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RESEARCH ARTICLE

Monitoring Ovarian Cycle Activity Via Progestagens in Urine and Feces of Female Mountain Gorillas: A Comparison of EIA and LC–MS Measurements

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Understanding the reproductive biology of endangered mountain gorillas (*Gorilla beringei beringei*) is essential for optimizing conservation strategies, determining any demographic impact of socioecological changes, and providing information for comparative studies of primates. Non-invasive techniques have been used to assess the reproductive function of many primates and the importance of validating the measurements of hormones metabolites is widely recognized because they may vary even within closely related species. To determine if it is possible to non-invasively monitor ovarian activity in wild mountain gorillas, we used enzyme immunoassays (EIA) to quantify both urinary and fecal excretion of immunoreactive pregnanediol-3-glucuronide (iPdG), defined as all metabolites detected by a pregnanediol-3-glucuronide immunoassay (PdG EIA). Simultaneously, we performed the liquid chromatography mass spectrometry (LC–MS) to quantify the excretion of pregnanediol in urine and feces. Samples were analyzed over nine cycles of five females from the habituated gorillas monitored by Karisoke Research Center, Rwanda. As an additional indicator for ovulation timing, estrone conjugates (E1C) were measured in a subset of urine samples. The concentrations of iPdG and pregnanediol measured in the same samples were significantly correlated. Urinary concentrations of iPdG and pregnanediol fluctuated over the menstrual cycle but did not reveal any cyclic pattern, whereas a typical preovulatory urinary E1C surge and postovulatory increases of fecal iPdG and pregnanediol were detected. The luteal peaks of iPdG and pregnanediol levels in feces were on average 2.8 and 7.6 times higher, respectively, than averaged levels in the corresponding follicular phase. The relative number of days with observed matings was higher within the presumed fertile window than in the preceding period. Overall, the results indicate that fecal analysis of iPdG and pregnanediol is suitable for detecting ovulation in female mountain gorillas. Urinary measurements using both EIA and LC–MS appeared to be uninformative for monitoring ovarian activity in this primate. Am. J. Primatol. 76:180–191, 2014.

Key words: ***Gorilla gorilla beringei*; ovulation; urinary and fecal progestagens**

INTRODUCTION

Monitoring cyclic activity in wild living primates with non-invasive methods has become a widespread practice in recent years. A wide array of assays for measuring steroid metabolites in feces, urine or saliva have been established [Heistermann, 2010] and have helped to provide species-specific information on estrus cycles (e.g., length, fertile window, abnormalities, postpartum resumption, and cessation) and pregnancies (e.g., start, duration, and losses) [Deschner et al., 2003; Shideler et al., 1993; Ziegler et al., 2009].

In most of those studies, immunoassays (EIA or RIA) were used to characterize excretory profiles of ovarian hormones. Hormonal data allow researchers to either pinpoint the day of ovulation defined via the increase in progestagen levels or by a marked

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estrogen peak at the end of the follicular phase, or to simply indicate regular cyclic activity as opposed to pregnancy or lactational amenorrhea. Generally progestagens produce more consistent results than estrogens [Hodges & Heistermann, 2011].

Liquid chromatography mass spectrometry (LC–MS) has been recently validated as an alternative method of investigating steroid metabolites in urine and feces of primates [Hauser et al., 2008; Weltring et al., 2012]. This method offers the advantage of allowing a highly specific simultaneous measurement of different compounds in the same sample. However, both acquisition and operating costs of LC–MS still limit its applications mainly to industrial and clinical research. While the use of LC–MS for the routine measurement of steroids in samples from free living primates might be impractical for most situations, it offers a good opportunity to validate EIA methods. In particular, LC–MS may be used instead of the traditional approaches of method validation such as radiometabolism studies that are not often possible because the species of interest cannot be kept in captivity or the conservation status of that species prevents such experiments.

Several assays for different metabolites have been validated for the use of urine and fecal extracts to evaluate ovarian function of primates [see Hodges et al., 2010; Hodges & Heistermann, 2011]. Among the great apes, cyclic activity has been successfully monitored in chimpanzees, bonobos, and captive western lowland gorillas using both urine [e.g., Bellem et al., 1995; Czekala et al., 1987, 1991, 1988a; Deschner et al., 2003, 2004; Emery & Whitten, 2003; Lasley et al., 1982; Mitchell et al., 1982] and fecal extracts [Atsalis & Margulis, 2008; Emery Thompson, 2005; Heistermann et al., 1996; Reichert et al., 2002]. In orangutans cyclic activity has been monitored in urine [Czekala et al., 1988b; Knott et al., 2010; Shimizu, 2005]. In contrast, hormonal measurements to monitor cyclic activity in mountain gorillas are limited. In one study, urinary estrone conjugates (E1C) and pregnanediol-3-glucuronide (PdG) profiles were determined using EIA techniques in only one cycle of three females. Although an estrogen peak was detected in each cycle, an increase in progestagen levels was found only in one female after conception [Czekala & Sicotte, 2000]. The authors concluded that urinary PdG levels in mountain gorillas allow for the detection of pregnancy but not for estimating the day of ovulation. In comparison with results on other great apes, these findings are intriguing because they indicate that mountain gorillas might differ from other closely related species in their metabolism and excretion pathways [Czekala & Robbins, 2001; Czekala et al., 1988b].

More practically, it would be useful to determine whether the observed patterns are related to cross-reactivities of the antibody creating so much noise that no clear cyclic pattern can be measured or whether indeed PdG occurs in such erratic concentrations that no clear cyclic pattern can be observed. Therefore it would be important to investigate if measuring progestagen metabolites in fecal extracts might allow for a clearer monitoring of cyclic patterns in this species. While in contrast to urine, the fecal analysis of progestagens metabolites involves extraction procedures [Wasser et al., 1988, 1994] and uses group-specific immunoassays [Schwarzenberger, 2007], the collection of fecal samples from mountain gorillas is much easier than urine in the wild and more practical for long-term studies.

The development of suitable laboratory-based methods is necessary for directly assessing the reproductive function of mountain gorillas. Nulliparous females begin to mate when they are approximately 6–7 years of age [Gerald, 1995; Watts, 1991] and exhibit small labial swellings at regular intervals [Czekala & Sicotte, 2000]. Following a long period of lactational amenorrhea (approx. 2–3 years), parous females enter a phase of cyclic activity that lasts typically 3–6 months until they conceive [Watts, 1990, 1991]. During this time period they show regular but short bouts of copulatory activity (1–4 days of mating behavior approximately every 28–30 days) [Watts, 1990, 1991]. While behavioral observations indicate that females primarily mate only at the likely time of conception or while pregnant [Robbins, 1999; Watts, 1990, 1991], endocrine data are needed to estimate if matings occur primarily during the fertile period in mountain gorillas.

In captive western gorillas, the different phases of the ovarian cycle were determined by using urinary concentrations of estrogens [Czekala et al., 1987, 1988a, 1991; Lasley et al., 1982; Mitchell et al., 1982] and sometimes the magnitude of follicular peak of urinary E1C was used solely to distinguish ovulatory from non-ovulatory cycles [Czekala & Sicotte, 2000]. However, a combined examination of estrogens and progestagen profiles provides more accurate information on the occurrence of ovulation and the competency of the luteal function [Heistermann & Hodges, 1995; Heistermann et al., 1993, 1995].

The goal of this study was to determine a suitable method using hormones for estimating the time of ovulation and the fertile period in wild mountain gorillas. Specifically, we sought to determine if it is possible to monitor cyclic activity of female mountain gorillas, using pregnanediol-3-glucuronide immunoassay (PdG EIA). For this, we performed both EIA and LC–MS to quantify immunoreactive pregnanediol-3-glucuronide (iPdG) and pregnanediol respectively in feces and urine, and compared their excretion patterns throughout the menstrual cycle. Furthermore, we used urinary estrone conjugates (E1C) profiles assessed by EIA to cross-check on the validity of using fecal analysis of iPdG to predict the time of ovulation and the fertile period in mountain gorillas.

METHODS

Data Collection

Data were collected from a total of 12 habituated female mountain gorillas monitored by Karisoke Research Center (KRC) in Rwanda's Parc National des Volcans. For the purpose of validation, we established menstrual cycle profiles of iPdG and Pregnanediol in a subset of five females during a time period where regular cycles were known to occur. These females were less than 30 years of age and their dependent infants were more than 2.5 years of age [Robbins et al., 2006; Watts, 1990, 1991]. Subsets of samples from the other females including pregnant and lactating ones were used for different method validation procedures (see below).

During each day of observation we recorded if the target female was seen copulating during focal and ad libitum observations conducted routinely by KRC field staff including S.H. We defined and recorded copulations as reported previously by Robbins [1999].

Samples Collection and Preparation

Urine and fecal samples were collected on an ad libitum basis during 239 "female days," defined as days that the study individuals were the focus of urine and/or fecal collection. For each individual, we aimed to get daily samples for at least a one month period to encompass a full cycle. Overall, we had a collection rate of 0.73 urine samples and of 0.81 fecal samples per day. Urine samples were pipetted from the ground or vegetation, and transferred into cryovials kept cold on ice packs. Upon arrival at the field station, the vials were immediately frozen at −20°C until they were transported on dry ice to the laboratory. Fresh fecal samples were collected in plastic bags, labeled, and frozen at −20°C until they were dried at 100°C [Emery Thompson, 2005].

Steroid hormones were extracted from dried feces as described by Heistermann et al. [1995]. Briefly, 3 ml of 80% methanol were added to 0.1 g of dried feces. Resultant suspension was shaken for 15 min and centrifuged at 4 400 rpm for 10 min. The supernatant was decanted and the procedure repeated with another 3 ml of 80% MeOH.

Prior to LC–MS measurements, fecal steroids were extracted as described in detail by Weltring et al. [2012]. In brief, we added deuterated internal standards such as progesterone-d9 (to quantify pregnanediol and progesterone) and estrone-d4 (to quantify estrone, estriol, and estradiol) to methanol extracts. Solid phase extraction was performed by using polymer-based reversed phase cartridges (Chromabond® HR-X, 30 mg, Macherey-Nagel, Düren, Germany). Steroids were eluted, dried, and reconstituted in phosphate buffer. Free steroids were separated from conjugated steroids by liquid–liquid extraction with *tert*-butyl methyl ether. The aqueous layer containing the conjugates was solvolysed using a mixture of ethyl acetate/sulfuric acid followed by liquid–liquid extraction to extract the free steroids. The combined steroid extracts were measured by LC–MS/MS. For more details on the solvolysis and liquid–liquid extraction, see Hauser et al. [2008]. Urinary steroids were extracted as stated by Hauser et al. [2008]. In short, internal standard solution was added to the samples, as above. The samples were then hydrolyzed by using β-glucuronidase (Sigma Chemical Co., St. Louis, MO). Lastly, we carried out solid phase extraction, liquid–liquid extraction, and solvolysis, as described for the method of extracting fecal steroids.

Hormone Analysis

Pregnanediol-3-glucuronide assay

We used a pregnanediol-3-glucuronide (PdG) EIA containing the antibody R13904 (provide by Coralie Munro, UCD, Davis, CA). This assay has been used already for the monitoring of female cyclic activity in humans [De Souza et al., 2010; Schlaff et al., 2004; Schwartz et al., 2012] and baboons [O'Connor et al., 2011]. Serial dilutions of urine samples from one female and fecal extracts from another female collected during regular cycles and pregnancy were assayed and resulted in curves parallel to the standard curve. Sensitivity of the assay at 80% binding was 25 pg/50 μl. The quality control showed intraassay coefficients of variation of 10.94% (high, $N = 66$) and 12.5% (low, $N = 66$) and interassay coefficients of variation of 11.99% (high, $N = 24$) and 14.67% (low, $N = 24$). PdG antibody cross-reacts with pregnanediol-3-glucuronide (100%), 20 α-hydroxyprogesterone (44–82%), 20 β-hydroxy-progesterone (3.16%), progesterone (0.2%), estradiol (0.04%), cortisol (0.06%), and testosterone (0.2%) [O'Connor et al., 2011]. We assessed the specificity of the assay using an immunogram. For this particular part of the study, we used fecal samples collected from eight females including the five females with regular cycles and additional two cycling and one lactating female to prepare one pooled sample that was separated into 33 HPLC fractions. As indicated in the description of LC–MS measurements, the same methods and gradient were used to determine different progesterone metabolites (progesterone and pregnanediol) in those fractions.

The pregnanediol peak fell within the peak of elevated immunoreactivity of the PdG EIA (Fig. 1). However, since the area of elevated immunoreactivity of the PdG EIA was considerably broader than the fractions in which pregnanediol was measured with LC–MS and since an additional peak of immunoreactivity was detected for the PdG EIA in fraction 32, we conclude that the PdG EIA cross-reacted with a number of unidentified metabolites other than PdG. We refer to all metabolites detected by the immunoassay as the

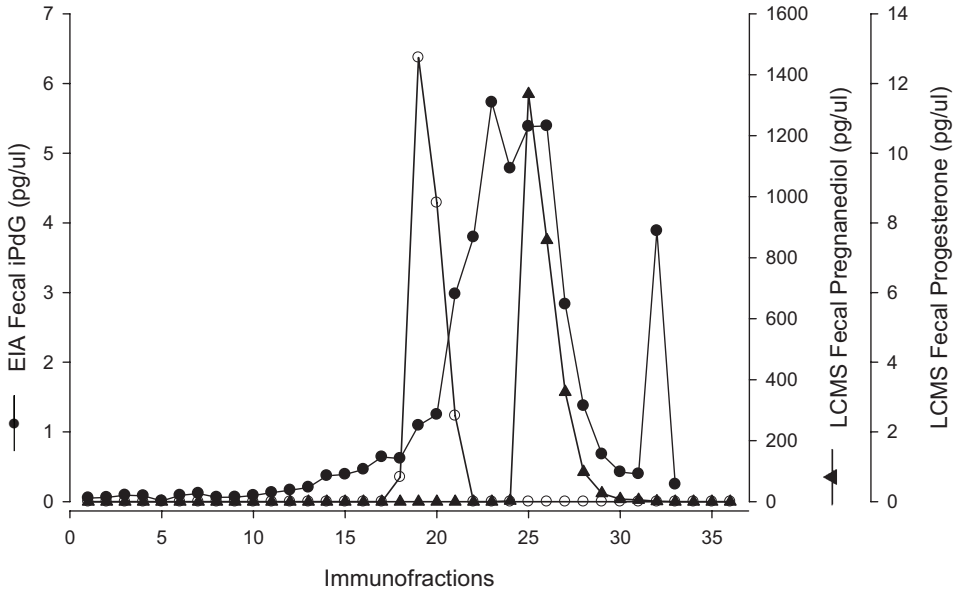

Fig. 1. Profile of HPLC immunoreactivity measured by PdG EIA in fractions of a pool sample of fecal extracts from eight non-pregnant females. Pregnanediol and Progesterone levels were measured by LC–MS in the same fractions. Note the different scale of the *y*-axes.

immunoreactive PdG (iPdG). Urinary iPdG and pregnanediol levels were indexed with creatinine concentrations [Deschner et al., 2004]. Fecal iPdG and pregnanediol levels were given as ng/g dry weight.

Estrone conjugate (E1C) assay

E1C antibody and enzyme-label were provided by the Deutsche Primatenzentrum GmbH (Göttingen, Germany). We followed the enzyme immunoassay procedures previously detailed in Heistermann & Hodges [1995]. Urine samples were diluted (1:10 to 1:200) in assay buffer and applied in duplicate aliquots of 50 μl to the assay. Serial dilutions of pooled samples collected from five pregnant and seven cycling females during regular cycles and pregnancy gave parallel displacement to the standard curve. Intra- and inter-assay coefficients, calculated from quality controls, were 9.2% ($n = 58$) and 5.2% ($n = 9$) (high), and 14.95% ($n = 64$) and 11.95% ($n = 9$) (low), respectively.

LC–MS measurements

LC–MS measurements of pregnanediol were carried out as described by Weltring et al. [2012]. In short, we used Waters Alliance 2695 separation module comprising a quaternary pump and a column oven (Waters, Milford, MA).

The separation was done at 30°C on a reverse phase C18 column (Gemini C18, 150 mm × 2 mm, 3μm, Phenomenex, Torrance, CA) protected by a guard column (Security Guard, 4 mm × 2 mm, 5μm, Phenomenex). Eluents A and B contained both 0.1% formic acid and were respectively composed with water/acetonitrile 95/5 (v/v) and 5/95 (v/v). The gradient elution was 30% B (0 min), linear increase to 70% B (0–20 min), and 30% B (24–34 min). After separation, the compounds were analyzed on a Quattro Premier XE tandem mass spectrometer with Z spray ESI interface (Micromass, Manchester, UK). Data were processed with MassLynx 4.1 (Waters).

Statistical analysis

For statistical analysis, we used a total of 134 fecal samples and 35 urine samples for which both values of pregnanediol and iPdG were measured. Only, those urine samples were included in statistical analysis because they were collected around conception from one of the study females (MAHIRWE) and therefore they were good candidates to reflect typical hormonal variation of an ovulatory cycle.

Statistical tests were conducted using within-individual hormonal levels to avoid the problem of pseudo-replication [Hurlbert, 1984; Machlis et al., 1985]. Spearman rank-correlation tests were performed to compare the levels of iPdG and pregnanediol yielded from fecal and urinary measurements. Tests of small sample sizes were exact [Mundry & Fischer, 1998; Siegel & Castellan, 1988]. Fisher's omnibus test was used to account for multiple testing of correlations between iPdG and pregnanediol levels in cycles of each of the five females. This test combines a number of P values into a single chi-square distributed variable, with degrees of freedom equaling twice the number of P values [Haccou & Meelis, 1994]. All tests were calculated by using R [R Development Core Team, 2012]. All P values were two-tailed.

Interpretation of Hormone Profiles and Definition of Fertile Phase

As in studies on other primate species [e.g., Dubuc et al., 2012; Higham et al., 2008; Ziegler et al., 2000], we used fecal iPdG to investigate hormonal changes in relation to different cycle phases in female mountain gorillas. We presumed that ovulation had occurred when a rise of more than two standard deviations of iPdG levels sustained for more than 2 days above the mean of the three to five preceding values [Deschner et al., 2003]. The day of ovulation was then defined as the day of the significant rise in fecal iPdG corrected by a time lag of 2 days, based on the findings on captive western lowland gorillas showing a mean gut retention time of 50 hr [Remis, 2000] and a time lag of 43–68 hr between the stressor and the excretion peak of fecal glucocorticoid metabolites [Shutt et al., 2012]. The fertile phase was delineated to a 5-days period comprising 3 days preceding the ovulation, the day of ovulation and 1 day to account for potential error in the timing of ovulation [Deschner et al., 2003; Heistermann et al., 2008; Van Belle et al., 2009].

For each female cycle we calculated the number of days the female was seen copulating during her fertile period (day −1 to day −5 relative to the day of the significant PdG rise; see below) and divided this value by five (days in the fertile period). We then calculated the average over the nine cycles from the five females. For comparisons with the non-fertile period, the same calculation was repeated for each 5 day period preceding and following the fertile period (day −6 to day −10 and day 0 to day 4 relative to the day of the significant PdG rise).

The research reported in this manuscript complied with protocols approved by the department of Tourism and Conservation of Rwanda Development Board, adhered to the legal requirements of Rwanda, and to the American Society of Primatologists (ASP) Principles for the Ethical Treatment of Non-Human Primates.

RESULTS

iPdG and Pregnanediol in Feces

To detect and compare excretory patterns of progesterone metabolites in feces, we measured iPdG by EIA and pregnanediol by LC–MS in the same samples. The average iPdG levels were 9.79-fold lower than pregnanediol levels. Within-individual iPdG and pregnanediol concentrations were significantly correlated (Fisher's omnibus test combining P values of Spearman correlations: $\chi^2 = 105.24$, df = 10, $P < 0.001$; Table I). Across individuals, pregnanediol levels were more variable than iPdG levels (Fig. 2). Each of the composite profiles of fecal iPdG and pregnanediol showed a cyclic pattern characterized by consistently low hormonal levels during

TABLE I. Cycles Analyzed and Spearman Correlations Between LC–MS and EIA Measurements Per Study Female

	Reproductive history and cycle characteristics of females						Spearman correlations between fecal iPdG (ng/g feces) and pregnanediol (ng/g feces) levels.		
Female	Start of hormonal analysis	Months between the loss of the dependent infant and the start of hormonal analysis	Months between the birth of the following infant and the start of hormonal analysis	Female days	*N* fecal samples (EIA)	*N* urine samples (EIA)	*N* (EIA and LC–MS)	Rho	*P*-value
UMWANA	28-May-10	NA[a]	NA[b]	74	64	NA[c]	14	0.69	0.008
BUKIMA	12-Aug-10	0.93	8.57	17	17	16	16	0.58	0.022
MAHIRWE	10-Jan-11	NA[a]	9.73	47	34	36	34	0.85	<0.001
MUGANGA	23-Dec-10	NA[a]	NA[b]	42	41	36	39	0.9	<0.001
IMVUNE	29-Nov-10	6.53	15.73	59	38	32	31	0.74	<0.001

NA, data not available.
LC–MS and EIA measurements per individual were significantly correlated ($P < 0.001$).
[a]The dependent infant was alive at the time of sampling.
[b]The female did not give birth within 12 months following the start date of hormonal analysis.
[c]No urine samples were analyzed.

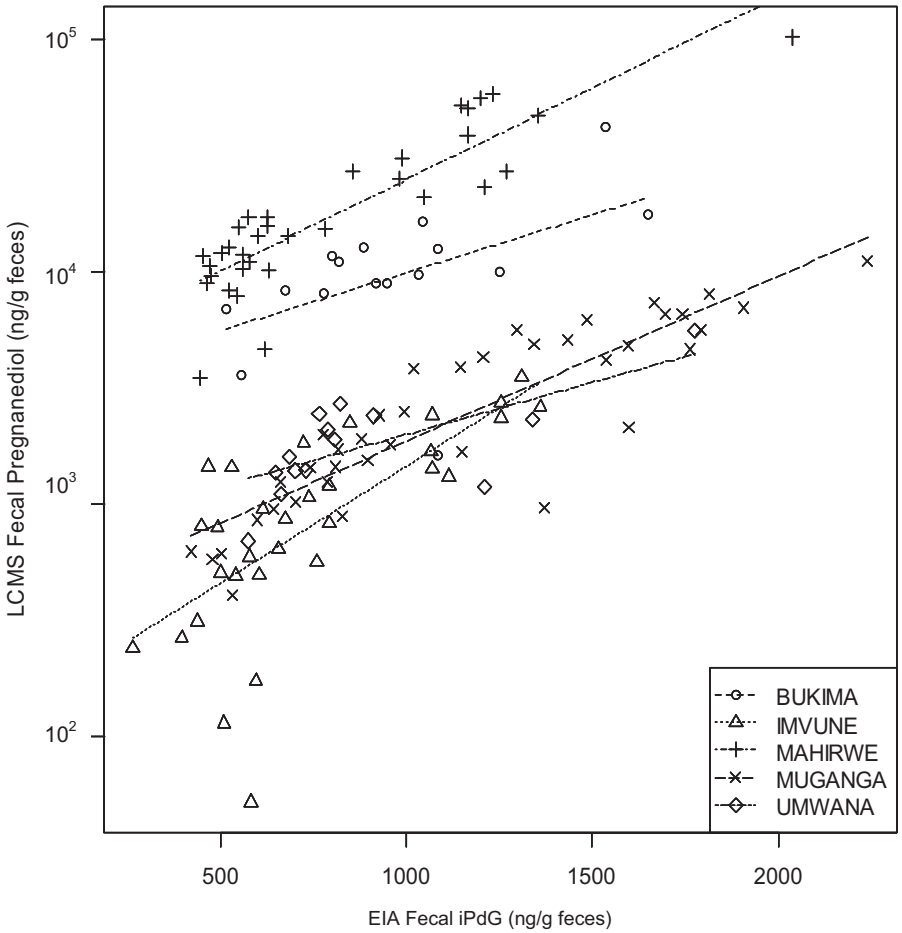

Fig. 2. Within-individual correlations between measures of fecal iPdG and pregnanediol for five females. The lines represent the regression lines for each individual.

the follicular phase (mean iPdG = 675.71 ± SD 185.2 ng/g feces and mean pregnanediol = 3304.24 ± SD 3940.16 ng/g feces) and elevated concentrations in the luteal phase (iPdG peak = 1889.56 ± SD 207.66 ng/g feces and pregnanediol peak = 25269.99 ± SD 23776.74 ng/g feces) (Figs. 3 and 4).

iPdG and Pregnanediol in Urine

In contrast to fecal analysis, iPdG levels in urine samples collected from the same female during the same time period were 2.5-fold higher when measured by EIA than pregnanediol measured by LC–MS. In the follicular phase, average iPdG and pregnanediol levels were 375.52 ± SD 316.94 ng/mg CREA and 166.51 ± SD 131.78 ng/mg CREA, respectively. iPdG measurements using EIA and pregnanediol measurements using LC–MS in urine were correlated significantly (Spearman's rho = 0.766, $N = 35$, $P < 0.001$) but neither showed any pattern matching with the cyclic pattern of the fecal hormonal profiles (Fig. 4). Pregnanediol concentrations ranged between 10.57 and 646.98 ng/mg CREA.

Urinary E1C Levels

To assess the possibility of using fecal measurement of progestagens to predict the time of ovulation, we measured urinary E1C to identify estrogen preovulatory surges in four cycles. E1C values ranged from 3.07 to 413.89 ng/mg CREA. Mean E1C concentrations increased from a baseline of 19.68–25.71 ng/mg CREA on days −9 to −14 (early follicular phase) and peaked (mean E1C = 168.94 ng/mg CREA) 1 day before the significant rise of EIA fecal iPdG (Fig. 3).

Correlates Between Hormonal Profiles and Observed Copulations

Copulations were observed on 28 of 239 female days. The likelihood of mating increased from 6.67% (3 of 45) during the 5 days preceding the fertile period to 24.44% (11 of 45) during the presumed 5 fertile days (Fig. 5). The likelihood of mating during the 5 days after the fertile period was 26.67% (12 of 45). In 7 of the 9 cycles, females were seen copulating within their estimated fertile window. The copulatory period lasted 1–4 consecutive days per cycle.

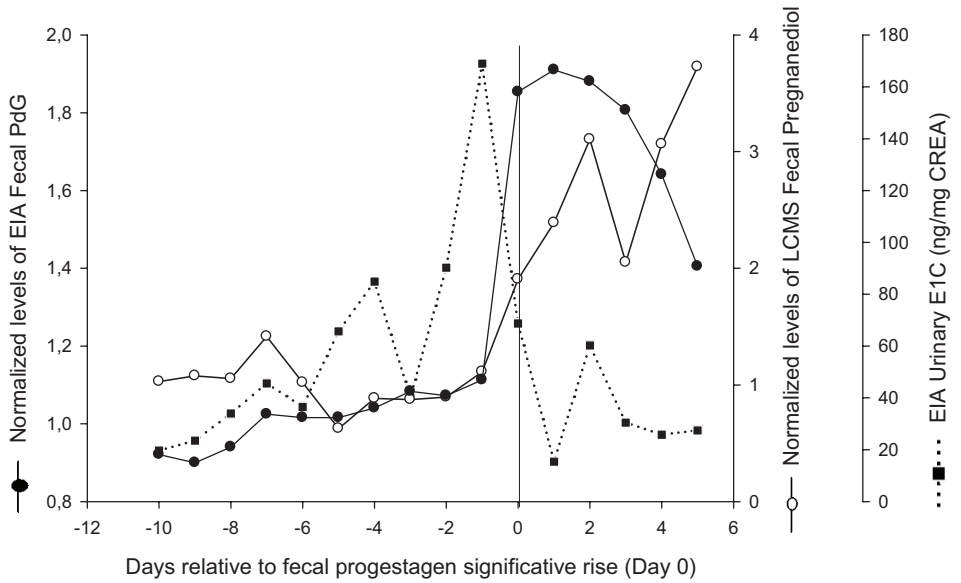

Fig. 3. Composite profiles of fecal progestagens and urinary E1C. All values are aligned to the significant rise of fecal hormonal levels (Day 0). Normalized levels correspond to the ratios between the hormonal concentration of a sample on a given day and the average of hormonal concentrations in the follicular phase of the relevant cycle. Closed and open circles indicate normalized levels of EIA fecal iPdG in nine cycles from five gorillas and normalized levels of LC–MS fecal pregnanediol in seven cycles from five gorillas, respectively. Closed squares represent urinary E1C measured in four cycles from three gorillas (BUKIMA, MAHIRWE, and MUGANGA). Note the different scale of the *y*-axes.

DISCUSSION

Non-invasive monitoring of ovulatory cycles in wild primates provides valuable information for correlations with behavioral patterns and other parameters of a species' reproductive biology. In contrast to other studies that have found that the measurement of progestagen and/or estrone levels in urine provide the most reliable estimate of cyclic activity in primates and other animals, our results indicate that when comparing EIA measures against LC–MS measures, cyclic activity in mountain gorillas can be best monitored by measuring iPdG with EIA in fecal extracts. Furthermore, we failed to find clear cyclic patterns of either iPdG and pregnanediol levels in urine of the same females during the same cycles.

Fecal Progestagens

As demonstrated in other studies, the quantification of fecal progestagens by using different immunoassays yields remarkable differences in absolute levels [e.g., Heistermann et al., 1993; Shideler et al., 1993; Ziegler et al., 1996]. Seemingly, the magnitude of postovulatory rise in fecal progestagens varies with the type of immunoassay and reflects the differences in progesterone metabolism across species. In mountain gorillas, we found that peak levels of fecal iPdG were on average 2.8 times higher than average levels during the respective follicular phase. Comparable increases of fecal iPdG in the luteal phase were found in other primates (e.g., *Macaca fascicularis* [Shideler et al., 1993], *Papio hamadryas anubis* [Higham et al., 2008], *Presbytis entellus* [Ziegler et al., 2000]) for which the ratios between luteal peak and follicular baseline vary on average between 3 and 4. The measurement of fecal pregnanediol with LC–MS allowed us to determine larger differences between follicular and luteal phases but the overall magnitude of pregnanediol rise in mountain gorillas remained lower than luteal increase of pregnanediol quantified by EIA in feces of some other primate species (e.g., *P. entellus* [Heistermann et al., 1995], *Cebus apella* [Carosi et al., 1999], Callitrichidae [Heistermann et al., 1993; Ziegler et al., 1996]). In similar studies, progesterone (P4) immunoassays were largely used to detect cycling patterns of progestagen metabolites in feces [Hodges & Heistermann, 2011]. Lowland gorillas display the smallest increase of fecal progesterone (P4) during luteal phase (e.g., *Gorilla gorilla* [Atsalis & Margulis, 2006; Miyamoto et al., 2001] compared to *Pan paniscus* [Heistermann et al., 1996], *Pan troglodytes* [Emery & Whitten, 2003], callitrichidae [Heistermann et al., 1993], and *C. apella* [Carosi et al., 1999]). Together with our findings, those data suggest that fecal measurement is appropriate to monitor the menstrual cycle in Gorilla sp., with mountain gorillas displaying lower luteal increase of progestagen metabolites than lowland gorillas.

The comparisons of the iPdG and pregnanediol levels obtained through the EIA and LC–MS showed that the LC–MS is a valuable method to validate the suitability of EIAs for detecting inter-individual variation in fecal progestagens in female mountain

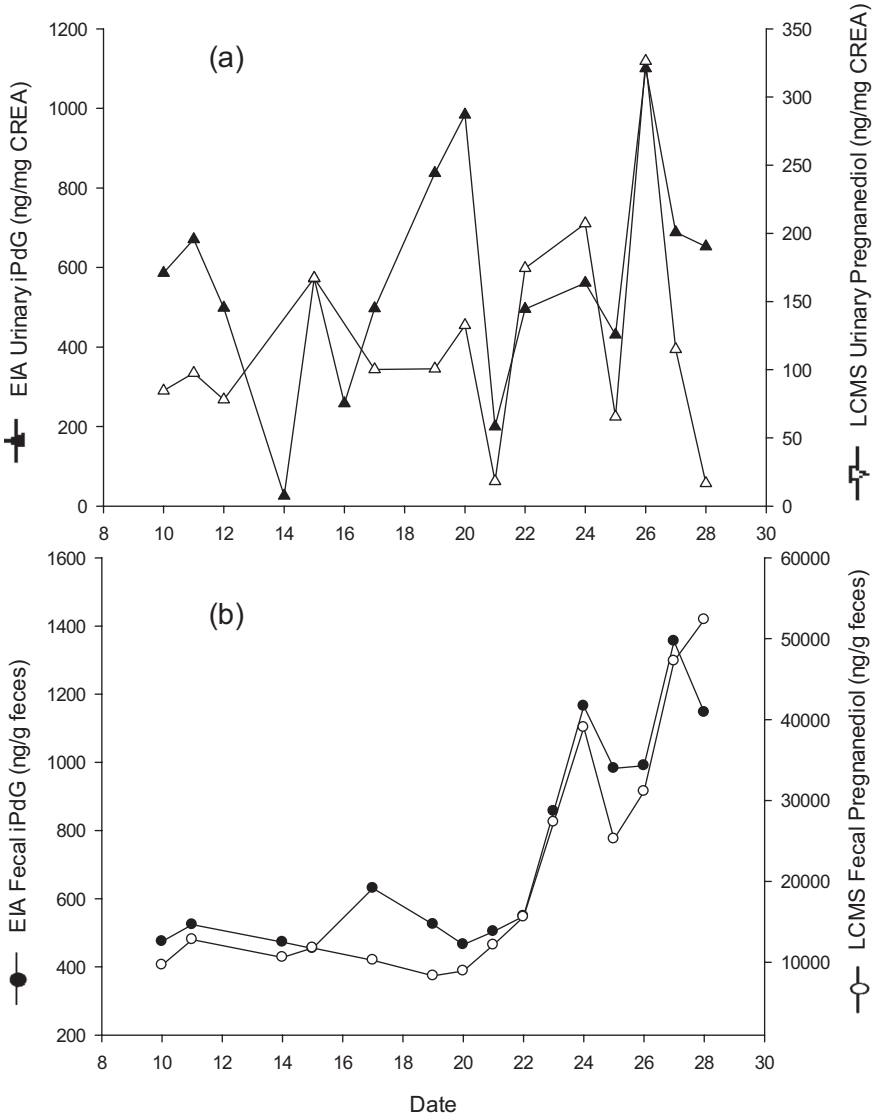

Fig. 4. Example of a cycle profile of iPdG and pregnanediol levels measured in urine (**a**) and fecal samples (**b**) from the female MAHIRWE in January 2011. Closed triangles: EIA urinary iPdG; open triangles: LC–MS urinary pregnanediol; closed circles: EIA fecal iPdG; open circles: LC–MS fecal pregnanediol.

gorillas. A significant correlation was found between iPdG levels from the EIA and pregnanediol levels from LC–MS measured in the same samples when correlations were carried out within a given female. However, the levels of fecal pregnanediol varied greatly across females. Apparently, LC–MS detected a generally higher progestagen levels in cycles of females that were closer to conception, based on known birth dates of infants by these females (Table I). One indication that this might have caused the observed pattern is that the two females with the highest pregnanediol levels were in their conceptive cycle or one cycle before it. Second, two other parous females which had very low levels of fecal pregnanediol still had not given birth more than 1 year after the time of this study, which means that the cycles analyzed occurred at least 16 months before the next conception. The variation of hormonal concentrations around conception has been investigated in only a few primates' species and progestagen levels were found to be sometimes lower in non-conceptive cycles than in conceptive cycles of *M. fascicularis* [Shideler et al., 1993], *Pan troglodytes schweinfurthii* [Emery Thompson, 2005], and humans [Baird et al., 1997], suggesting that the lower concentrations of fecal pregnanediol are indicative of reduced fertility in some cycles in female mountain gorillas.

An alternative explanation for the high between-female variability of progestagen levels could be

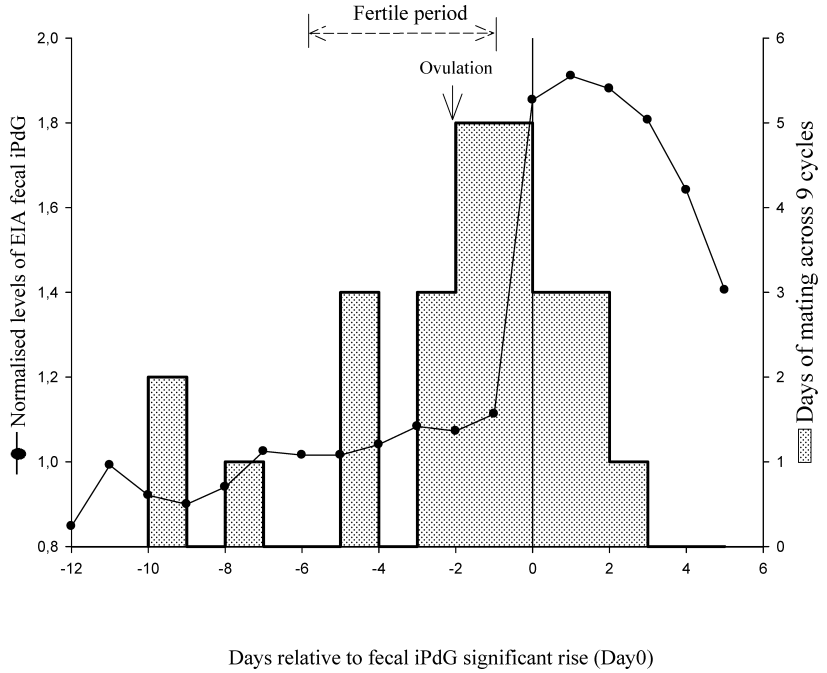

Fig. 5. Distribution of copulations (shaded area) in relation to the fertile phase. Closed circles: normalized levels of EIA fecal iPdG in nine cycles from five females; vertical line: significant rise of EIA fecal iPdG.

individual differences in progesterone metabolism of the gut flora [for review see Goymann, 2012]. There are many intestinal microorganisms involved in metabolic reactions of steroids [Groh et al., 1993], and changes in gut flora may affect subsequently the metabolism of progesterone and the patterns of its excreted forms in feces [Martin et al., 1975]. Differences in the gut microbial communities among female mountain gorillas could have caused large differences in absolute levels of pregnanediol measured with LC–MS. On the other hand, iPdG values measured with EIA might have not been impacted as strongly by those differences in metabolism, because the EIA antibody might have cross-reacted with a wide range of progesterone metabolites excreted across individuals, thereby dampening the dramatic differences when focusing on one metabolite only. However, although it is unclear whether inter-individual variations of hormonal concentrations indicate cycle quality or merely differences in metabolism of progesterone by the gut flora, EIA, and LC–MS measurements used in this study clearly reflected ovulation-related changes in progesterone production. Therefore, fecal analysis of both pregnanediol and iPdG remain suitable for monitoring cyclic activity in mountain gorillas.

Urinary Progestagens

Urinary progestagens were not a reliable indicator of ovulation because there was no significant and sustained increase in iPdG levels. Since iPdG levels measured by EIA correlated significantly with urinary pregnanediol levels measured with LC–MS, it is unlikely that the absence of a clear cyclic pattern in iPdG levels was caused by cross-reactivities of the antibody with metabolites that do not stem from the progestagen metabolism. We suggest that the absence of a detectable cyclic pattern and generally low levels of urinary progestagens in mountain gorillas found in this study and the only other study to investigate ovulatory cycles in wild gorillas [Czekala & Robbins, 2001; Czekala & Sicotte, 2000] in comparison to closely related species (e.g., *Pan troglodytes verus* [Deschner et al., 2003], *P. paniscus* [Heistermann et al., 1996], *Pongo pygmaeus* [Shimizu, 2005], humans [Munro et al., 1991], *Gorilla gorilla gorilla* [Bellem et al., 1995; Czekala et al., 1988a; Lasley et al., 1982; Mitchell et al., 1982]) were caused by a urinary progestagen metabolism that might be strikingly different in mountain gorillas. Simultaneously collected serum and urine would be necessary to determine the specific metabolic pathways of progestagens excreted in mountain gorilla urine, which is not possible given there are so few of this endangered species in captivity. However, a study that compares urinary and fecal levels of a wider array of different progestagens could shed some light into this intriguing problem.

Occurrence of Copulations

The females copulated primarily around the day of ovulation, which was estimated by using hormonal data obtained from fecal analysis of progestagens. Our results showed that the likelihood of mating was almost the same in the five days immediately after

the fertile period (particularly the first day after the presumed fertile period) than during the fertile period. It is possible that our estimation of the fertile window might be slightly shifted from reality if mountain gorillas have a faster gut passage time than captive western gorillas. However, the observed distribution of copulations across the menstrual cycle suggests that male mountain gorillas may be able to perceive signals related to ovulation or that females are receptive primarily during this fertile window. In chimpanzees, a species in which females exhibit prominent sexual swellings for more than a week around the time of ovulation, dominant males mate predominantly around the time of ovulation, while subordinate males are forced to mate during days when conception is less likely [Deschner et al., 2004]. Small sexual swellings have been reported as well from nulliparous female mountain gorillas [Czekala & Sicotte, 2000]. However, the females of this study were all parous and therefore did not exhibit sexual swellings. How precisely dominant male mountain gorillas are able to estimate the female fertile phase and if dominant males are not able to prevent subordinate males from copulating on days of highest probability of conception remains unclear. Detailed analyses of behavioral interactions between female and male mountain gorillas in relation to the timing of ovulation are the subject of another study in preparation.

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