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Application of Urinary and Fecal Steroid Measurements for Monitoring Ovarian Function and Pregnancy in the Bonobo (*Pan paniscus*) and Evaluation of Perineal Swelling Patterns in Relation to Endocrine Events[1]

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ABSTRACT

Estrone conjugates (E_1C), pregnanediol glucuronide (PdG), and estriol (E_3) in urine, and immunoreactive E_1C, E_3, pregnanediol (Pd), and progesterone (P_4) in feces were determined along with records of perineal sex skin swelling throughout 7 nonconception cycles and 3 full-term pregnancies of 4 adult female bonobos (*Pan paniscus*). A typical preovulatory urinary E_1C surge and postovulatory increase in urinary PdG were seen during the menstrual cycles. Fecal progestin levels were significantly correlated with those of PdG in urine in all cycles, while E_1C measurements in feces were significantly correlated with those in urine in only 3 cycles. On the basis of hormone profiles, a variable follicular phase of 17–40 days and a relatively constant luteal phase of 11–15 days was found, resulting in cycle lengths of 31–51 days. All urinary and fecal hormones were markedly elevated during pregnancy. Measurement of E_1C in both urine and feces was most useful for early pregnancy diagnosis, while E_3 was of value in confirming pregnancy and assessing fetal viability. The period of perineal swelling during the cycle comprised on average 66.3% of cycle length, half of which was associated with a phase of maximum tumescence. Ovulation usually occurred within the maximum swelling phase, but timing of ovulation within this period was highly variable and was more closely associated with the end rather than the onset of maximum tumescence. The data presented here are of great practical value in the captive breeding management of bonobos and offer new opportunities for investigating basic questions of bonobo reproductive biology both in captivity and in the wild.

INTRODUCTION

The bonobo (*Pan paniscus*) was the last species of great ape to be described, and because of its close phylogenetic relation to man, it has subsequently attracted considerable interest in a wide range of research areas, such as anatomy and morphology (e.g., [1, 2]), phylogeny and evolution (e.g., [3, 4]), ecology and behavior (e.g., [5–7]) and genetics and reproduction (e.g., [8–11]). With the exception of the mountain gorilla, the bonobo inhabits the smallest known range of the great apes and is restricted to the tropical rain forests of the central Zaire basin [1, 12]. Because of extensive destruction of the native habitat [13, 14], distribution of the species is fragmented, and the free-ranging population now occupies only a small percentage of its historical range [12, 14]. Although a detailed survey is lacking, the number of wild living bonobos has decreased considerably in the last few decades [14], and with an estimated population of 10 000–20 000 individuals [15], the species is listed as endangered under Appendix I of the Convention on International Trade in Endangered Species of Flora and Fauna (CITES) [16]. In captivity there are only about 100 animals worldwide, which represents the smallest captive population of all great ape species [16].

In order to promote population growth and demographic stability as well as to preserve genetic diversity in captivity, 90% of the worldwide captive population of bonobos has, since 1988, been included in a coordinated international breeding program. Although this effort has resulted in an increase in the number of captive births, management problems such as a high first-year infant mortality still exist [16, 17]. Moreover, the different founder animals are not equally represented in the gene pool of the current captive population, and some of them have never bred [16, 17]. The causes of this asymmetry in reproductive success between the different animals are not clear since there is practically no information on the reproductive endocrinology of the species. Knowledge in this area would not only be important for assessing the fertility status, but would also provide the basis for enhancing reproduction by assisted reproductive technologies. However, to date there is only a single report on estrogen excretion during an incomplete pregnancy [18]. No information exists on endocrine characteristics of the menstrual cycle, and a reliable method for monitoring female reproductive status has hitherto not been described.

As seen in a number of other primate species such as the chimpanzee [19], macaque [20–22], and baboon [23, 24], bonobos show regular changes in swelling of the sex skin (or perineum) during the ovarian cycle. In comparison to other species, however, the period of perineal swelling in bonobos is extremely prolonged, comprising as much as 77% of the intermenstrual interval [10, 25–27]. Furthermore, perineal swelling in bonobos is characterized by an unusually long phase of maximum tumescence, which often extends well beyond 20 days [10, 25, 27], and by a gradual rather than abrupt detumescence phase [10, 26]. The significance of this pattern is not clear, particularly since, in the absence of information on underlying hormonal changes, the timing of ovulation within the cycle and its relation to perineal swelling is not known. Thus, endocrine information is not only important for characterizing the bonobo's ovarian cycle and for timing of reproductive events but also for assessing the practical value of using changes in perineal swelling patterns as an external marker of a female's reproductive status. Moreover, knowledge in this area would provide a basis for a better understanding of the role of perineal swelling in the regulation of male-female interactions concerning socio-sexual and mating behavior. This is a major topic of interest in this species in

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particular with respect to interpreting the phenomenon of extended female receptivity as well as female sexual signalling and the likeliness to conceal ovulation [9, 10, 28].

The aims of the present paper are therefore 1) to describe hormonal changes throughout the menstrual cycle and pregnancy in captive bonobos by measurement of estrogen and progesterone (P_4) metabolites in urine, 2) to verify the reliability of fecal hormone analysis as an alternative noninvasive approach suitable for application in the field, and 3) to examine the temporal relationship between perineal swelling patterns and time of ovulation.

MATERIALS AND METHODS

Animals

The study was performed on 4 wild-born, unrelated adult female bonobos with estimated ages of 14–22 yr. Menstrual histories of the females had been recorded for 2–16 yr before the present study and were found to be within the normal range reported for the species. Three of the females were maintained in a mixed social group together with one adult and two subadult males and one 2-yr-old infant, in the Animal Park of Planckendael, Belgium. The female with the infant was lactating during the study period. The animals were housed in an indoor enclosure of 600 m^3 with access to a semi-natural outdoor island (3000 m^2) on days on which temperature exceeded 12°C. The fourth female lived together with two adult males in the Zoological Garden of Leipzig, Germany. Animals were housed in an indoor cage of approximately 130 m^3 in size with a connected outdoor enclosure of approximately 90 m^2. With the exception of the nulliparous female housed in Leipzig, all other females were multiparous. All females were mated regularly during the study period, and in three of the four animals this resulted in conception followed by term pregnancies.

Sample Collection and Scoring of Perineal Swelling

Matched urine and fecal samples were collected during 7 nonconception cycles (6 ovulatory and 1 anovulatory) and throughout 3 full-term pregnancies. Sample collection occurred 5–7 days per week during conception and nonconception cycles and the first 2 mo of pregnancy, and 1–2 times per week for the remainder of gestation. For collection of samples, females were separated in individual cages, and urine and feces were collected separately from the cleaned cage floor. For the Planckendael females, sample collection usually occurred between 1500 h and 1700 h, whereas samples from the animal housed in Leipzig were collected between 0800 h and 0930 h. Cross-contamination of urine and fecal samples could not always be avoided, but only uncontaminated samples were taken for analysis. Samples were frozen immediately at −20°C and stored without preservatives until assayed.

Daily records of menstruation were taken, and visual inspections of sexual skin swelling were carried out at a distance of a few meters while the animals were in the group or separated for sample collection. In contrast to the study of Dahl et al. [10], the anatomical event of "labial occlusion" (occlusion of the labia minora, their key criterion for assessing anogenital swelling in bonobos) could not be used in the present study because it was not applicable to all our study females; e.g., in some animals the labia minora were always occluded. In this study, according to the method used by Furuichi [25], therefore, the degree of wrinkling and the size of the labia minora were taken as main parameters for evaluating changes in sex skin swelling during the menstrual cycle. Three major stages of tumescence were thus recorded as the most suitable and reliable measures of swelling under the study conditions described: 1) no swelling: minimal size and maximal degree of wrinkling; 2) partial swelling: relative increase/decrease in size and loss/appearance of wrinkles compared to stage 1 or 3; 3) maximum swelling: maximum size with no wrinkles and tight appearance, e.g., the period of maximum turgidity.

Sample Preparation and Hormone Assays

Urine samples were analyzed for immunoreactive estrone conjugates (E_1C; dilutions 1:20–1:100, depending on the reproductive status) and pregnanediol glucuronide (PdG; dilutions 1:50–1:5000) by direct assays, and for estriol (E_3; dilutions 1:2–1:300) after enzyme hydrolysis. Fecal samples (total amount collected) were lyophilized and pulverized as described by Heistermann et al. [29]. A sample of the resulting powder representing 0.05–0.10 g dry weight was then extracted with 5 ml 40% methanol in water by vortexing for 15 min in a 15-ml polypropylene tube, followed by centrifugation at 2200 × *g* for 10 min. The supernatant was decanted into a clean glass tube and, after appropriate dilution in assay buffer (0.04 M PBS containing 0.1% BSA, pH 7.2), was taken directly to assay. Individual extraction efficiencies were monitored by the recovery of [^{3}H]progesterone (50 000 cpm; NEN Du Pont, Bad Homburg, Germany), which was added to the fecal powder before extraction. [^{3}H]Progesterone was routinely used as a recovery tracer irrespective of which hormone was to be assayed since all measurements were conducted from the same extract. The overall mean ± SD recovery was 66.3 ± 6.1% (n = 524). Since P_4 was the least polar of all hormones measured, recovery values were lower compared to the other hormones (75–85%). Reported values for fecal E_1C and E_3 are therefore slightly overestimated.

Microtiterplate enzymeimmunoassays (EIA) previously characterized by Heistermann and Hodges [30] were used to determine E_1C and immunoreactive PdG in urine. Relative to estrone-3-glucuronide (100%), significant cross-reactions were found only for unconjugated estrone (71%) and estrone-3-sulfate (17%), with values < 1% for all other steroids tested. Cross-reactions in the PdG assay relative to PdG (100%) were 22% for pregnanediol (Pd), 32% for 20α-hydroxyprogesterone, 14% for 5α-pregnane-20α-ol-3-one, and < 1% for all other steroids tested. For E_3 determinations, 50-μl aliquots of urine were hydrolyzed in 300 μl 0.1 M sodium acetate buffer (pH 4.7) containing 2500 U β-glucuronidase/sulfatase from Helix pomatia (no. G-1512; Sigma Chemie, Deisenhofen, Germany). Hydrolysis was carried out overnight in a shaking water bath at 37°C. After hydrolysis, samples were diluted with assay buffer and taken directly to assay (see below). The efficiency of each hydrolysis procedure was determined in parallel by monitoring the recovery of 10 000-cpm [^{3}H]estrone glucuronide (Dept. of Biochemistry, University College London, London, England) added to 5 control samples containing 50 μl urine from a late pregnant animal. The mean recovery of the controls (87.4 ± 4.3%, n = 25) after extraction was then used to correct the hormone values obtained in the E_3 assay. All hormone values were indexed by creatinine (Cr) to compensate for the variations in the volume and concentration of the voided urine [30].

Fecal methanol extracts were assayed for immunoreac-

tive E_1C (dilutions 1:2–1:50, depending on the reproductive status) in the same EIA used for the measurement of E_1C in urine. Measurements of E_1C were chosen for monitoring estrogen dynamics in feces because previous investigations showed that fecal E_1C showed a higher correlation with urinary E_1C than either unconjugated estrone or estradiol [31]. Fecal extracts were also assayed for immunoreactive P_4 (dilutions 1:2–1:100) and Pd (dilutions 1:5–1:500) in enzymeimmunoassays previously characterized by Heistermann et al. [29]. The cross-reactivity of the P_4 antiserum relative to P_4 (100%) was 74% for 11α-hydroxyprogesterone, 43% for 5-pregnen-3,20-dione, 14% for 5α-dihydroprogesterone, 2.4% for pregnenolone, and < 0.1% for all other steroids tested. The same assay as used for PdG in urine was used to measure Pd in feces, except that free Pd was used to make up the standard curve. Immunoreactive E_3 in hydrolyzed urine and nonhydrolyzed fecal extracts (dilution 1:2–1:100) was determined by RIA using an antiserum raised in sheep against estriol-6-CMO-ovalbumin (Guildhay Antisera Ltd., Guildford, UK) and [^{3}H]estriol as tracer (100 μl, 10 000 cpm; NEN Du Pont). The antiserum showed the following cross-reactivities relative to E_3 (100%): 3.9% for 16-epiestriol, 0.2% for 17-epiestriol, and < 0.2% for all other steroids tested, including estrone-3-glucuronide, estrone-3-sulfate, estradiol-17β, and estrone. In brief, hydrolyzed urine or fecal extracts were diluted in assay buffer (0.1 M PBS containing 0.1% gelatin; pH 7.0), and 100-μl aliquots were assayed in duplicate along with E_3 standard (100 μl, 7.8–1000 pg/tube) in an overnight incubation at 4°C. Dextran-coated charcoal (0.05% and 0.5%, respectively) was used to separate free from bound steroid. Sensitivity of the assay determined at 90% binding was 8 pg per tube.

Validation of Hormone Measurements

With the exception of E_1C in feces, serial dilutions of urine and fecal extracts from samples of the follicular and luteal phase and different stages of pregnancy gave displacement curves parallel to those obtained with the appropriate standards. Accuracy was assessed by determining the recovery of known amounts of pure hormone (five doses) added in quadruplicate to urine or feces containing low levels of endogenous hormones. Mean ± SD recovery values ranged from 85.5 ± 10.5% for urinary PdG to 133.4 ± 26.3% for fecal Pd; recovery values for all other steroids were within these ranges.

Intra- and interassay coefficients of variation calculated from replicate determinations of urine and fecal quality control pools gave values between 6.3% (urinary E_1C) and 16.2% (fecal Pd), and 8.1% (fecal P_4) and 14.9% (urinary E_1C), respectively. To assess specificity of fecal hormone measurements, HPLC of samples from the follicular and luteal phase of the menstrual cycle and early and late pregnancy was performed. Steroids were separated by reverse-phase HPLC using a Nova-Pak C18 column (3.9 mm × 150 mm; Millipore Corp., Milford, MA) and acetonitrile: water mixtures as eluents according to the method described by Heistermann et al. [29]. Profiles of immunoreactivity confirmed the presence of all steroids measured (indicated by co-elution with added tritiated authentic tracers), but indicated for each assay the presence of additional immunoreactive peaks, particularly for the progestin determinations. Measurements of all fecal steroids were thus nonspecific, and fecal hormone values reported throughout this study are therefore expressed as immunoreactivity.

Analysis of Data

The patterns of excreted hormones were used to define the lengths of the follicular and luteal phases of the menstrual cycles and the day of ovulation. The follicular phase was defined as the interval between the first day of menstruation until the day of the preovulatory urinary E_1C peak (Day 0), whereas the luteal phase comprised the period from the day after the E_1C peak until the day before the next menstruation. On the basis of the finding that the urinary E_1C peak in primates usually occurs 0–1 day before the LH peak in serum (e.g., [32–35]), the day of ovulation was defined as the day following the E_1C peak. A threshold value of two standard deviations (SD) above the mean of the preceding follicular phase values was taken in order to determine the day of the first significant postovulatory increase in urinary and fecal progestin concentrations. Mean hormone levels in conception cycles were considered significantly different from those of nonconception cycles when they were consistently greater than 2 SD of the corresponding mean value of nonconception cycles. An increase above this threshold value indicates a statistically significant rise with $p < 0.05$ [36, 37].

Pearson product-moment correlation coefficients were calculated for the relationships between urinary and fecal hormone concentrations during individual ovulatory cycles. In order to obtain information on a possible time lag in the excretion of fecal hormones compared to their urinary counterparts, calculations were conducted by aligning the values 1) by the calendar day on which samples were collected (unadjusted) and 2) by adjusting for a time lag of 1 and 2 days.

Changes in female perineal swelling patterns were analyzed with respect to the lengths of the different swelling stages and their relationship to periovulatory endocrine events. Three periods of swelling were defined: 1) premaximum swelling phase: extends from the first day of swelling (stage 2) to the day before the onset of maximum tumescence (stage 3); 2) maximum swelling phase: comprises the period in which the female shows maximum tumescence (stage 3); 3) detumescence phase: extends from the first day after the end of maximum swelling until the day before full detumescence (stage 1) is reached. In order to examine the temporal relationship between changes in sex skin swelling, timing of ovulation, and onset of the luteal phase, the onset of both maximum swelling and detumescence was related to the preovulatory urinary E_1C peak and the day of the significant postovulatory urinary PdG rise. The relationship between swelling scores and urinary E_1C concentrations during the follicular phase was examined by using the Spearman rank correlation test.

RESULTS

Endocrine Changes during the Menstrual Cycle

Representative profiles of immunoreactive urinary E_1C and PdG in relation to the pattern of perineal swelling during two complete menstrual cycles of an individual female are depicted in Figure 1 (left). All profiles revealed a cyclic pattern in which the follicular and luteal components of the cycle could be clearly distinguished, with the exception of the anovulatory cycle, which did not show a luteal phase progestin increase. Mean levels of E_1C calculated over the 7 cycles studied increased 5- to 7-fold during the follicular phase, from baseline levels of 20–30 ng/mg creatinine to preovulatory peak values of 140 ng/mg creatinine. There-

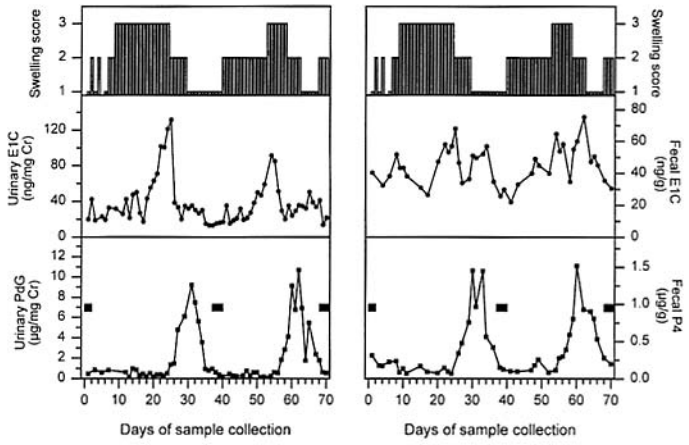

FIG. 1. Hormonal profiles of 1) E_1C and PdG in urine (left) and 2) immunoreactive E_1C and P_4 in feces (right) in relation to the stage of perineal swelling during two consecutive menstrual cycles in an individual female bonobo. Black bars indicate periods of menstruation.

after, concentrations declined rapidly to reach low follicular phase values within 3 days (see Fig. 1). In 5 of the 7 cycles a secondary increase in E_1C concentrations was seen during the luteal phase. PdG concentrations were consistently low during the follicular phase (0.5–1.0 µg/mg creatinine) but rose significantly 0–4 days (mean: 2 days) after the E_1C peak to reach maximum concentrations of 10–20 µg/mg creatinine on Days 8–10 of the luteal phase (Fig. 1, left). Thereafter, PdG levels began to decline and had returned to baseline values by the second or third day of menstruation.

The patterns of excretion of immunoreactive E_1C and P_4 in feces throughout the menstrual cycle are shown in Figure 1 (right) for the same two individual cycles for which urinary hormone profiles are depicted. Fecal P_4 measurements showed a well-defined pattern throughout the cycle with consistently low levels of 0.05–0.20 µg/g during the follicular phase and elevated concentrations with a maximum of 0.80–1.5 µg/g in the postovulatory period. Qualitatively similar profiles with a follicular/luteal phase shift of similar magnitude as observed for P_4 were obtained for fecal Pd excretion, but the data are not shown. Average *r* values for paired comparisons of urinary PdG levels to fecal P_4 and Pd were both highest (0.80 and 0.84, respectively) when adjusted for a time lag of 1 day (Table 1). For all individual cycles, progestin correlations were statistically significant, whether unadjusted or adjusted for a time lag, with the exception of cycle #DZ1 when adjusted for a 2-day lag. On the basis of the degree of correlation, the time lag between fecal P_4 and urinary PdG excretion was 0 days in two cycles, 1 day in three cycles, and 2 days in one cycle, whereas that between fecal Pd and urinary PdG excretion was 1 day in four cycles and 2 days in two cycles (Table 1).

The average *r* values for paired comparisons of urinary E_1C to fecal E_1C levels were 0.49 when unadjusted and 0.46 and 0.38 when adjusted for a time lag of 1 and 2 days, respectively (Table 1). Correlation coefficients between urinary and fecal estrogen concentrations, however, were statistically significant in only 3 of the 6 cycles. On the basis of the degree of correlation, a time lag of one day was found in 2 cycles, whereas in the third, the highest correlation coefficient was found for unadjusted values. Despite the significant correlation between urinary and fecal E_1C levels, however, because of a high day-to-day variability in all cycles studied, the pattern of fecal E_1C excretion was less clear, and a distinct preovulatory fecal E_1C peak could usually not be seen.

The intermenstrual intervals of the 6 ovulatory nonconception cycles and the lengths of their component phases are shown in Table 2. Whereas the cycles of the nonlactating females were between 31 and 37 days in length, with a follicular phase ranging between 17 and 26 days, the lactating female (HO) showed a prolonged cycle of 51 days due to an extended follicular phase of 40 days. A prolonged follicular phase length (34 days) was also seen in 1 of the 3 conception cycles. In contrast to the considerable variation in follicular phase lengths, the length of the luteal phase was relatively consistent, with a range of 11–15 days over the 6 menstrual cycles studied.

Endocrine Changes during Pregnancy

All three pregnancies were carried to term and resulted in the birth of healthy singleton offspring. However, the infant of the nulliparous female died 8 days after birth from starvation.

TABLE 1. Correlation coefficients (*r*) for the relationship between urinary and fecal hormone concentrations during individual menstrual cycles in bonobos.[a]

	Cycle identification number							
	KO	HO	HE	DZ1	DZ2	DZ3	Mean	SD
Urine PdG: Feces P_4								
Unadjusted[b]	0.65	0.71	0.69*	0.89	0.84	0.86	0.77	0.10
Adjusted for 1-day lag[c]	0.73	0.78	0.83	0.84	0.87	0.74	0.80	0.06
Adjusted for 2-day lag[c]	0.72	0.59	0.92	0.32+	0.65	0.61*	0.64	0.20
Urine PdG: Feces Pd								
Unadjusted	0.73	0.74	0.64*	0.82	0.64	0.82	0.73	0.08
Adjusted for 1-day lag	0.83	0.78	0.85	0.94	0.80	0.86	0.84	0.06
Adjusted for 2-day lag	0.80	0.57	0.95	0.39+	0.74	0.87	0.72	0.21
Urine E_1C: Feces E_1C								
Unadjusted	0.26+	0.13+	0.56+	0.79	0.71	0.48+	0.49	0.26
Adjusted for 1-day lag	0.59	0.21+	0.16+	0.83	0.57*	0.39+	0.46	0.25
Adjusted for 2-day lag	0.57*	0.27+	0.62+	0.44+	0.21+	0.17+	0.38	0.19

[a] All *r* values given for individual cycles are statistically significant at $p < 0.01$ except where otherwise indicated either by * = $p < 0.05$ or + = not significant.
[b] Unadjusted = urinary and fecal hormone concentrations are aligned by sample date.
[c] Adjusted = urinary and fecal hormone concentrations are aligned by a 1- or 2-day time lag in fecal hormone excretion.

TABLE 2. Menstrual cycle characteristics in the bonobo.

Cycle identification	Cycle length (days)	Follicular phase length (days)	Luteal phase length (days)
KO	33	18	15
HE	37	26	11
DZ1	31	18	13
DZ2	37	25	12
DZ3	31	17	14
HO[a]	51	40	11
Mean ± SEM	33.8 ± 1.4	20.2 ± 1.7	13.0 ± 0.7

[a] Female was lactating, data not included in calculation of means.

Figure 2 shows the composite profiles of urinary and fecal estrogen and P_4 metabolites during the 3 conception cycles in comparison to those of the 6 ovulatory nonconception cycles. Mean urinary E_1C and PdG levels in pregnant animals were significantly elevated ($p < 0.05$) compared with those in nonpregnant animals by Days 14 and 12 after the E_1C peak, respectively. Although the differences between levels in conception and nonconception cycles were less pronounced for fecal hormones, significant elevations in mean fecal hormone concentrations during conception cycles occurred by Days 19 (E_1C) and 13 (P_4) after the urinary E_1C peak ($p < 0.05$). Profiles of immunoreactive estrogen and P_4 metabolites in urine and feces throughout gestation are shown for one of the three pregnancies in Figure 3. Concentrations of all urinary and fecal steroids measured were clearly elevated during the course of pregnancy, with maximum levels occurring in the month before parturition. Levels of all hormones declined to values within the nonpregnant range within a few days after birth.

Although excretion patterns of estrogens and progestins in feces were highly correlated with those in urine throughout gestation, fecal steroids showed a more modest increase. Figure 4 shows mean + SD concentrations of urinary and fecal steroids during early, mid-, and late pregnancy for individual females in comparison to levels from nonpregnant animals. Concentrations of urinary hormones were similar between animals at each stage of gestation, whereas levels of fecal steroids showed a higher variation between animals, particularly during late pregnancy. Compared to nonpregnant levels, concentrations of E_1C in both urine and feces showed the most rapid rise during early pregnancy (10- to 20-fold in urine, 3- to 5-fold in feces over Days 10–30), whereas the sustained rise in E_3 occurred later (from Day 50) but was more pronounced throughout gestation (urine: 200- to 500-fold, feces: 20- to 80-fold). Quantitatively, E_3 was also the most abundant estrogen in both urine and feces in the last month of pregnancy in all 3 females. In comparison to estrogens, urinary and fecal progestins showed a more modest increase during gestation (urine: 10- to 15-fold,; feces: 3- to 25-fold). On the basis of the interval between the estimated day of conception (Day 1 after preovulatory urinary E_1C peak) to parturition, gestation lengths for the 3 females were 229, 234, and 238 days, respectively.

Relationship between Periovulatory Endocrine Changes and Perineal Swelling Patterns

In all 4 study females, the firmness and degree of wrinkling of the labia minora showed clear fluctuations during the cycle, although the maximal degree of wrinkling varied considerably between individuals. Regular cyclic changes were also observed for the size of the perineal swelling, and these accompanied the fluctuations in labial firmness. There was, however, a high degree of variation in the absolute size of the swelling seen in the 4 females, with the labia minora of the oldest female retaining considerable mass and size even when they were completely detumescent and extensively wrinkled. Despite considerable variability in female swelling characteristics, however, minimal, partial, and maximal stage of perineal tumescence could be reliably distinguished (see Fig. 1; for definitions see *Materials and Methods*). Table 3 presents the data on the durations of the different swelling phases and on the temporal relationship between changes in swelling patterns and the

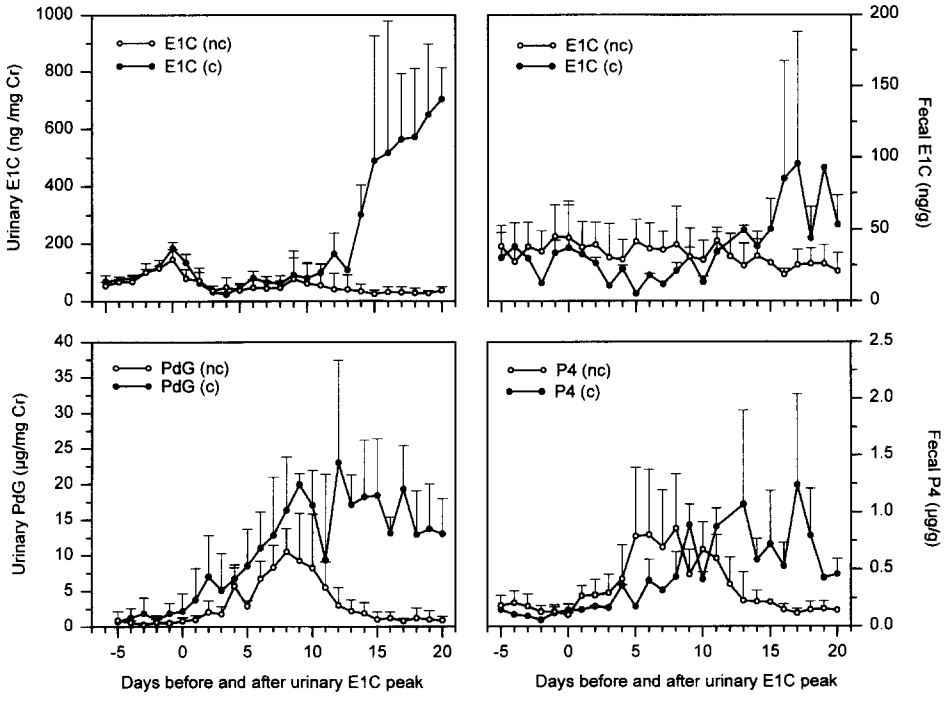

FIG. 2. Composite profiles (mean + SD) of 1) E_1C and PdG in urine (left) and 2) immunoreactive E_1C and P_4 in feces (right) during ovulatory nonconception cycles (nc, n = 6) and conception cycles (c, n = 3) in female bonobos. See text for explanations.

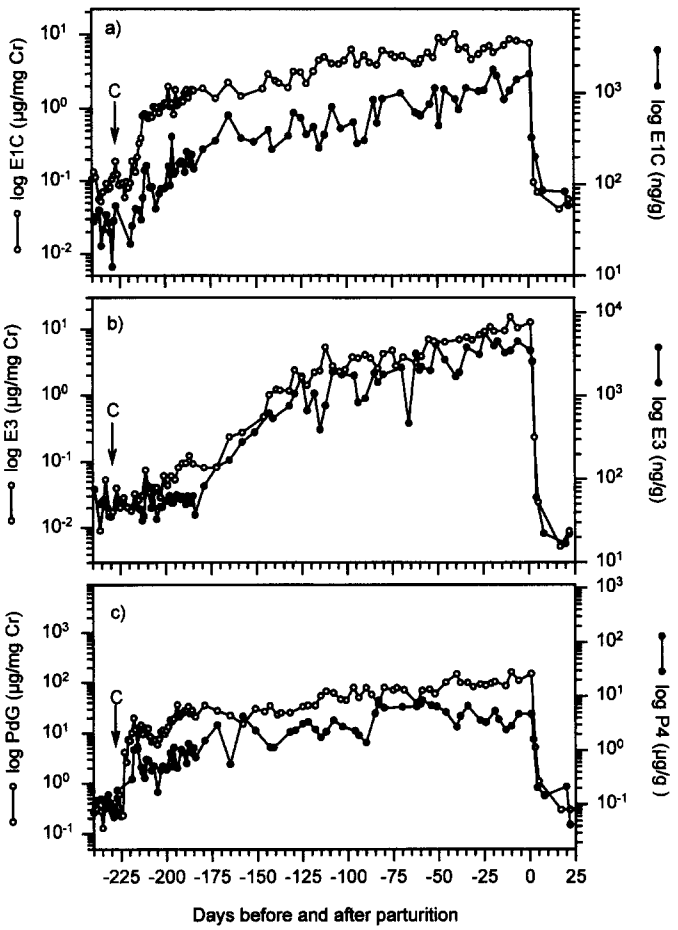

FIG. 3. Hormonal profiles of urinary and fecal **a**) immunoreactive E_1C, **b**) E_3, and **c**) PdG and P_4 during a full-term pregnancy in an individual female bonobo. Arrows indicate the presumed day of conception (C). Note the logarithmic scales for all hormone measurements.

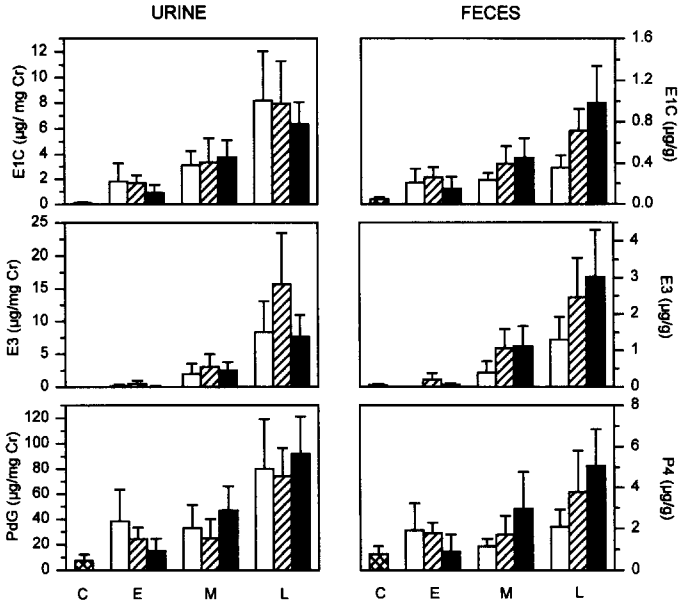

FIG. 4. Mean ± SD concentrations of immunoreactive urinary (left) and fecal (right) estrogen and P_4 metabolites during early (E), mid (M), and late (L) pregnancy in the 3 pregnant females are compared to late follicular phase concentrations (E_1C, E_3) and midluteal phase levels (PdG, P_4) of ovulatory nonconception cycles (C, n = 6). Each of the three stages of pregnancy comprised one third (76–79 days) of respective gestation periods for individual females.

periovulatory endocrine events. The first signs of swelling occurred on average 5.0 days (range: 1–15 days) after the onset of menstruation. Females were then partially swollen for a period of 3–18 days (mean: 9.3 days) before reaching the stage of maximum tumescence. Duration for which maximum swelling was recorded was highly variable, ranging from 4 to 26 days (mean: 11.5 days), but was significantly correlated ($r = 0.76$, $p < 0.05$) with the length of the follicular phase of the corresponding cycle. On average, the period of maximum tumescence comprised 34.4 ± 5.6% (range: 12–45%) of the length of the menstrual cycle. In the majority of cycles studied, detumescence did not occur abruptly, and some significant level of labial swelling was maintained for up to 7 days (mean: 4.1 days, range: 1–7 days). Thus, in total, the period in which a female showed signs of swelling was variable but extremely long (mean: 23.8 days, range: 17–39 days), representing on average 66.3 ± 3.5% (range: 55–74%) of total cycle length.

In relation to endocrine changes, timing of the onset of the maximal swelling phase varied considerably among females and cycles, occurring 1–21 days (mean: 8.4 days) before the preovulatory urinary E_1C peak. In contrast, timing of detumescence was less variable and more closely related to periovulatory endocrine events, occurring 0–7 days (mean: 3.1 days) after the E_1C peak and 0–3 days (mean: 1.0 days) after the significant luteal phase urinary PdG rise. In both duration of swelling phases and relationship of swelling to endocrine events, nonconception cycles did not apparently differ from conception cycles (see Table 3).

A plot of mean E_1C concentrations against the three swelling stages during the follicular phase of individual ovulatory (nonconception and conception) cycles is shown in Figure 5. Concentrations of E_1C and the degree of variation were similar for stages 1 (range: 15.4–55.7 ng/mg creatinine) and 2 (range: 25.0–66.4 ng/mg creatinine), while at the stage of maximum swelling, E_1C levels were highest but also more variable between cycles (range: 51.9–138.8 ng/mg creatinine). Least-square regression analysis between individual log E_1C concentrations and swelling scores revealed a correlation coefficient of 0.78, showing that there was a significant positive correlation ($p < 0.01$) between the two variables.

DISCUSSION

The present study provides the first detailed information on the reproductive endocrinology of the female bonobo and shows that the measurement of estrogen and P_4 metabolites in both urine and feces are reliable methods for assessing reproductive function in this species. More importantly, by generating comprehensive data on hormonal changes that accompany the phenomena of ovarian cyclicity, conception, pregnancy, and parturition and by relating endocrine changes to the pattern of perineal swelling, the present paper significantly extends our knowledge of the basic reproductive physiology of the species.

Measurements of E_1C and PdG in urine were used to assess endocrine changes during the ovarian cycle since these hormones are known to accurately reflect changes in corresponding circulating hormones in other species of great apes and in the human [32–34, 38]. Our results confirm that both hormones are abundant steroid metabolites excreted into the urine during the ovarian cycle and demonstrate that—as in other primates [20, 30, 33, 34, 39–

TABLE 3. Duration of perineal swelling phases and their relation to periovulatory endocrine changes in individual cycles of bonobos.

Cycle identification	Duration of premaximum swelling[a] (days)	Duration of maximum swelling[a] (days)	Duration of detumescence[a] (days)	Duration of total swelling (days)	Onset of maximum swelling in relation to E_1C peak (days)	Onset of detumescence in relation to	
						E_1C peak (days)	PdG rise (days)
KO1	12	4	2	18	−2	+2	0
HE	—[b]	14	7	21	−9	+5	+1
DZ1	3	14	6	23	−12	+2	0
DZ2	4	16	5	25	−16	0	0
DZ3	13	7	3	23	−1	+6	+3
HO[c]	8	23	5	36	−16	+7	+2
KO2[d]	9	7	1	17	−4	+3	—[d]
CC1[e]	6	26	7	39	−21	+5	+1
CC2[e]	18	4	2	24	−2	+2	+2
Mean ± SEM	9.3 ± 2.0	11.5 ± 2.7	4.1 ± 0.9	23.8 ± 2.4	−8.4 ± 2.6	3.1 ± 0.7	1.0 ± 0.4

[a] See *Materials and Methods* for definitions.
[b] No data available.
[c] Female was lactating, data not included in calculation of means.
[d] Anovulatory cycle, no urinary PdG rise following E_1C peak.
[e] Conception cycles, data for third conception cycle not available.

42]—their measurement provides reliable information on follicular development and luteal function. From the pattern of estrogen and progestin excretion, it was possible not only to confirm a cycle length of approximately 34 days (within the range previously reported: [10, 25–27, 43]) but also to provide for the first time information on the timing of ovulation and thus the length of the component phases of the menstrual cycle. A relatively constant postovulatory period (11–15 days) and a lengthy but highly variable preovulatory period (17–34 days) is in line with data from other species of primates [20, 33, 44], showing that the majority of variation in cycle length is primarily due to variation in the length of the follicular phase. The limited data on the lactating female, which, despite showing similar hormonal patterns, showed the longest follicular phase (40 days) resulting in a prolonged cycle (51 days), suggests that lactation might be one of the factors acting on follicular phase length variability in bonobos.

As recently shown for other primate species [29, 39, 41, 42, 45, 46], the present study demonstrates that monitoring of cyclic ovarian function in the bonobo is also possible by the measurement of hormones in feces. The patterns of immunoreactive fecal P_4 and Pd were similar in all cycles studied, and levels of both were significantly correlated with urinary PdG concentrations, indicating that in the bonobo, measurement of either steroid in feces can be reliably used to monitor corpus luteum function (see also [29, 39]). Concerning time lag of excretion, both fecal progestins showed on average a delay of 1 day compared to the excretion of PdG into the urine, although there was a slightly smaller range in the delay of fecal Pd excretion.

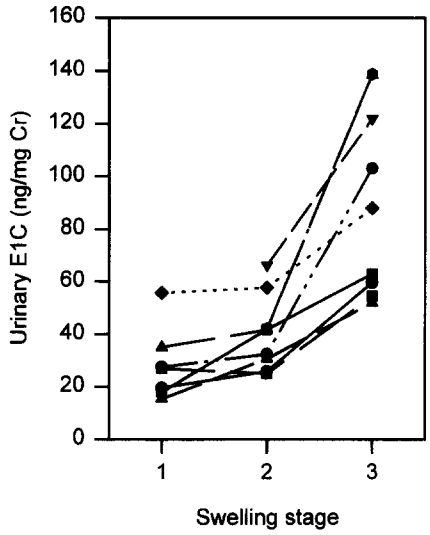

FIG. 5. Concentrations of urinary E_1C in relation to perineal swelling stages during the follicular phase of ovulatory cycles. Each symbol represents the value of an individual cycle.

In contrast to the high degree of correspondence between urinary and fecal progestins, measurements of E_1C in feces and urine were significantly correlated in only 50% of the cycles studied. Furthermore, because of a considerable day-to-day variability, the pattern of fecal E_1C excretion was generally not well defined, and a preovulatory E_1C peak could usually not be seen. There are four possible explanations for this finding. First, the E_1C measurements showed a high cross-reactivity with unconjugated estrone, which was present in fecal extracts but appeared not to be excreted in a cyclic pattern [31]. Second, parallelism was not obtained in fecal E_1C measurements, suggesting the interference of nonspecific compounds in the assay. Third, as in other primates [47, 48], estrogens in feces might represent only a small proportion of excreted total estrogens, the bulk being eliminated in the urine. And last, E_1C in feces might not, even measured with a more specific assay, best reflect estrogen secretory dynamics. Since measurements of unconjugated fecal estradiol were no better correlated with urinary E_1C profiles [31], future studies are needed to clarify whether a different fecal estrogen measurement provides more accurate information on ovarian function.

Apart from describing endocrine profiles during the ovarian cycle, the present study also provides useful data on urinary and fecal hormone excretion during pregnancy. Measurements of E_1C in both urine and feces showed the most rapid postconception increase and therefore were most useful in early pregnancy diagnosis. Furthermore, in the absence of any overlap between values in nonpregnant and pregnant animals beyond 4 wk after conception, E_1C measurements should be of diagnostic value and provide a useful alternative to chorionic gonadotropin measurements for detection of early pregnancy. Also of interest was increasing excretion of E_3 in both urine and feces from approxi-

mately Day 50 of gestation, a phenomenon characteristic of all anthropoid primates [49, 50]. E_3 was in fact the major estrogen during the last month of gestation, confirming the findings from a single incomplete pregnancy reported by Czekala et al. [18]. Since the formation of E_3 in anthropoid primates is largely dependent upon 16α-hydroxylase activity on fetal-derived dehydroepiandrosterone before aromatization [49], E_3 can be of diagnostic value in assessing fetal viability and for staging pregnancy [49–51]. Furthermore, since E_3 was barely detectable in nonpregnant animals and showed the most pronounced increase during gestation, measurement of E_3 excretion in urine or feces can be considered as most reliable in confirming that a female is pregnant. Since occasional measurements are sufficient to serve this function, E_3 determinations in feces are of particular value in diagnosing and monitoring pregnancy in free-ranging bonobos, from which frequent samples might be difficult to obtain.

The present study also examines for the first time the relationship between the pattern of female sex skin swelling and underlying hormonal changes during the ovarian cycle. This was done in order to obtain information on the timing of ovulation within the swelling period and thus on the reliability of perineal swelling as an external indicator of the menstrual cycle stage. Concerning the duration of perineal swelling, which on average was present during two thirds of the menstrual cycle, our results confirm the findings of previous reports [10, 25–27] that duration of swelling in the bonobo is variable but nevertheless markedly longer than in other primate species studied so far. In several animals, the majority of the overall swelling period comprised the phase of maximum tumescence, which was first observed in the mid- to late follicular phase and extended into the beginning of the luteal phase. The swelling scores in the follicular phase were significantly correlated with urinary estrogen levels, while the process of detumescence in the luteal phase was associated with rising urinary progestin concentrations. As found in other primates that exhibit conspicuous sex skin swelling [10, 20, 52–54], perineal tumescence in bonobos thus also appears to be estrogen-related, while detumescence appears to be enhanced by rising progestin concentrations.

By using the pattern of urinary E_1C and PdG as independent markers of the female's cycle stage, it was possible to relate the characteristic changes in perineal swelling patterns of each cycle to the time of presumed ovulation. In all but one cycle, the preovulatory urinary E_1C peak occurred within the phase of maximum tumescence, indicating that ovulation in bonobos usually takes place within this swelling stage. Timing of ovulation (defined as the day after the urinary E_1C peak) during the maximum swelling period, however, was highly variable and was more closely associated with the end rather than with the onset of maximum tumescence. On the basis of the present data it thus appears that daily observations of changes in the size and degree of wrinkling of the sex skin alone are not reliable for detecting or predicting the time of ovulation in the bonobo. It is possible that a more detailed assessment of swelling changes, using other criteria and a more graduated scoring system (e.g., [10]) than the one used in this study, might reveal a closer relationship between anogenital swelling changes and endocrine events, as seen in other primate species such as the chimpanzee [10, 19], gibbon [54], and baboon [24]. Continuous observations of the pattern of perineal swelling as described here, however, are nevertheless useful for estimating the stage of the menstrual cycle and for retrospectively indicating the periovulatory period, since onset of detumescence occurred on average 3–4 days after the preovulatory urinary estrogen peak and 1 day after the luteal phase urinary PdG rise.

In association with the extended period of perineal sex skin swelling, female bonobos show a prolonged period of sexual activity during the menstrual cycle [6, 9, 25, 27]. This has been taken to suggest that ovulation in bonobos might be concealed [28]. The poor temporal relationship between the onset and end of maximum swelling and the time of ovulation found in the present females would support this contention, at least if one assumes that the approach to ovulation in bonobos is advertised by changes in the swelling, as it is in chimpanzees [10]. Although there is some evidence from both captive [9] and wild animals [25] that reproductive behavior is predominantly associated with the phase of maximum swelling, a detailed study on the distribution of mating and socio-sexual behaviors throughout the cycle and their relation to the different pattern of swelling and particularly the time of ovulation has not been conducted. This, however, would be needed to answer the question whether male bonobos are able to detect the time of ovulation in the female or whether ovulation is in fact concealed. Unless this information is available, the function of female bonobo perineal swelling and its significance in the regulation of male-female interactions remains unanswered. With the information and practical tools provided by the present study, however, this question can now be appropriately investigated. Furthermore, the use of perineal swelling as an external indicator of a female's cycle stage offers the opportunity to more effectively plan and carry out timed matings between animals whose genes are underrepresented in the captive gene pool. By temporarily separating particular animals from the group to create a preferred mating combination during the presumed periovulatory period, it is possible to control the paternity and thus enhance the genetic diversity, which is the most important aspect to be managed in the captive bonobo population [55]. The breeding control through this simple and relatively noninvasive procedure will allow bonobos to be kept in more natural groups while at the same time ensuring the long-term genetic health of the captive population.

In conclusion, the availability of reliable noninvasive methods for monitoring reproductive function in female bonobos offers new opportunities for improving the captive breeding management and, furthermore, combining behavioral and physiological studies on both animals in captivity and in the wild. This will hopefully lead to a better understanding of the reproductive biology of this rare and endangered species of great apes.

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