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ORIGINAL ARTICLE

Chronic levonorgestrel treatment in *Macaca sylvanus*: effects on perineal swelling size and fecal sex steroid excretion

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Abstract

OBJECTIVE: Administered levonorgestrel enables contraception under normal cyclicity of the ovaries and does not suppress the development of cyclic perineal swellings. This study investigated the influence of levonorgestrel on fecal gonadal steroid excretion rates and the expression of perineal swelling size.
SETTINGS: Two groups of Barbary macaque females were observed: Twenty-four implanted individuals under semifree conditions and five non-implanted individuals under caged conditions. Eight of the implanted females had large expressions of the perinea and sixteen reduced. The non-implanted group had no perineal swellings.
METHODS: To determine the individual steroid excretion rates an enzyme-immunoassay for fecal samples was established.
RESULTS: Estradiol excretion rates did not differ in distinctively swollen implanted females, but were increased when compared to non-implanted individuals (df=2; p=0.0002). Implanted females with large perineal swellings had lower progesterone concentrations in the feces compared to individuals with reduced swellings and did not differ from the non-swollen group (df=2; p=0.054). Females with large perineal swellings showed a higher calculated estradiol to progesterone ratio index than the other groups (df=2; p=0.0005). Non-implanted individuals showed increased testosterone excretion rates (df.=2; p=0.0001).
MAIN FINDINGS: The results indicate a positive relationship between the perineal swelling size and levonorgestrel implantation.
CONCLUSION: The ratio of fecal estradiol and progesterone titers can be judged as an endocrine indicator for the expression rate of perineal swelling size.

INTRODUCTION

The contraceptive function of the synthetic gestagen levonorgestrel (LNG) has proven to be an effective agent in humans and primates. Its contraceptive effect can be exerted in different ways, such as the inhibition of follicular development and therefore ovulation, or by changes in the cervical mucus negatively influencing sperm transport or implantation suppression via endometrial changes [1]. Beyond these effects, its influence on the sex steroid secretion rate is not yet perfectly understood. Studies in LNG-implanted humans suggest that serum estradiol and progesterone changes may be the endocrine mechanism responsible for suppression of ovulation. Low estradiol levels were observed during the first two years of use, while the percentage of normal fluctuating estradiol levels increases with duration of use. These effects may be due to persistent or absent follicular development, which maybe followed irrespectively by luteal activity or inactivity. Even cellular luteinization along with elevated progesterone levels are not reliable markers for ovulation in LNG users. However, during exposure to LNG, physiological interference with sex hormone-binding globulins (SHBG) is detectable resulting in free estradiol and testosterone concentrations [2–7].

Morphological effects of LNG on secondary sex characteristics such as perineal swelling in non-human primates remain also partially unclear. During the follicular phase in intact promiscuously living primate species, the perineum of gradually increases in tumescence, culminating in its maximum extension at ovulation. This is usually followed by a relatively rapid detumescence under normal ecological and cycling conditions. Enlargement is linked to the estradiol secretion and detumescence to progesterone secretion [8]. In chimpanzees LNG appears to shorten the period of full swelling size while lengthening the period of partial swelling. LNG provides effective contraception without inhibiting the ovarian steroid secretion rates of estradiol and progesterone [4].

The present study investigates LNG-implanted (housed under semifree conditions) and non-implanted (housed under caged conditions) Barbary macaque females during their non-sexual phase of the year. The Barbary macaques are seasonal breeders. The females show cyclic changes in perineal swelling size during their active mating season in autumn and winter [9, 10]. The implanted individuals of our study group were characterized by conspicuous variation in perineal swelling size during the non-sexual phase of the year after implantation. The non-implanted group showed no signs of perineal enlargement during sexual inactivity but had the expected cyclic changes in perineal swelling size during the mating season. This report focuses on the question of whether LNG affects sex steroid concentrations and whether these effects are possibly linked to perineal swelling expression.

In order to make frequent and noninvasive endocrine measurements, the sex steroid levels were assessed from fecal samples. Enzyme immunoassays were used to determine the quantities of immunoreactive estradiol, progesterone, and testosterone.

METHODS

Subjects

The implanted study group consisted of 24 adult Barbary macaque females (from a group of 45 adult females and 31 adult males) living under semifree conditions in Affenberg Salem, Germany. The group was studied from March to July 1995, during the non-sexual phase of the year. The mean age of focal females was 10.5 (±4.6 SD) years. The diet of the animals consists of plants like beech, fir, pine trees and grass. Once a day this diet was supplemented with food such as wheat, fruits, vegetables and pellets. Water was available ad libitum.

The 24 adult females were implanted with the contraceptive levonorgestrel (Norplant control. no. 100006191; manufactured by Leiras, Turku, Finland). The subdermal implants were soft, flexible, opaque white silastic silicon capsules with a total length of about 34 mm; sealing plugs did not measure less than 1 mm. The hormone column length was 28.5–31.5 mm, with an almost white levonorgestrel powder column. Each column contained 70 mg of the synthetic progestin. The mean release rate was 14 μg/capsule/day. The capsules give effective contraceptive protection for five years. The inter-individual range of implantation duration was between one and four years.

The second group lived under caged conditions in Schönbrunn Zoo (Austria). This group consisted of five intact adult females (mean age 9.2±3.9 SD), one adult male, as well as two juvenile and two subadult males. The study was conducted from March to July 1994. The animals were fed daily with fruits, vegetables and a mixture of cooked corn. Once a week animal proteins (milk and eggs) and semolina-corn were supplemented. Water was available ad libitum. As both groups were not observed under experimental conditions the zoo group does not represent a control group. No manipulation of the groups was done irrespective allowed by the head of the park and zoo. Age and feeding patterns were comparable in both groups. Analogous to the study by Alvares et al. [2] the two groups can be categorized as users and non-users of LNG.

Swelling Measurement

Perineal swellings of all non-implanted and of the 24 implanted focal animals were assessed weekly – those of other females in the implanted group were measured monthly – for lateral and posterior video images. These Hi8 video camcorder images of the individuals were transferred to hard disk (using the Macintosh software DESKTOP TV 2.1).

The lateral and posterior areas were quantified pixelwise by using the software package IMAGE SXN (Macintosh). For each animal per week we obtained two ratios – the cross section of the swelling to the whole

body surface (skipping the perineal swelling) – once laterally and once posteriorly and summed these two values. This method allowed us to detect the frontal and lateral expression of the swellings relatively to the body surface irrespective of the distance between the camera position and the filmed individual. Both views, the lateral and the posterior, were strictly filmed frontal. The sum for all measurements of these two ratios was averaged for each individual, expressing the extent of the perineal swelling. The swelling size of each focal female stayed constant during the observation period.

In the implanted group we observed that 16 females had statistically significant smaller swellings (mean 16.9±1.4 SE) compared to the remaining 8 (mean 32.4±1.6 SE). Individuals of the non-implanted group showed no perineal expression (mean 8.2±0.5 SE) and were statistically different to the implanted females (one-way ANOVA and post hoc Bonferroni tested, df=2; $p<0.001$). The latter value includes only the measurements from the non-swollen circumanal area (posterior view), because laterally no swelling expression was detectable. Therefore, this value can be described as "background noise" of the measurement method.

Fecal sample collection

Feces of focal females in the LNG group were collected once a week from the ground between 01:00 p.m. and 03:00 p.m. immediately after excretion. Afternoon samples were preferred as these were not mixed with urine. In the zoo group the feces were collected three times per week between 01:00 p.m. and 03:00 p.m. These samples were also not mixed with urine. A total of 210 samples were collected from the semifree living group and 270 from the zoo group. After collection all samples were immediately frozen at –30 °C.

Extraction of feces and enzyme immunoassay

0.5 g feces per sample were extracted with 1.5 mL double distilled water and 3 mL methanol. The samples were vortexed for 30 min, followed by centrifugation at 1,500 × g for 15 min. A 10 µL aliquot of the supernatant was used for the assay. The analyses were performed using enzyme immunoassays.

The following antibodies and enzyme labels were used: for the estradiol assay, the antibody (17β-estradiol-17-HS:BSA) was diluted 1 : 200,000, the biotin label was (17β-estradiol-17-glucuonid : DAOO-biotin) diluted $1:10^7$. The estrone standard curve range used was from 80 pg to 0.32 pg (Palme and Möstl, 1993). For the testosterone assay the antibody used (testosterone-3-CMO : SA) was diluted 1 : 75,000, the enzyme label (5α-androstane-3α, 17β-diol-3-HS : DAOO-biotin) was diluted $1:5\times10^6$. The range of the tesosterone standard curve was between 80 pg and 0.32 pg. For the progesterone assay, the antibody used (5α-pregnane-3β-ol-20-one-3HS : BSA) was diluted $1:15\times10^3$, the enzyme label used (4-pregnene-3, 20-dione-3CMO : DAOO-biotin) was diluted $1:600\times10^3$. The standard curve range was between 500 pg and 2.05 pg. The detailed characteristics of the cross reactions are described elsewhere [11,12].

For pooled fecal samples, the intraassay coefficient of variation (CV) for estradiol was 11.7%, for testosterone 9.8%, and for progesterone 10.2% (n=35), while the interassay CV for estradiol was 15.1%, for testosterone 11.8%, and progesterone 12.4%, (n= 16).

High performance liquid chromatography (HPLC)

This method was used to validate the measured steroid concentrations. For HPLC separation 0.5 g of pooled fecal samples were extracted with methanol (4 ml, 80%) and the supernatants were evaporated under nitrogen. The residue was reconstituted with a 0.5 ml water/$NaHCO_3$ solution (5%) and re-extracted with diethylether (5 ml). The extracts were introduced into a Lichrosorb 10 µm Si60 25×0.4 cm^2 column (from Forschungszentrum Seibersdorf, Austria) and separated using a mixture of n-hexane/chloroform/methanol (60 : 40 : 1; v/v/v; linear methanol gradient up to 6%; flow rate 2 ml/min). Within 36 minutes, 75 fractions were collected. After separation, the organic solvents were evaporated and the residues redissolved in assay buffer. The concentrations of immunoreactive substances were measured in each fraction using immunoassays for estradiol, testosterone and progesterone.

Estradiol (E) - Progesterone (P) index

This index was taken from Hinde and Atkinson [13] and Hill [14], and calculated with the following formula:

$E\times(E+P)^{-1}\times100$

Values above the 50% level indicate proportionally higher E, those below 50% higher P levels.

Statistical Analysis

The hormonal data were averaged and means are given with the standard error of the mean. Comparisons between categories were made using one-way ANOVA with the post hoc Bonferroni test. Correlations were determined using the Pearson correlation coefficient (two-tailed) and the partial correlation coefficient. Statistical significance was assumed to be achieved at the 95% confidence level.

RESULTS

The estradiol immunoassay (Figure 1a) produced three peaks over the pattern of elution (fractions 21, 31, 34). The two major substances cross reacting with the assay corresponded to ^{3}[H] estrone (fraction 21) and ^{3}[H] 17ß-estradiol (fraction 34). Small amounts of 17α-estradiol (fraction 31) were measurable. The progesterone immunoassay showed three major peaks (Figure 1b). The cross reacting steroid in fraction 16 had a elution pattern similar to ^{3}[H] progesterone. None of the four eluted testosterone (Figure 1c) immunoreactive substances showed the same elution pattern as ^{3}[H] testosterone.

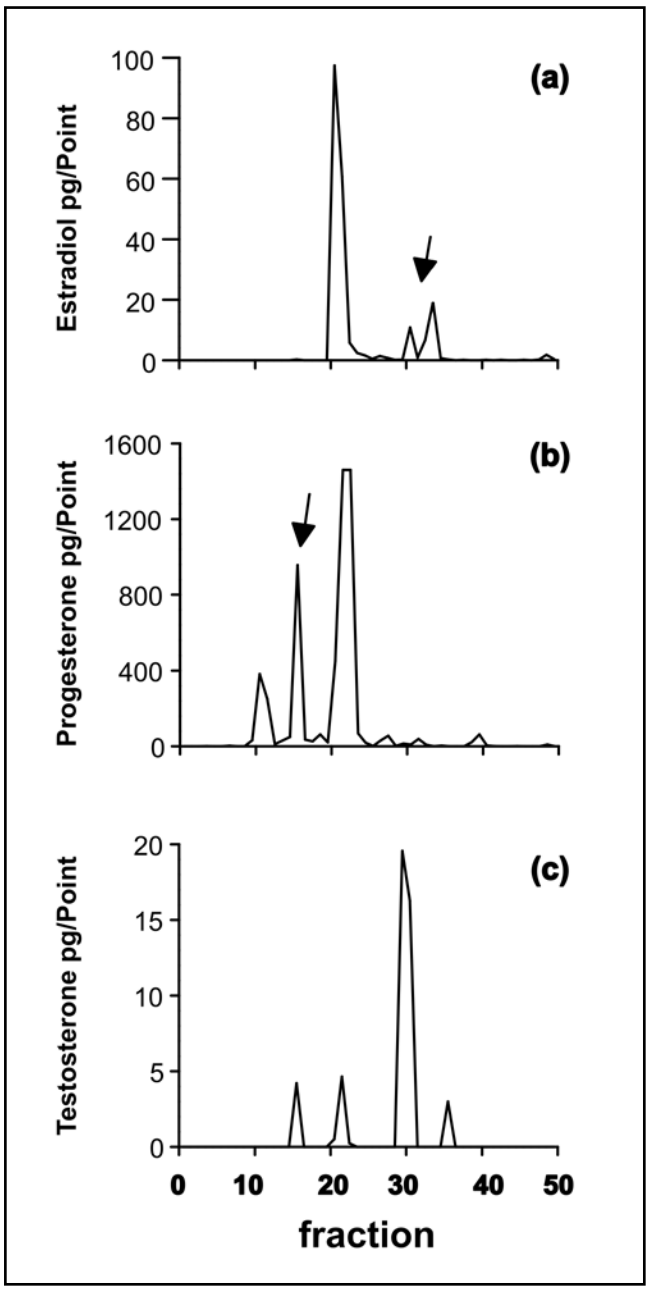

Figure 1. HPLC separation of immunoreactive amounts of (**a**) estradiol, (**b**) progesterone, and (**c**) testosterone steroids. Each fraction was analyzed with EIAs for estradiol, progesterone, and testosterone. (Arrows indicate in (**a**) elution patterns similar to ^{3}[H] 17ß and 17α-estradiol, in (**b**) elution patterns similar to ^{3}[H] progesterone.)

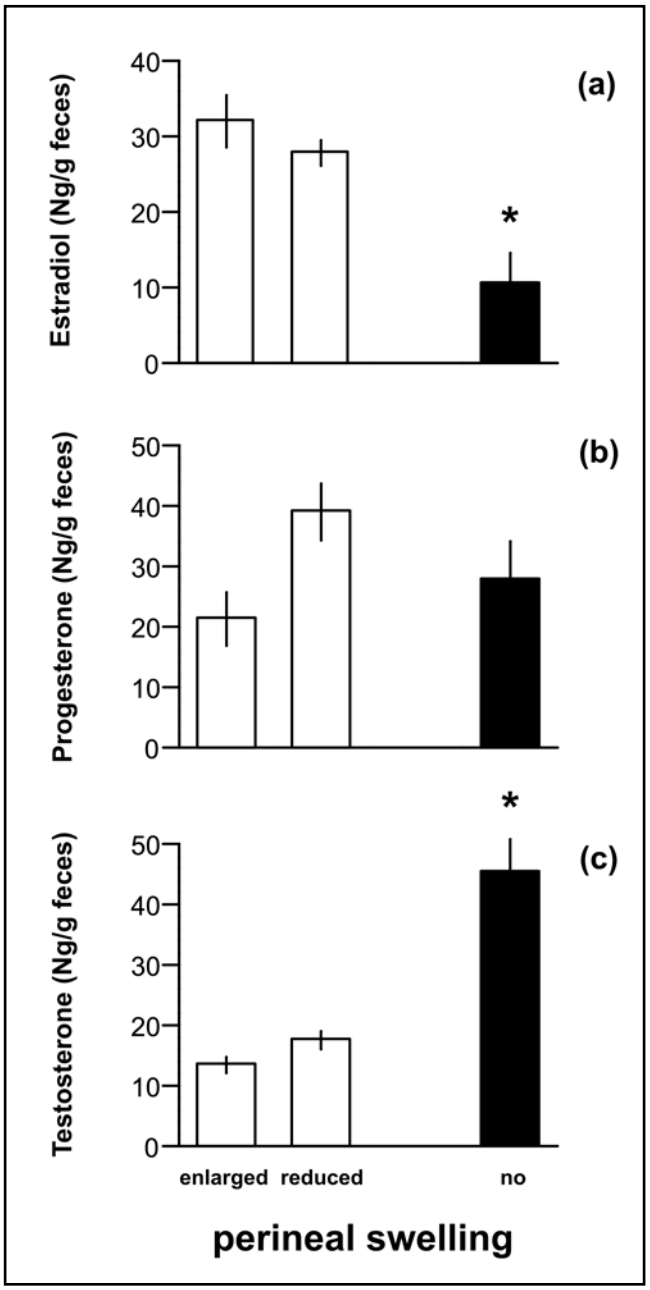

Figure 2. Mean differences between excreted (**a**) estradiol, (**b**) progesterone and (**c**) testosterone levels in LNG-implanted and non-implanted females. (Values are mean ± SE; one-way ANOVA; df=2; p<0.05; Bonferroni test; *statistically significant at 95% confidence level).
☐ - implanted group, ■ - non implanted group.

A comparison of fecal gonadal steroid excretion rates between the implanted and non-implanted group is shown in Figure 2. Both categories of implanted females, those with enlarged and reduced perinea, exhibited significantly higher fecal estradiol concentrations than the non-implanted zoo group (Figure 2a. df=2; F=12.616; p=0.0002; one-way ANOVA; enlarged vs. no, p<0.05; reduced vs. no, p<0.05; Bonferroni test). There was a marginally significant difference in the excreted fecal progesterone concentrations among the three groups (Figure 2b. df=2; F=3.329; p=0.054; one-way ANOVA). With regard to the testosterone metabolites, there was a highly significant difference between both implanted categories and the non-implanted zoo group (Figure 2c. df=2; F=37.146; p=0.0001; one-way ANOVA; enlarged vs. no, p<0.01; reduced vs. no, p<0.01; Bonferroni test). Non-implanted individuals excreted more fecal testosterone.

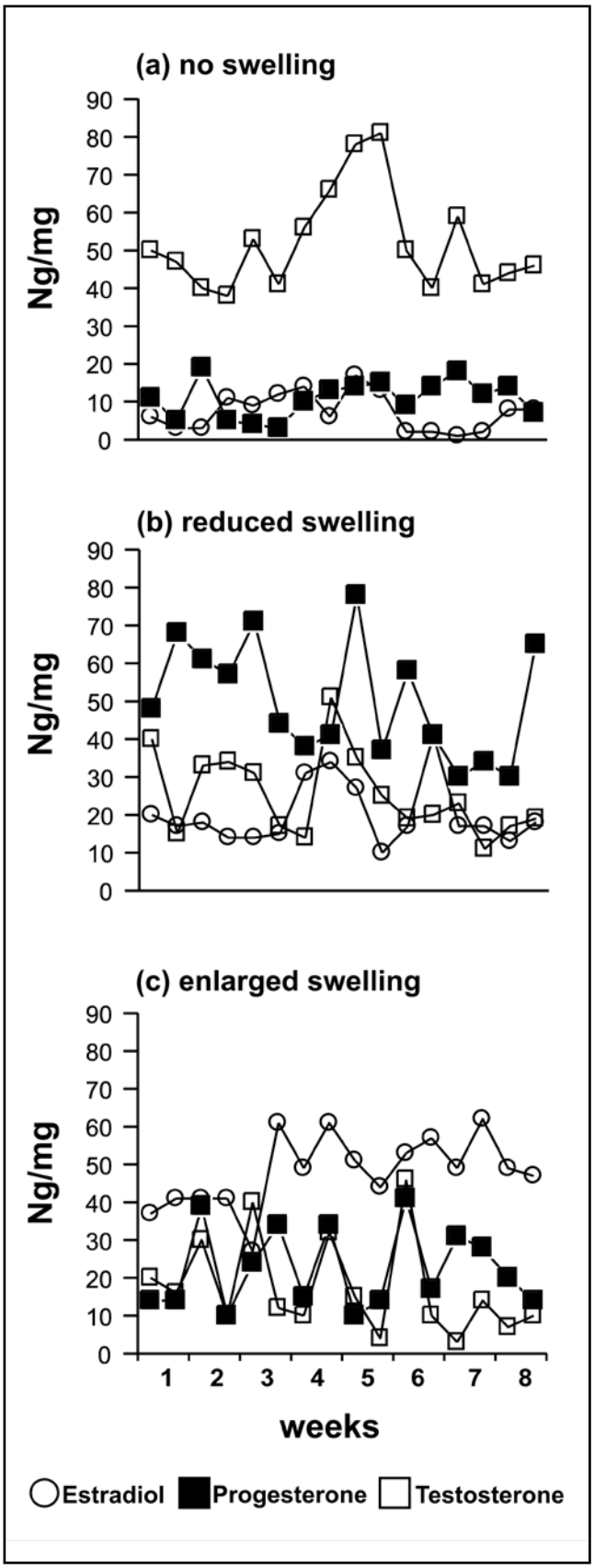

Figure 3. Individual gonadal steroid profiles of one female with enlarged, one with reduced, and one with no perineal swelling expression. Due to comparable sampling time and number of fecal samples, data were only available for a two month period (May, June 1994 for a non-implanted and May, June 1995 for two implanted females; values are mean).

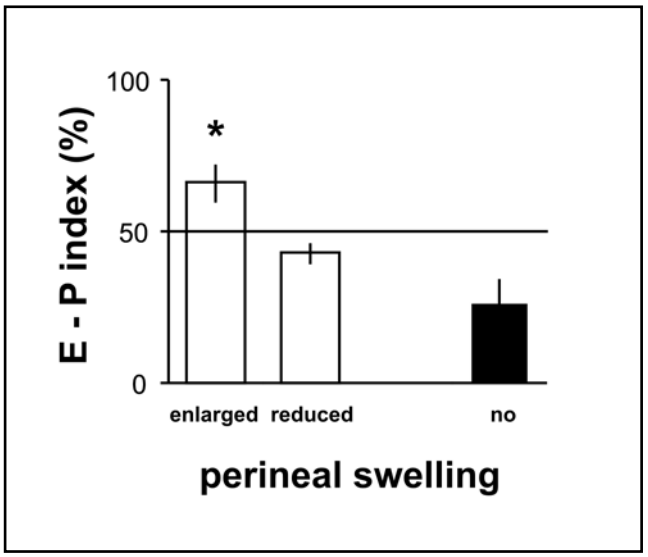

Figure 4. Calculated estradiol to progesterone (E-P) index for females with different perineal swelling expressions. (Values are mean ± SE; one-way ANOVA; df=2; p<0.05; Bonferroni test; * statistically significant at 95 % confidence level).
☐ - implanted group, ■ - non implanted group.

The individual patterns of fecal steroid excretion rates are interesting (Figure 3). There was no apparent fluctuation in the estradiol and progesterone excretion patterns in the non-implanted group (Figure 3a). In contrast, the testosterone excretion pattern showed signs of variation. On the other hand, estradiol varied pronounced in the implanted female with enlarged swelling expression whereas progesterone concentrations varied most in the implanted female with lower amounts of swelling (Figure 3b, c).

The calculated mean E-P index for females with enlarged perineum was over 50%, i.e. in this category the fecal estradiol components were more concentrated than the progesterone components (Figure 4). In both the reduced perineum (implanted) group and the non-implanted zoo group the index was below 50%. The latter groups had a statistically significantly lower index than the enlarged group (df=2; F=11.062; p=0.0005; one-way ANOVA; enlarged vs. reduced, p<0.05; enlarged vs. no, p<0.05; Bonferroni test).

The partial correlations in Table 1 showed a significant negative relationship between the progesterone excretion rate and the perineal swelling size (r=–0.499; p<0.05; corrected for age and implantation time).

Finally, we found no relationship between elapsed period of implantation and fecal progesterone excretion rates (Table 1) as had been indicated in human studies by Brache et al. [5]. The duration of implantation was not related to the relative fecal steroid hormone concentrations (E/P, r=–0.083; E/T, r=–0.281; P/T, r=–0.251). No relationship between the age of the females and their swelling size (r=0.353) was detectable.

Table 1. Partial correlation coefficients between steroid hormones and the independent variables swelling size, age and implantation time.

	swelling size[1]	age[2]	implantation time[3]
E	0.054	–0.039	–0.234
P	–0.499*	0.184	–0.095
T	–0.219	0.142	–0.252
E-P index	–0.349	0.466	–0.182

(E=estradiol, P=progesterone, T=testosterone)
[1]corrected for age and implantation time;
[2]corrected for swelling size rank, implantation time;
[3]corrected for swelling size and age;
*statistically significant at 95% confidence level

DISCUSSION

This study focused LNG effects on fecal sex steroid excretion rates and its coherence on the expression of perineal swellings during the non sexual phase in Barbary macaque females. Both groups the implanted and the intact differed mainly in their swelling expression and excretion rates of estradiol and testosterone metabolites in the feces.

Methodically, the enzyme immunoassays for estradiol and progesterone showed comparable reactivity to excreted non-conjugated metabolites analyzed in fecal samples of *Papio cynocephalus cynocephalus* and *Pithecia pithecia* [15,16]. In the assays, estradiol and progesterone were detectable in small amounts and the immunoreactivity was determined by analyzing metabolites of these hormones. Whitten and Russell [17] described estradiol and progesterone as the main metabolites in the feces of the sooty mangabey. This apparent contrast to our results seems to be related to the specificity of the antibodies used in the cited study. On the basis of the immunization process at position 3 of the 4-pregnane molecule [12] cross reactivity of our progesterone antibody with LNG metabolites is unlikely. The cross reactivity of the testosterone antibody in the elutions demonstrated that metabolites other than testosterone contributed to the observed immunoreactivity. This result can be seen as analogous to analyzed fecal testosterone masses in *Propithecus verreauxi* [18] and in humans [19]. Because of these results the excreted fecal steroid values can be qualified as equivalents to estradiol, progesterone, and testosterone molecules.

The fecal steroid concentrations show that implanted females had elevated estradiol and decreased testosterone concentrations compared to intact females. These results may reflect interferences caused by the interactions between the LNG and the hypothalamo-pituitary-gonadal system, the follicular development/luteal complex or sex steroid availibility modulated by the sex-hormone-binding globulins (SHBG). The last effect appears to be the most plausible explaination, to date. Koritnik and Marschke [7] found that LNG treated *Macaca fascicularis* females had decreased SHBG levels and elevated free estradiol concentrations as other studies have documented in humans [20,21]. Hence, SHBG suppression caused by LNG is directly related to the binding capacity of estradiol and testosterone to the protein [20]. The negative interaction between LNG and excreted testosterone in our study is partially supported by other studies. Several researchers have documented a 30% to 50% decrease in total and/or free testosterone as seen in our study, while other studies have shown unchanged percentages of free testosterone [22–25]. Nonetheless, the comparison of individual gonadal steroid profiles (Figure 3a–c) support our interpretation that LNG effects occur via estradiol and testosterone actions in implanted individuals.

The variation of excreted progesterone levels within the implanted group is higher than between the implanted and the intact groups. Alvarez et al. [2] found generally decreased plasma concentrations of progesterone in women administrated with LNG compared to controls. Within the implanted women the variation of progesterone in the second half of the cycle differed considerably. Sonographic images of follicles documented three types of LNG users, one group with ovulatory activity and relatively elevated progesterone titers, a second group with luteinized unruptured follicles with intermediate progesterone secretion rate and a group with persistent follicles representing the lowest progesterone concentrations. The results of excreted fecal progesterone in implanted and intact Barbary females are difficult to interpret. The mean concentrations between all three investigated groups do not differ significantly in pair wise calculations. Implanted females with reduced swellings show a tendency of elevated progesterone excretion rates. This could be a result of luteinized unruptured follicles in their ovaries; whereas females with the largest expressions of swellings have the lowest progesterone excretion titers, and therefore may represent individuals with persistent follicles. Hence, in comparison to humans with a monthly cycle Barbary macaques are seasonal breeders and in both observed groups no sexual activity was shown. In particular the individual profiles indicate that LNG affects the estradiol and progesterone excretion rate while intact females show negligible fecal hormonal concentrations during sexual inactivity.

The regulating factor for the perineal swelling size in the implanted group seems to be the progesterone concentration. From these results we conclude that the ratio of fecal estradiol and progesterone can be judged as indicator for the expression rate of perineal swelling size. Both arguments the absolute levels of gonadal steroids and their ratio have been discussed in the literature as main regulating factors for perineal swelling expression in chimpanzees [26,27].

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