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Validation of an enzyme immunoassay for assessing adrenocortical activity and evaluation of factors that affect levels of fecal glucocorticoid metabolites in two New World primates ☆

Rebecca Rimbach [a,b,*], Eckhard W. Heymann [a], Andrés Link [b,c], Michael Heistermann [d]

[a] Behavioral Ecology and Sociobiology Unit, German Primate Center, Kellnerweg 4, D-37077 Göttingen, Germany
[b] Fundación Proyecto Primates, Cra. 11a No. 91-55, Apartamento 202, Bogotá, Colombia
[c] Laboratorio de Ecología de Bosques Tropicales y Primatología, Departamento de Ciencias Biológicas, Universidad de Los Andes, Cra. 1 No. 18a-12, Bogotá, Colombia
[d] Endocrinology Laboratory, German Primate Center, Kellnerweg 4, D-37077 Göttingen, Germany

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ABSTRACT

Non-invasive methods to assess stress hormone output via fecal glucocorticoid metabolites (FGCMs) have become a powerful tool in behavioral studies and conservation biology because they allow exploring the link between behavior, an animal's socio-ecological environment and its adrenocortical activity. However, FGCM levels are influenced by numerous other factors which often confound their interpretation. Thus, before applying these methods, knowledge on the impact of these factors is important. In this study we investigated the effect of (1) time of day, (2) age, (3) sex and (4) female reproductive state on FGCM levels in brown spider monkeys (*Ateles hybridus*) and red howler monkeys (*Alouatta seniculus*). Initially, we validated a 11β-hydroxyetiocholanolone enzyme immunoassay for monitoring the physiological stress response via fecal analysis in both species. We determined FGCM levels in fecal samples collected from two and six groups of wild spider monkeys ($n = 461$ samples) and howler monkeys ($n = 166$ samples), respectively. Our analyses revealed a strong effect of time of day on FGCM levels in spider monkeys, but no effect in howler monkeys. Adults of both species had significantly higher FGCM levels than subadults. In neither of the two species we found a sex-effect on FGCM output. Reproductive condition strongly affected FGCM levels in female spider monkeys which showed increasing concentrations with progressing gestation. This was not investigated in female howler monkeys due to an insufficient sample size. Our data indicate that the influence of the tested factors on fecal glucocorticoid metabolite output is species-specific, and that these variables need to be considered when interpreting FGCM levels in the species.

1. Introduction

In recent years, there has been a substantial increase in the number of studies that investigate the interactions between animal behavior and steroid hormone levels in vertebrates of all major taxa (e.g., (Bonier et al., 2009; Cavigelli, 1999; Engh et al., 2006; Ganswindt et al., 2003; Goymann et al., 2003; Kenagy and Place, 2000; Reeder et al., 2004)). These studies help to gain insight into the proximate factors underlying and modulating behavioral variation, life history traits, fitness, and survival of animals. Measurement of hormones is also employed in conservation research to asses and monitor the physiology, health and well-being of populations of endangered species in the wild (Chapman et al., 2006; Cyr and Romero, 2008; Franceschini et al., 1997; Hodges and Heistermann, 2003; Tarlow and Blumstein, 2007; Van Meter et al., 2009; Wikelski and Cooke, 2006; Wingfield et al., 1997) as well as to facilitate and ensure the propagation and welfare of animals in captivity (e.g., Dehnhard et al., 2008; Graham et al., 2002; Heistermann et al., 2004; Pirovino et al., 2011).

In the latter contexts, glucocorticoids (cortisol and corticosterone) have received most attention. As front hormones of the vertebrate stress response that reflect physiological stress loads of individuals and populations, they have proven as an important biomarker when assessing the physiological consequences of anthropogenic disturbances and habitat fragmentation for individual and population health (Chapman et al., 2006, 2007; Franceschini et al., 1997; Martínez-Mota et al., 2007; Rangel-Negrín et al., 2009; Thiel et al., 2011; Wasser et al., 1997; Wikelski and Cooke, 2006; Wingfield et al., 1997). Generally, glucocorticoids and their

* Corresponding author at: Behavioral Ecology and Sociobiology Unit, German Primate Center, Kellnerweg 4, D-37077 Göttingen, Germany.
E-mail addresses: rrimbach@dpz.eu (R. Rimbach), eheyman@gwdg.de (E.W. Heymann), a.link74@uniandes.edu.co (A. Link), mheistermann@dpz.eu (M. Heistermann).

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metabolites can reliably be measured in blood, urine and feces using enzyme immunoassays (EIAs) (for detailed reviews see (Romano et al., 2010; Sheriff et al., 2011)). When studying stress physiology in wildlife, however, non-invasive methodologies based on the measurement of GC metabolites (GCM) in excreta (urine, feces) is the preferred approach because blood sampling is usually not feasible (and undesirable) in wild animals. Moreover, excreted GCM levels in urine and feces provide a more integrated measure of adrenocortical activity than point serum samples and thus diminish the influence of the pulsatile and episodic patterns of GC secretion (Whitten et al., 1998a). Since excreta can also be collected much more regularly than blood, analyses of urinary and fecal hormone metabolites provide the most suitable way to obtain longitudinal information on endocrine activity.

The metabolism and excretion route of glucocorticoids can differ substantially between species, even closely related ones (Bahr et al., 2000; Palme et al., 2005). Thus, prior to applying urinary or fecal analysis, it is crucial to biologically validate the respective method for each new species to assure that the data to be generated will be biologically meaningful (Goymann, 2012; Heistermann et al., 2006; Palme et al., 2005; Schwarzenberger, 2007; Touma and Palme, 2005; Whitten et al., 1998b). In this respect, the validation should not only demonstrate that the GCM measurement reliably detects adrenocortical endocrine activity in response to a stressor, but should also evaluate the specificity of the measurement when immunological detection methods are used (Goymann, 2012; Heistermann et al., 2006). The latter is particularly important given that metabolites of glucocorticoids and other steroids (e.g., testosterone) can be structurally very similar (Ganswindt et al., 2003). Since antibodies used for the quantification of glucocorticoids can potentially cross-react with those metabolites of different origin and function (Ganswindt et al., 2003; Heistermann et al., 2006; Palme et al., 2005), such cross-reactions can have major and distorting effects on the results obtained (see Ganswindt et al., 2003; Goymann, 2012). Further, glucocorticoid metabolism can differ even between sexes within a given species (e.g., (Baltic et al., 2005; Touma et al., 2003)), making comparisons of GC levels between males and females potentially problematic and meaningless unless the immunological specificity of the assay used is demonstrated (for a detailed review see (Goymann, 2012)).

In many species basal stress hormone levels are affected by a variety of intrinsic factors (for review see (Goymann, 2012; Keay et al., 2006; Millspaugh and Washburn, 2004)) such as age (Sapolsky, 1992; Seraphin et al., 2008), sex (Raminelli et al., 2001; Sapolsky, 1992; Touma et al., 2003), reproductive state (Carnegie et al., 2011; Cavigelli, 1999; Setchell et al., 2008; Weingrill et al., 2004; Ziegler et al., 1995) and body condition (Charbonnel et al., 2008), and they also often show diurnal variation (Bosson et al., 2009; Chapman et al., 2006; Raminelli et al., 2001). All these factors may confound interpretation of GC levels generated in contexts such as behavioral studies or conservation research. Knowledge about whether and in which specific way these variables have an impact on stress hormone output in a given species is therefore of high importance when GC data is collected for such research questions.

The way and extent to which such factors influence adrenocortical activity appears to be species-specific, emphasizing the importance to assess their impact in every previously unstudied species. For example in Columbian ground squirrels (Bosson et al., 2009), common marmosets (Raminelli et al., 2001) and red colobus monkeys (Chapman et al., 2006) a diurnal rhythm of glucocorticoid secretion is reflected in fecal glucocorticoid metabolite (FGCM) levels, while such variation is absent in other species (e.g. white rhinoceros (Turner et al., 2002), baboons (Beehner and Whitten, 2004), western lowland gorillas (Shutt et al., 2012)). Correspondingly, adult male chimpanzees (Seraphin et al., 2008) and Assamese macaques (Ostner et al., 2008) (but only during the breeding season) have higher GC levels than subadult males and in rats glucocorticoid levels increase with increasing age (Sapolsky, 1992), whereas there is no age-effect in other species (e.g. spiny mice (Nováková et al., 2008)).

In addition to these biological sources of variation, GC levels from feces can also be affected by methodological issues, in particular the way how samples are collected and stored (Khan et al., 2002; Lynch et al., 2003; Shutt et al., 2012).This presents a serious challenge especially for researchers that work in remote areas where there is no access to freezers. One solution to this problem is the immediate extraction of steroids from feces using on-site extraction methodologies (Beehner and Whitten, 2004; Murray et al., 2012; Shutt et al., 2012) in combination with validated methods to store extracts under tropical conditions (Santymire and Armstrong, 2010; Shutt et al., 2012). However, to date it remains unclear how versatile such methods are, i.e. to what extent they can be applied across multiple species.

As part of a larger project that investigates the impact of anthropogenic disturbances and habitat fragmentation on the stress physiology of wild brown spider monkeys (*Ateles hybridus*) and red howler monkeys (*Alouatta seniculus*) in Colombia, we examine here the effect of time of day, age, sex and female reproductive condition on fecal glucocorticoid excretion, information that does not exist for either of the two species. Brown spider monkeys are endemic to Colombia and Venezuela (Defler, 2003). Due to their restricted distribution, their long inter-birth intervals (32–50 months) (Di Fiore and Campbell, 2007), severe habitat loss and high hunting pressure the species is critically endangered (Urbani et al., 2008) and belongs to the 25 most endangered primate species in the world (Mittermeier et al., 2012). As other spider monkey species, they are mainly frugivorous, although young leaves can make up to 50% of their diet (Galvis et al., 2012). In contrast, red howler monkeys have a much wider distribution (Brazil, Ecuador, Peru, Venezuela and Colombia) and are not threatened with extinction (Boubli et al., 2008). They are highly folivorous and can persist even in extremely small forest fragments (Estrada and Coates-Estrada, 1996; Gilbert, 2003; Lovejoy et al., 1986).

For the present study we initially validated an EIA for assessing adrenocortical activity non-invasively from fecal samples of the two species and tested for potential storage effects on FGCM levels in fecal extracts stored for six month at high temperatures. For validation we used 1) the physiological stress response to anesthesia (e.g., Martínez-Mota et al., 2008; Sapolsky, 1982; Whitten et al., 1998b) in zoo-housed and wild animals to test the suitability of four different EIAs in reflecting the stress-related FGCM increase in feces and 2) evaluated the specificity of the most suitable EIA in both sexes of both species by characterizing the pattern of immunoreactive metabolites measured using HPLC analysis.

Thus, with this study, we procure the methodological basis for studying adrenocortical activity non-invasively in *A. hybridus* and *A. seniculus* and provide important comparative baseline information on the influence of several intrinsic variables on FGCM levels in these two endocrinologically unstudied species of New World primates.

2. Material and methods

2.1. Study sites and animals

2.1.1. Field

We collected fecal samples of two wild groups of brown spider monkeys (SJ1, SJ2) and six groups of red howler monkeys (C0, C1, C2, C3, C7, I) in which all individuals were individually recognized and fully habituated (Table 1). All groups ranged in a forest fragment located within the private cattle ranch "Hacienda San Juan