Ultra TMB, Thermo Fisher Scientific Inc., Rockford, USA) was added and the plates incubated at room temperature in the dark for another 45–60 min. The enzyme reaction was finally stopped by adding 50 µl of 2 M H_2SO_4 to each well and absorbance measured at 450 nm (reference 630 nm) in a spectrophotometer (see above).

Cross-reactivities for T-EIA I were reported in Palme and Möstl (1994), while those for T-EIA II were reported in Kersey et al. (2010). Serial dilutions of samples of both diluted neat urine and urine following hydrolysis and extraction showed displacement curves that run parallel to the respective standard curve in both assays. Assay sensitivities at 90% binding were 0.3 pg for both EIAs. Intra-assay coefficients of variation (CV) of high and low value quality controls (pool samples of standard preparations) were < 10% in both assays. Respective inter-assay CVs were 9.0% (high, N = 10 assays) and 10.7% (low, N = 10 assays) for T-EIA I and 8.5% (high, N = 3 assays) and 14.5% (low, N = 3 assays) for T-EIA II.

Urinary steroid concentrations (from both EIA and LC–MS) were

corrected for creatinine, measured as described by Bahr et al. (2000), to account for differences in urine concentration and are expressed as ng/mg creatinine (ng/mg Cr).

In this paper, we refer to the urinary cortisol, testosterone and DHEA measurements by LC–MS as $uCort_{LC–MS}$, $uT_{LC–MS}$ and $uDHEA_{LC–MS}$, respectively. By contrast, we refer to immunoreactive urinary cortisol and testosterone measurements by EIA as $iuCort_{EIA}$ and iuT_{EIA}, respectively. We would like to emphasize the fact that while LC–MS accurately quantifies the levels of each hormone in urine, the measurements from EIA are influenced by potential cross-reactivity of the antibody with other compounds.

2.5. Statistical analysis

All analyses were conducted in R statistical software version 3.5.1 (R Core Team, 2018). For all analyses we used non-parametric tests and computed exact p-values where appropriate (Mundry and Fischer, 1998).

To compare hormone levels measured by EIA and LC–MS and to detect any potential cross-reactivity in the EIAs used, we correlated EIA and LC–MS results using a Spearman's rank correlation. We used a bootstrapping procedure to avoid pseudo-replication due to having multiple samples from each individual. Using an R script, we randomly selected one urine sample from each male and calculated a correlation coefficient across all males. We repeated this procedure 1000 times, saving all correlation coefficients. If we only had one sample for an individual, then this sample was re-used in all correlations. To avoid the issue of multiple testing we did not calculate p-values. Instead, to infer significance of the correlations, we calculated 95% confidence interval of all correlation coefficients and if the interval did not include 0, we deemed the correlation to be significant. We report the mean correlation coefficient (rho) from all correlations.

To compare testosterone levels between adult and immature males, we used a Mann-Whitney *U* test. Mean hormone levels per individual were used when we had multiple samples from the same individual. The significance value was set to $p < 0.05$.

3. Results

The pattern of conjugation of cortisol and testosterone, as revealed by LC–MS, was very similar between adult and immature males. Both steroids were primarily excreted as glucuronides with sulfates making up only a small portion (Fig. 1). For cortisol, a substantial proportion was also excreted unconjugated, i.e., in free form, while free testosterone made up only a small portion of the total amount of urinary testosterone (Fig. 1).

3.1. Steroid levels in urine

For both age classes, $uCort_{LC–MS}$ was the most abundant, followed by $uDHEA_{LC–MS}$, and then $uT_{LC–MS}$ (Fig. 2).

Absolute concentrations of $iuCort_{EIA}$ and iuT_{EIA} were higher than $uCort_{LC–MS}$ and $uT_{LC–MS}$, respectively (cortisol adults: 2 times, cortisol immature: 3 times; testosterone adults EIA I: 4 times and EIA II: 5 times, immature EIA I: 19 times, and EIA II: 18 times; Fig. 2). Absolute concentrations of iuT_{EIA-I} and iuT_{EIA-II} were very similar in both adult and immature males (Fig. 2).

3.2. Correlations between urinary steroids measured by LC–MS and EIA

We found that $iuCort_{EIA}$ as measured from *unprocessed* urine correlated strongly, positively and significantly with $uCort_{LC–MS}$ in both adult and immature males (Tables 1 and 2). This suggests that $iuCort_{EIA}$ measurements accurately reflect urinary cortisol levels.

We found that iuT_{EIA-I} and iuT_{EIA-II} as measured from *unprocessed* urine did not correlate significantly with $uT_{LC–MS}$ in neither adult nor immature males (Tables 1 and 2). This suggests that iuT_{EIA} measurements from *unprocessed* urine do not accurately reflect urinary testosterone levels. Both iuT_{EIA-I} and iuT_{EIA-II} as measured from *hydrolyzed* urine correlated strongly, positively and significantly with $uT_{LC–MS}$ in adult but not in immature males (Tables 1 and 2). This suggests that iuT_{EIA} measurements from hydrolyzed urine do accurately reflect urinary testosterone levels in adult but not in immature males.

To determine if the cross-reactivity of the cortisol-antibody with testosterone or DHEA were influencing results, we correlated $iuCort_{EIA}$ with $uT_{LC–MS}$ and $uDHEA_{LC–MS}$. We found that $iuCort_{EIA}$ did not correlate significantly with $uT_{LC–MS}$, in neither adult nor immature males (Tables 1 and 2). This suggests that cross reaction of the cortisol antibody with urinary testosterone did not have a strong impact on results. However, $iuCort_{EIA}$ did correlate significantly with $uDHEA_{LC–MS}$ in both adult and immature males. To tease apart whether this is a cross-reactivity issue or a true correlation between both adrenal steroids, we also correlated $uCort_{LC–MS}$ with $uDHEA_{LC–MS}$ and again found a significant positive correlation in both adult and immature males (Bootstrapped Spearman rank correlations: adult: N = 13, rho = 0.40, 95% CI = [0.10, 0.68]; immature: N = 8, rho = 0.70, 95% CI = [0.24, 0.68]). Taken together, this suggests that the cross-reactivity of the cortisol-antibody with DHEA does not significantly impact $iuCort_{EIA}$ measurements, but rather that both cortisol and DHEA reflect adrenocortical activity in a similar manner.

To determine if the cross-reactivity of the T-antibodies of EIA I and EIA II with cortisol or DHEA were influencing results, we correlated iuT_{EIA-I} and iuT_{EIA-II} with $uCort_{LC–MS}$ and $uDHEA_{LC–MS}$. Both iuT_{EIA-I} and iuT_{EIA-II} as measured from *unprocessed* urine correlated significantly and positively with $uCort_{LC–MS}$ and $uDHEA_{LC–MS}$ in both adult and immature males (Tables 1 and 2). This suggests that cross-reactivity of both T-antibodies with urinary cortisol and DHEA confound iuT_{EIA} measurements from *unprocessed* urine. By contrast, iuT_{EIA-I} as measured from *hydrolyzed* urine did not correlate significantly with $uCort_{LC–MS}$ or $uDHEA_{LC–MS}$ in neither adult nor immature males (Tables 1 and 2). This suggests that the cross-reactivity of the T-antibody of EIA I with urinary cortisol or DHEA does not significantly influence iuT_{EIA-I} measurements in neither adult nor immature males when *hydrolyzed* urine is used. Similarly, iuT_{EIA-II} as measured from *hydrolyzed* urine also did not correlate significantly with $uCort_{LC–MS}$ or $uDHEA_{LC–MS}$ in adult males (Table 1). However, it correlated significantly and positively with $uCort_{LC–MS}$ and $uDHEA_{LC–MS}$ in immature males (Table 2). This suggests that the cross-reactivity of the T-antibody of EIA II with urinary cortisol or DHEA does not significantly influence iuT_{EIA-II} measurements in adult males but does so in immature males when *hydrolyzed* urine is used.

3.3. Comparison of testosterone levels between age classes

We found that $uT_{LC–MS}$ levels were significantly higher in adult males than immature males (Mann-Whitney *U* test: N adults = 13, N immatures = 8, U = 6, $p < 0.001$, mean adults = 5.7 ± SD 7.3 ng/mg Cr, mean immatures = 0.9 ± SD 0.7 ng/mg Cr). However, this was not the case for iuT_{EIA} measured from *hydrolyzed* samples (T-EIA I: N adults = 13, N immatures = 8, U = 34, p = 0.210, mean adults = 22.2 ± SD 12.5 ng/mg Cr, mean immatures = 16.9 ± SD 5.1 ng/mg Cr; T-EIA II: N adults = 10, N immatures = 8, U = 19, p = 0.068, mean adults = 27.5 ± SD 17.1 ng/mg Cr, mean immatures = 15.9 ± SD 5.8 ng/mg Cr).

4. Discussion

In this study, we validated the use of cortisol and testosterone EIAs in the urine of adult and immature male Barbary macaques by comparing results to those from LC–MS measurements. We found that $iuCort_{EIA}$ measurements from unprocessed urine correlated strongly with $uCort_{LC–MS}$ in both adult and immature males. This indicates that

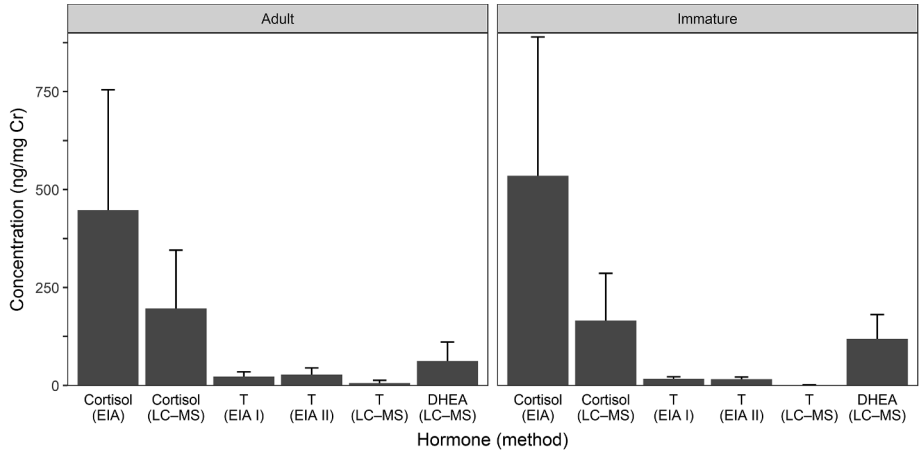

Fig. 2. Mean urinary hormone levels as measured by LC–MS and EIA. N = 13 adult males (except T-EIA II, N = 10), N = 8 immature males. Error bars indicate standard deviation. Cortisol-EIA used unprocessed urine. Testosterone-EIA used hydrolyzed urine. LC–MS used hydrolyzed and solvolyzed urine.

Table 1
Bootstrapped Spearman rank correlations (rho) between steroids measured by EIA and LC–MS in adult males. Significant correlations (95% Confidence Interval (CI) does not include 0) in bold.

				95% CI	
EIA	LC–MS	N	rho	Lower	Upper
Cortisol (unprocessed)	**Cortisol**	**13**	**0.95**	**0.90**	**0.99**
	Testosterone	13	−0.02	−0.35	0.30
	DHEA	**13**	**0.54**	**0.31**	**0.73**
Testosterone (unprocessed, EIA I)	**Cortisol**	**13**	**0.69**	**0.49**	**0.83**
	Testosterone	13	0.12	−0.30	0.50
	DHEA	**13**	**0.76**	**0.58**	**0.90**
Testosterone (unprocessed, EIA II)	**Cortisol**	**9**	**0.35**	**0.12**	**0.58**
	Testosterone	9	−0.09	−0.57	0.40
	DHEA	**9**	**0.70**	**0.57**	**0.87**
Testosterone (hydrolyzed, EIA I)	Cortisol	13	0.25	−0.18	0.66
	Testosterone	**13**	**0.78**	**0.51**	**0.95**
	DHEA	13	0.23	−0.10	0.52
Testosterone (hydrolyzed, EIA II)	Cortisol	10	−0.03	−0.37	0.47
	Testosterone	**10**	**0.76**	**0.45**	**0.95**
	DHEA	10	0.37	−0.04	0.64

Table 2
Bootstrapped Spearman rank correlations (rho) between steroids measured by EIA and LC–MS in immature males. Significant correlations (95% Confidence Interval (CI) does not include 0) in bold.

				95% CI	
EIA	LC–MS	N	rho	Lower	Upper
Cortisol (unprocessed)	**Cortisol**	**8**	**0.88**	**0.71**	**1.00**
	Testosterone	8	−0.03	−0.52	0.36
	DHEA	**8**	**0.90**	**0.64**	**1.00**
Testosterone (unprocessed, EIA I)	**Cortisol**	**8**	**0.73**	**0.43**	**0.93**
	Testosterone	8	0.22	−0.33	0.64
	DHEA	**8**	**0.83**	**0.67**	**0.95**
Testosterone (unprocessed, EIA II)	**Cortisol**	**7**	**0.87**	**0.71**	**1.00**
	Testosterone	7	−0.12	−0.68	0.39
	DHEA	**7**	**0.85**	**0.64**	**0.96**
Testosterone (hydrolyzed, EIA I)	Cortisol	8	0.52	−0.14	0.93
	Testosterone	8	0.36	−0.36	0.88
	DHEA	8	0.36	−0.26	0.83
Testosterone (hydrolyzed, EIA II)	**Cortisol**	**8**	**0.83**	**0.62**	**0.98**
	Testosterone	8	−0.04	−0.62	0.60
	DHEA	**8**	**0.90**	**0.83**	**0.93**

the direct measurement of cortisol by EIA is a reliable method for assessing adrenocortical activity in adult and immature male Barbary macaques. By contrast, iuT_{EIA} measurements only correlated with $uT_{LC–MS}$ if steroids in the urine were enzymatically hydrolyzed prior to analysis and only in adult males. In immature males, even iuT_{EIA} measurements from hydrolyzed urine did not correlate significantly with $uT_{LC–MS}$. This lack of correlation is likely due to cross-reactivity of the testosterone antibody with other adrenal steroids such as cortisol and DHEA, which are present at much higher concentrations relative to testosterone in immature males. Furthermore, $uT_{LC–MS}$ – but not iuT_{EIA} – levels were significantly higher in adult than immature males, biologically validating the measurement of testosterone by LC–MS but not by EIA. Taken together, our results suggest that the testosterone EIAs are suitable to assess gonadal endocrine activity from urine analysis in adult but not immature male Barbary macaques, and only if a hydrolysis is conducted prior to analysis.

4.1. Pattern of conjugation of cortisol and testosterone

Both cortisol and testosterone were primarily excreted as glucuronide conjugates and only a small proportion was excreted as sulfates. Cortisol was also excreted in substantial quantities in its free (i.e. unconjugated) form, but testosterone was not. This is consistent with other studies that found that these steroids are primarily excreted in conjugated form, and mainly as glucuronides, in the urine of primate (Bahr et al., 2000; Möhle et al., 2002; Ziegler et al., 2000) and non-primate species (Teskey-Gerstl et al., 2000).

4.2. Steroid levels in urine

We found that both $iuCort_{EIA}$ and iuT_{EIA} levels were significantly higher than $uCort_{LC–MS}$ and $uT_{LC–MS}$, respectively, a result consistent with findings from other studies that have compared levels of these two steroids in males, as measured from immunoassays and LC–MS (Baid et al., 2007; Hsing et al., 2007; Preis et al., 2011; Welker et al., 2016). Indeed, these “inflation” in absolute hormone concentration as measured by EIA is not surprising given that, in contrast to LC–MS measurements, immunological hormone measurements are generally non-specific as they are influenced by cross-reactivity characteristics of the antibody used. In line with this argument, the difference between EIA and LC–MS measurements is larger in urine samples than in blood samples (Preis et al., 2011). As previously mentioned, in blood, steroids primarily circulate in their native, unconjugated form at much higher proportions than in urine so the impact of cross-reactivity of the antibody is lower in blood samples than in urine (Preis et al., 2011; Venturelli et al., 1995). It is important to note that the cross-reactivity of the antibody in immunoassays are not necessarily disadvantageous. As long as the cross-reacting metabolites originate from the same parent hormone, this may actually be advantageous as such so-called group-

specific assays may have a higher sensitivity to detect a biologically meaningful change in hormone levels (e.g. Heistermann et al., 2006; Shutt et al., 2012).

4.3. Correlations between urinary cortisol measured by EIA and urinary steroids measured by LC–MS

We compared cortisol EIA and LC–MS results to determine if the EIA measurements truly reflect urinary cortisol levels and to determine if cross-reactivities with testosterone or DHEA were confounding results. We found that $iuCort_{EIA}$ measured from unprocessed urine correlated strongly and significantly with $uCort_{LC–MS}$ for both adult and immature males. This strong correlation suggests that the $iuCort_{EIA}$ measurements accurately reflect urinary cortisol levels and was achieved without the need to perform deconjugation steps in the urine samples prior to analysis by EIA. It is likely that cortisol was excreted in sufficiently high quantities in its unconjugated form to be measured by the EIA, despite the fact the most of it was excreted as glucuronides. Alternatively, but not mutually exclusive, our cortisol antibody may potentially also cross-react with and measure cortisol in its conjugated forms. The markedly higher absolute levels of cortisol measured by EIA compared to LC–MS (see above) would be in line with such an assumption. Since the deconjugation step is time-consuming and costly, it is worthwhile to check if this analytical step can be avoided prior to any urinary hormone analysis (like in this study) to still produce reliable results.

Cross-reactivities of our cortisol-antibody with urinary testosterone and DHEA were negligible. We found that $iuCort_{EIA}$ did not correlate significantly with $uT_{LC–MS}$ in neither adult nor immature males but did have a significant positive correlation with $uDHEA_{LC–MS}$ in both adult and immature males. Rather than this correlation being caused by a cross-reactivity problem, our results indicate that the correlation was caused by simultaneous secretion as $uCort_{LC–MS}$ and $uDHEA_{LC–MS}$ were also significantly correlated. The strength of the correlation between $uCort_{LC–MS}$ (or also $iuCort_{EIA}$) and $uDHEA_{LC–MS}$ was stronger in immature than adult males, possibly indicating a stronger interrelationship between these two adrenal steroids in immature compared to adult males.

Generally, urinary cortisol (or corticosterone) analysis is often used as a measure of adrenocortical activity in various species of mammals (Aronsen et al., 2015; Brown et al., 2010; McCallister et al., 2004; McLeod et al., 1996; Schmid et al., 2001; Touma et al., 2003). In this respect, the cortisol-EIA tested in this study has been shown to successfully pick up the urinary cortisol response to an ACTH challenge in African elephants (Ganswindt et al., 2003). It has also been demonstrated to measure urinary cortisol levels in other nonhuman primates (Bahr et al., 2000), indicating its suitability to non-invasively assess cortisol secretion in various mammalian species. Therefore, we envisage that the measure of urinary cortisol in unprocessed urine as described here is likely suitable for tracking adrenocortical activity in male Barbary macaques, although, to support these results, an accompanying physiological or biological validation would be desirable.

4.4. Correlations between urinary testosterone measured by EIA and urinary steroids measured by LC–MS

We compared two testosterone EIAs with LC–MS results to determine if the EIA measurements truly reflect urinary testosterone levels and to determine if cross-reactivity of the antibodies with steroids of adrenal origin, such as cortisol or DHEA, were potentially confounding results. Both iuT_{EIA-I} and iuT_{EIA-II} measurements from unprocessed urine did not correlate significantly with $uT_{LC–MS}$ in neither adult nor immature males. Since > 80% of testosterone was excreted as glucuronide conjugate, we assessed whether enzymatic hydrolysis would result in stronger correlations between our T-EIA and LC–MS measurements. Indeed, this extra analytical step largely improved the correlations between the two data sets so that both iuT_{EIA-I} and iuT_{EIA-II} measurements from hydrolyzed urine now correlated strongly with $uT_{LC–MS}$ in adult males but not immature males. It is likely that cross-reactivity of the testosterone antibodies with cortisol and DHEA, and probably also other adrenal steroids, are confounding the results generated from unprocessed urine in adult and immature males and from hydrolyzed urine in immature males. This is supported by the fact that $uCort_{LC–MS}$ and $uDHEA_{LC–MS}$ were both positively correlated with iuT_{EIA-I} and iuT_{EIA-II} when measured from unprocessed urine in both age classes, and with iuT_{EIA-II} – but not iuT_{EIA-I} – when measured from hydrolyzed urine in immature males.

Obviously, the cross-reactivities of the antibodies in the T-EIAs with other metabolites are constant and do not change whether being used with unprocessed or hydrolyzed urine. The observed changes in the strength of correlations with LC–MS measurements are caused by the differences in the ratio of unconjugated testosterone to cross-reacting adrenal steroids. For instance, adult males have $uCort_{LC–MS}$ and $uDHEA_{LC–MS}$ concentrations approximately 35 and 11 times higher than $uT_{LC–MS}$, respectively (Fig. 2). By contrast, immature males have approximately 186 and 134 times higher $uCort_{LC–MS}$ and $uDHEA_{LC–MS}$ levels than $uT_{LC–MS}$, respectively (Fig. 2). In both T-EIAs, the cross-reactivity of the antibody with cortisol and DHEA is < 0.1% (Kersey et al., 2010; Palme and Möstl, 1994). Thus, < 4% and < 1% of the EIA results from hydrolyzed urine can be attributed to cross-reactivity with cortisol and DHEA, respectively, in adult males. This relatively low level of co-measurement of the two adrenal hormones does not obviously confound the T-EIA measurement in adult males as evidenced by the strong positive correlations that both iuT_{EIA-I} and iuT_{EIA-II} have with $uT_{LC–MS}$ when measured from hydrolyzed urine samples. However, in immature males the picture looks different. Around 19% and 13% of the T-EIA results from hydrolyzed urine can be attributed to cross-reactivity with cortisol and DHEA, respectively, a proportion sufficiently large enough to confound testosterone levels measured by EIA in hydrolyzed urine of immature males. Since the vast majority of testosterone was excreted as testosterone glucuronide in male Barbary macaque urine (Fig. 1), the absolute concentration of unconjugated testosterone in unprocessed urine was apparently low enough that even in adult males, cross-reactivity with other compounds became a problem. Similar to our results, testosterone immunoassays that have been validated for use in males did not produce reliable results in females, likely due to lower concentration of testosterone and higher proportion of cross-reacting adrenal steroids in females (Goymann, 2005; Preis et al., 2011).

Importantly, cortisol and DHEA are only two examples of hormones coming from a non-gonadal, i.e. the adrenal pathway, whose cross-reaction with the testosterone antibody, potentially affects the measurements of urinary testosterone with the tested T-EIAs. However, there are many other candidate urinary steroid metabolites with a similar potential. For example, one study in chimpanzees found that just among the limited number of potential glucocorticoid and androgen metabolites measured, there were 10 steroids present in the urine of males at higher concentrations than testosterone (Hauser et al., 2008a). This potentially leads to a situation where the overall sum of cross-reactivities with all these metabolites, even if the cross-reactivity with each one is very small, will have a much bigger effect than what we describe in this study. This danger is more and more prominent the lower the ratio of the target hormone to confounding metabolites. Concordantly, cortisol, as the most abundant steroid measured in this study, is the least affected by this problem and produced the strongest correlations between EIA and LC–MS measurements. As we show in this study, one approach to mitigate the impact of antibody cross-reactivity in urine samples is to increase the ratio of the target hormone through deconjugation.

4.5. Comparison of testosterone levels between age classes

To assess whether our EIA and LC–MS measurements were able to

capture biologically meaningful differences in hormone levels, we compared testosterone and DHEA levels of adult and immature males. As predicted, uT_{LC-MS} was significantly higher in adult males compared to immature males, biologically validating the measurement of testosterone by LC–MS in male Barbary macaques. However, there was no significant difference in iuT_{EIA} levels between age classes as measured by either EIAs from hydrolyzed urine. As discussed above, this is likely because in immature males, a substantial co-measurement of adrenal steroids in both T-EIAs increases the concentrations of immunoreactive testosterone and thereby obscures the existing difference in "real" testosterone levels between the two age classes. Similarly to our results, others have found that a testosterone antibody cross-reacting with DHEA metabolites has masked the predicted difference in testosterone levels between adult males and females or castrated males (Goymann, 2005; Möhle et al., 2002). Taken together, our results suggest that measuring testosterone with either EIA from hydrolyzed urine is suitable to assess gonadal endocrine activity in adult but not immature male Barbary macaques. This study highlights that even when a biological validation of a testosterone EIA fails, the tested EIA might still be suitable for specific age and sex classes. At the same time, just because an EIA reliably measures testosterone in one age sex class, e.g. adult males, does not necessarily make it suitable for all other age sex classes. By not only comparing EIA measurements across age sex classes but also correlating EIA with LC–MS measurements we were able to determine in which age class results were reliable and in which they were unreliable.

5. Conclusions

Combining LC–MS and EIA approaches, we identified two main issues to consider when measuring steroid hormones from urine samples using immunoassays: pattern of conjugation and cross-reactivities of the antibody. Both the pattern of conjugation and cross-reactivity are most likely to confound results when the absolute concentration of the target hormone is low relative to other cross-reacting metabolites. As cortisol was excreted in sufficiently high concentrations in its native form in our study subjects, results from EIA and LC–MS were strongly correlated in both adult and immature males, making deconjugation steps unnecessary. Deconjugation of urinary steroid hormones can also be avoided if the antibodies used have a substantial cross-reactivity with the conjugated forms (Al-Dujaili, 2006; Eastman et al., 1984; Heistermann and Hodges, 1995). Testosterone was excreted in urine in much lower concentrations than cortisol and a higher proportion of it was conjugated, primarily as glucuronides, necessitating hydrolysis of steroids to ensure that a strong correlation of EIA compared to LC–MS results was obtained in adult male samples. However, even after hydrolysis, EIA measurements of testosterone in immature male urine samples were still not reliable, most likely due to cross-reactivity with adrenal steroids, which occur at much higher concentrations than testosterone in immature males. Thus, to generate data on gonadal endocrine activity in immature Barbary macaques, LC–MS analysis may be the most reliable approach. We envisage that immunological assays may potentially also be applied successfully on immature samples if highly specific testosterone (metabolite) antibodies with almost no cross-reactivities with steroids of adrenal origin are available. However, given the generally low production of testosterone in immature males, it is possible that most immunoreactive androgens in the urine of immatures are breakdown products of androgens of adrenal origin. To confirm that urinary measurements of androgens in immature males (and possibly also females), whether done with LC–MS or EIA, are indeed stemming from serum testosterone of gonadal origin, requires a comparison between serum and urine levels. Measurement of fecal testosterone metabolites may provide an alternative to urinary measurements (Rincon et al., 2017), although similar validation work as described here for urine analysis is necessary to firmly confirm this. Taken together, both testosterone EIAs can be used in studies assessing gonadal activity in adult but not immature male Barbary macaques. Even within the same sex, age-related changes in androgen production can cause the same immunoassay to be reliable in one but not another age class. Given differences between steroids in terms of conjugation patterns and absolute levels, preparatory steps in terms of deconjugation and extraction will vary between different EIAs and hormones.

Declaration of Competing Interest

None.

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