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Measuring fecal testosterone in females and fecal estrogens in males: Comparison of RIA and LC/MS/MS methods for wild baboons (*Papio cynocephalus*)

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ABSTRACT

The development of non-invasive methods, particularly fecal determination, has made possible the assessment of hormone concentrations in wild animal populations. However, measuring fecal metabolites needs careful validation for each species and for each sex. We investigated whether radioimmunoassays (RIAs) previously used to measure fecal testosterone (fT) in male baboons and fecal estrogens (fE) in female baboons were well suited to measure these hormones in the opposite sex. We compared fE and fT concentrations determined by RIA to those measured by liquid chromatography combined with triple quadropole mass spectrometry (LC/MS/MS), a highly specific method. Additionally, we conducted a biological validation to assure that the measurements of fecal concentrations reflected physiological levels of the hormone of interest. Several tests produced expected results that led us to conclude that our RIAs can reliably measure fT and fE in both sexes, and that within-sex comparisons of these measures are valid: (i) fT_{RIA} were significantly correlated to $fT_{LC/MS/MS}$ for both sexes; (ii) fT_{RIA} were higher in adult than in immature males; (iii) fT_{RIA} were higher in pregnant than non-pregnant females; (iv) fE_{RIA} were correlated with 17β-estradiol (fE_2) and with estrone (fE_1) determined by LC/MS/MS in pregnant females; (v) fE_{RIA} were significantly correlated with fE_2 in non-pregnant females and nearly significantly correlated in males; (vi) fE_{RIA} were higher in adult males than in immature males. fE_{RIA} were higher in females than in males, as predicted, but unexpectedly, fT_{RIA} were higher in females than in males, suggesting a difference in steroid metabolism in the two sexes; consequently, we conclude that while within-sex comparisons are valid, fT_{RIA} should not be used for intersexual comparisons. Our results should open the field to important additional studies, as to date the roles of testosterone in females and estrogens in males have been little investigated.

1. Introduction

Testosterone (T) has traditionally been viewed as a male hormone and estrogens (E) as female hormones, even though T and E are present in both sexes. However, interest has been building in recent years about possible roles of T in females and E in males. For example, T has been postulated to play an important role in female sexual behavior (Davis et al., 1995; Sherwin and Gelfand, 1987; Shifren et al., 2000), as well as in aggression in a variety of animal species (Beehner et al., 2005; Dabbs et al., 1988; Gill et al., 2007; Glickman et al., 1992; Rosvall, 2013; von Engelhardt et al., 2000; Woodley and Moore, 1999). The presence of androgen receptors in female neural and peripheral tissues suggests that the effect of T on female behavior extend beyond the conversion of T into E (Staub and DeBeer, 1997). T may also have an important role in women's health, particularly in bone formation and bone mineral density (Davis et al., 1995; Raisz et al., 1996). Similarly, E has been shown to have an important role in male sexual function

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and behavior in several vertebrate species (Kacker et al., 2012). E may also have important health consequences in men, especially for bone density and for lipid and glucose metabolism (Barrett-Connor et al., 2000; Cohen, 2008; Gillberg et al., 1999; Khosla et al., 2001; Oettel, 2002; Tomaszewski et al., 2009).

Measuring hormone concentrations in wild animal populations has become increasingly feasible in recent decades with the development of non-invasive methods such as fecal determination (Touma and Palme, 2005; Whitten et al., 1998). This method presents the advantage of not interfering with the animal's behavior and allowing for repeated sampling of the same animal. Due to its integrative nature, fecal determination provides a measurement of hormone concentrations over a longer period of time than samples from blood, which vary considerably with time of day (Touma and Palme, 2005). However, one pitfall of steroid hormone determination in feces is that the circulating hormone is degraded to several metabolites, and usually only a small quantity is present in its original form (Heistermann et al., 2006). Therefore, when using fecal hormone determination it is essential to validate the immunoassay, and verify that the chosen antibody only cross-reacts with metabolites of the original hormone and not with metabolites of other hormones (Goymann, 2005; Heistermann et al., 2006).

Traditionally, three methods have been used to verify that the level of the hormone measured is biologically relevant. The first one consists of injecting the animal with radiolabeled hormone, measuring its metabolic products in feces by high-performance liquid chromatography, and verifying that the major radioactive peaks found by this method exhibit strong immunoreactivity with the antibody used (Goymann et al., 2002; Möhle et al., 2002; Möstl et al., 2005; Palme et al., 2005; Touma et al., 2003; Wasser et al., 1994, 2000). The second method involves stimulating or suppressing the circulating hormone pharmacologically (using for example adrenocorticotropic hormone or gonadotropin-releasing hormone to stimulate glucocorticoid or testosterone production, respectively), and then verifying that the metabolites measured in feces reflect that stimulation or suppression (Möstl et al., 2005; Touma and Palme, 2005; Wasser et al., 2000). The third method consists of verifying that fecal hormone concentrations follow physiological patterns previously determined in blood (e.g., elevated T concentrations during the breeding season, cyclic variation of E and progesterone (P) during the menstrual cycle, elevated E and P concentrations during pregnancy) (Goymann, 2005; Hirschenhauser et al., 2005).

The metabolism and route of excretion of fecal steroids vary considerably among species, and previous studies have emphasized the necessity of a separate validation for each species (Goymann, 2005; Heistermann et al., 2006; Hirschenhauser et al., 2012; Möhle et al., 2002). A recent review by Goymann (2012) also underlines that excretion route and metabolite types may vary between the sexes (see also: Goymann, 2005; Hirschenhauser et al., 2012; Palme et al., 2005; Touma and Palme, 2005; Touma et al., 2003). For example, Touma et al. (2003) reported that in mice, the percentage of GC metabolites recovered in feces was greater for males than females (73% and 53%, respectively). In contrast another study by Dantzer et al. (2011a,b) reported that in red squirrels, while the percentage of T metabolites excreted in feces was slightly lower for males than females (44% and 56%, respectively), these differences did not reach significance. The type of GC and T metabolites excreted in feces was also shown to be different for each sex (Dantzer et al., 2011a,b; Goymann, 2005; Touma et al., 2003). Because the antibody used in an immunoassay will only recognize some of these metabolites, this could lead to a disconnect between concentrations of excreted hormones and serum levels (Goymann, 2012). Also, Möhle et al. (2002) showed that two hormones of different origin, T (gonadal) and DHEA (adrenal), can be degraded in similar metabolites when excreted in feces and urine. When a T antibody cross-reacts with metabolites that are common to both hormones, this could lead to an apparent absence of difference between male and female T levels, as shown for several species of primates and birds (Dittami et al., 2008; Goymann, 2005; Möhle et al., 2002).

In this study, we investigated whether hormone specific radioimmunoassays (RIAs) previously validated in baboons (Khan et al., 2002; Lynch et al., 2003), and used to measure fecal testosterone (fT) in males (Beehner et al., 2009; Gesquiere et al., 2011) and fecal estrogens (fE) in females (Gesquiere et al., 2005, 2007) are well suited to measure fT in females and fE in males. We compared fE and fT concentrations determined by RIA to those determined by high-pressure liquid chromatography combined with triple quadrupole mass spectrometry (LC/MS/MS). In contrast to RIA, where the antibody can cross-react with several metabolites, LC/MS/MS is a highly specific method that measures the mass to charge ratio of individual steroids (Hauser et al., 2008). We also compared fE and fT concentrations between the sexes, and across reproductive state (females) or age (males) to verify that the hormone concentrations measured in the feces reflect physiological levels.

2. Methods

2.1. Field site and subjects

The males and females in the present study were the individually identified members of five social groups in the Amboseli baboon population that have been monitored for reproductive, demographic, and behavioral events on a near-daily basis for over four decades (e.g., Alberts and Altmann, 2012; Gesquiere et al., 2007; see www.amboselibaboons.nd.edu for a complete bibliography and the Baboon Project Monitoring Guide, which outlines the data collection protocols). Since December 1999, physiological data have been obtained through non-invasive collection of freshly deposited feces from known individuals and subsequent analysis of steroid hormone metabolites extracted from the feces.

All data collection procedures were non-invasive, and adhered to the laws and guidelines of Kenya (Research Permit MOEST 13/001/C351 Vol. II) and were approved by the Animal Care and Use Committee at Princeton University (IACUC 1821) and at Duke University (IACUC A028-12-02).

2.2. Demographic and reproductive data

Age was known for all females and immature males and for all adult males born in study groups. For immigrant males, age was estimated based on coat condition, degree of scarring, body carriage (e.g., degree of straightness versus curvature of the spine, how the head is carried, etc.), and canine tooth condition when they first joined the study population (see Alberts and Altmann, 1995 for details).

Female sexual swelling state (turgescent or deturgescent) and size, presence of menstrual blood, and the color of the paracallosal skin, were recorded for all adult females in a group each time an observer was with that group (Altmann, 1970; Gesquiere et al., 2007; see also the Amboseli Baboon Research Project (ABRP) Monitoring Guide at www.amboselibaboons.nd.edu for details on data collection protocols). Female reproductive state was assigned subsequently based on these records. Menstrual cycles in female baboon are easy to identify by the successive turgescence (follicular phase) and deturgescence (luteal phase) of the sexual skin. Failure to cycle after 40 days and the absence of menstrual blood usually indicates that the female is pregnant (Beehner et al., 2006). Pregnancy is then confirmed by the change of color of the