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Influence of Gestation on Uterine Endometrial Steroid Receptor Concentrations in the African Elephant, *Loxodonta africana*[1]

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ABSTRACT

The modulatory effects of gestational age and circulating concentrations of progesterone, 5α-pregnane-3,20-dione, and estradiol-17β on the uterine sex steroid hormone receptor levels of the African elephant were investigated. Uterine tissue biopsies and blood samples were obtained from animals culled in the Kruger National Park. Estrogen and progesterone receptor concentrations were determined in uterine biopsies from subadult, lactating, early-, mid-, and late-pregnant elephants, by equilibrium binding assays. Circulating estradiol-17β and progesterone concentrations were measured by means of RIAs, while plasma concentrations of 5α-pregnane-3,20-dione were determined with an amplified ELISA. Significant inverse correlations of the concentrations of estrogen and progesterone receptors with the gestational stage of the elephants were observed. Pregnant uterine horns of individual animals contained lower levels of estrogen and progesterone receptors than the nonpregnant horns of the same animals. A strong positive correlation existed between uterine estrogen and progesterone receptors levels. Circulating concentrations of 5α-pregnane-3,20-dione and progesterone decreased with an increase in the concentrations of progesterone receptors as well as with fetal age. We conclude that the progesterone receptor concentrations are down-regulated with progressing gestation in the African elephant. This down-regulation appears to be linked to an increase in circulatory 5α-pregnane-3,20-dione concentration in the plasma of pregnant animals.

INTRODUCTION

In the search for an alternative to culling, information on the interaction between reproductive steroid hormones and their respective receptors in the uterus may be of importance for the development of a contraceptive agent for the management of African elephant populations [1, 2]. The African elephant differs from most other mammals in that luteal and circulating concentrations of 5α-pregnane-3,20-dione (5α-DHP) are higher than those of progesterone during pregnancy [3–5]. Research into the interaction of progestins, estrogens, and their respective receptors is limited in the African elephant [6]; and the relationship of cyclic changes in receptor concentrations to changes in the reproductive status of the African elephant has not previously been reported on. Research on the influence of circulating progesterone and 5α-DHP on uterine progesterone receptor levels also provides valuable information on the biological importance of this and other 5α-reduced metabolites that circulate at relatively high concentrations in the African elephant [3].

Steroid hormones may antagonize the activities of one another through negative regulation of receptor concentrations [7]. Estradiol and progesterone show negligible binding to each other's receptors, but progesterone nevertheless influences the action of estrogen by controlling nuclear retention of the estrogen-receptor complex [8]. The ratio of estrogen to progesterone concentrations is very important in determining the synergistic and/or antagonistic effects on different tissues in the reproductive tract. The present paper describes the correlation of changes in estrogen and progesterone receptor concentrations with changes in reproductive status in the African elephant and investigates apparent heterogeneity in the distribution of each receptor type within the uterus. The relationships between circulating estradiol-17β, progesterone, 5α-DHP, and receptor concentrations were also examined.

MATERIALS AND METHODS

Animals and Tissue Collection

Endometrial tissue was collected from female African elephants killed as part of the management activities of the National Parks Board in the Kruger National Park during May 1995. Uterine tissue biopsies were removed onto crushed dry ice within 30 min of death. Samples were divided into those taken from the caudal and corpus regions of the uterus horn in both pregnant and nonpregnant animals. All the samples were wrapped in aluminum foil and immediately snap-frozen in liquid nitrogen. Subsequently, the samples were transported to the laboratory on dry ice and stored at −70°C until processed.

Blood was collected from the jugular vein into heparinized glass tubes and stored on ice for transport to the laboratory, where it was centrifuged and the plasma removed. All plasma samples were kept at −20°C until assayed.

Gestational age was determined as $t = 106w^{1/3} + 138$, where t is the age of the fetus in days and w is the fetal mass in kilograms [9]. The animals from which tissue was collected were classified as early- (1–7 mo, n = 6), mid- (8–14 mo, n = 2), and late- (15–22 mo, n = 4) pregnant. Samples were also collected from subadult (n = 4) and lactating (n = 6) cows.

Equilibrium Binding Assays

Estrogen and progesterone receptor assays were conducted simultaneously (in duplicate), and tissues from individual elephants were analyzed separately. Cytosol fractions (protein concentration: 2–5 mg/ml) of a particular tissue type were prepared separately, and validated and assayed according to the method of Potgieter et al. [10] as described in Greyling et al. [6]. Endometrial cytosols, prepared from the nonpregnant and pregnant uterine horns of a pregnant cow (gestational age: 7 mo) were stripped with

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0.16% dextran-coated charcoal (E. Merck, Darmstadt, Germany; Dextran T70 from Pharmacia, Uppsala, Sweden) before receptor analyses. All radiolabeled compounds were made up in $TEDAG_{10}$ buffer containing 10 mM Tris HCL, 1.5 mM EDTA, 1 mM dithiothreitol, 1 mM sodium azide, and 10% (w:v) glycerol with the pH adjusted to 7.4 at 4°C, to final concentrations of 2 nM, 4 nM, and 8 nM. The Bradford method [11] was used to determine the protein concentrations using the Bio-Rad protein assay kit with BSA as a standard (Chemlab, Johannesburg, S.A.).

Hormone Assays

To determine the levels of progesterone in the blood, duplicate aliquots of 100 μl plasma were extracted with 4 ml petroleum ether (analytical grade, distillation range: 40–60°C from Saarchem, Krugersdorp, S.A.). Dried extracts were reconstituted in 100 μl PBS (0.1% w:v gelatin, pH 6.8–7). The concentration of progesterone was then measured using the RIA procedure described by Van Aarde [12]. The progesterone antibody (AS 1529), supplied by Prof. R.P. Millar of the Department of Chemical Pathology, University of Cape Town, South Africa, was raised in a goat against progesterone-11-succinyl-BSA. Cross-reactions with other steroids, as determined by the supplier, were as follows: 11α-hydroxyprogesterone: 85%; 17α-hydroxyprogesterone: 12.5%; 5β-pregnane-3,20-dione: 12.5%; 5α-pregnane-3,20-dione: 3%; 5β-pregnane-3-ol-20-one: 1.73%; 11-deoxycorticosterone: 1.1%; 5α-pregnane-3-ol-20-one: 1%; and 20α-hydroxypregn-4-ene-3-one, 20β-hydroxypregn-4-ene-3-one, 11-deoxycortisol, testosterone, androstenedione, pregnenolone, 5β-pregnane-3,20-diol, and estradiol-17β: < 0.7%. The intra- and interassay coefficients of variation were 5.8% (n = 6) and 13.5% (n = 6), respectively, and the mean recovery of progesterone (1.25 ng/ml) added to stripped plasma was 93 ± 5.2% (n = 4). The slope of the line relating percentage binding and serial volumes of plasma was parallel to that of the slope of the line of the standard curve ($F_{1,8}$ 0.423, $p > 0.05$). The sensitivity of the assay, defined as two standard deviations of the buffer blank, ranged from 0.005 to 0.030 ng/ml.

To determine the plasma concentrations of estradiol-17β, duplicate aliquots of 200 μl plasma were extracted and assayed in the same manner as described for the progesterone extraction, except that 3 ml of analytical-grade diethyl ether (Saarchem) was used instead of petroleum ether. The labeled steroid that was used in this case was [2,4,6,7-^{3}H]estradiol with a specific activity of 75 Ci/mmol from Amersham International (Buckinghamshire, UK). Estrogen antibody (E29BI), provided by Prof. R.P. Millar from the Department of Chemical Pathology at the University of Cape Town, South Africa, was raised in a rabbit against a conjugate of estradiol-6-(*O*-carboxymethyl)oxime:BSA. Cross-reactivities with other steroids, as determined by the supplier, were as follows: 17β-estradiol: 100%; estrone: 0.01%; cortisol: 0.005%; deoxycorticosterone: 0.002%; corticosterone: 0.001%; and 17-OH-pregnenolone, androstenedione, progesterone, and testosterone: < 0.001%. Inter- and intraassay coefficients of variation were 5.2% (n = 4) and 5.3% (n = 4), respectively. Serially diluted plasma yielded a curve parallel to that of the standard ($F_{1,8}$ 3.44, $p > 0.05$). The mean recovery of known amounts (2.5 pg/ml) of estradiol-17β from stripped plasma was 92.5 ± 1.5% (n = 6). The detection limit of the assays ranged from 0.51 to 3.90 pg/ml.

For determination of the 5α-DHP concentrations, duplicate aliquots (50 μl, 100 μl, and 200 μl, depending on reproductive status) of plasma were extracted with diethyl ether (Rectapur, Prolabo, Fontenay-sous-Bois, France) as described above. The concentrations were determined using an amplified enzyme-linked immunoassay similar to that described by Hamon et al. [13]. Briefly, all the wells of an assay plate (Nunc-Immuno Plate; Nunc, Roskilde, Denmark) were coated at 4°C overnight with 100 μl 11α-hydroxy-dihydroprogesterone conjugated to BSA (0.5 μg/ml), diluted 1:2000 with PBS (pH 7.0–7.5). The plates were washed four times with 0.05% Tween-20 (Sigma, St. Louis, MO), after which 50 μl of standard or plasma extracts, reconstituted in PBS, was added to the wells. The antiserum, a monoclonal antibody raised in a rat and kindly supplied by M. Hamon of the Babraham Institute, Cambridge, UK [13], was diluted 1:400 in PBS, and 50 μl was added to each well. The plates were incubated at 4°C for 1–2 h, after which they were washed and 100 μl of conjugate (goat anti-rat IgG labeled with alkaline phosphatase, Sigma Immuno Chemicals), diluted 1:10 000, was added to each well. After an incubation of 20 min at 4°C, the plates were washed again. NADPH, which served as a substrate for the alkaline phosphatase, was added (100 μl per well). After a further incubation for 10 min at room temperature, amplifier (diaphorase and alcohol dehydrogenase) for the reaction was added to each well, and color development was terminated by the addition of 100 μl of 0.3 M H_2SO_4 after approximately 7 min. The optical density was measured at 490 nm in a V_{max} kinetic microplate reader (Molecular Devices, Cambridge, UK) against an assay buffer blank. The cross-reactivities of the 5α-DHP antibody, described by Hamon et al. [13], were as follows: 5α-dihydroprogesterone: 100%; progesterone: 56%; 5α-pregnane-3-hydroxy-20-one: 21%; 5β-dihydroprogesterone: 17%; pregnenolone: 5%; 20α-dihydroprogesterone: 1.2%; and 20β-dihydroprogesterone, 5α-pregnane-20α-hydroxy-3-one, 5α-pregnane-3β,20α-diol, Δ^5-pregnane-3β,20α-diol, Δ^5-pregnane-3β,20β-diol, equilin, and equilenin: < 0.01%. Intra- and interassay coefficients of variation were 9.4% (n = 4) and 16.8% (n = 4), respectively, and the mean recovery of 5α-DHP (5 ng/ml) added to stripped plasma was 76 ± 4% (n = 6). The slope of the line relating percentage binding and serial volumes of plasma was parallel to that of the standard curve ($F_{1,7}$ 2.91, $p > 0.05$). The limit of detection of the assays ranged from 0.05 to 0.09 ng/ml.

Statistical Analysis

Scatchard and saturation curves with the Rosenthal correction for nonspecific binding were drawn by using the COMBICEPT 2000CA software program (Packard Instrument Company, Downers Grove, IL). Receptor concentrations are given in femtomoles per milligram of protein (mean ± SEM). The Kruskal-Wallis H and Mann-Whitney U tests [14] were used to determine statistical differences in receptor concentrations in animals of different reproductive stages. Correlations between estrogen and progesterone receptor concentrations and the circulating concentrations of their appropriate ligands were determined by means of the Pearson Product-Moment Correlation (r) and multiple regression analysis [14]. Nonlinear regressions were obtained by determining correlation matrices for the power transformations in the ladder of powers [15]. The difference between the slopes of regression lines and the significance of each slope was tested by means of t-tests [16]. Significance was taken at the 95% level. Results from Scatchard

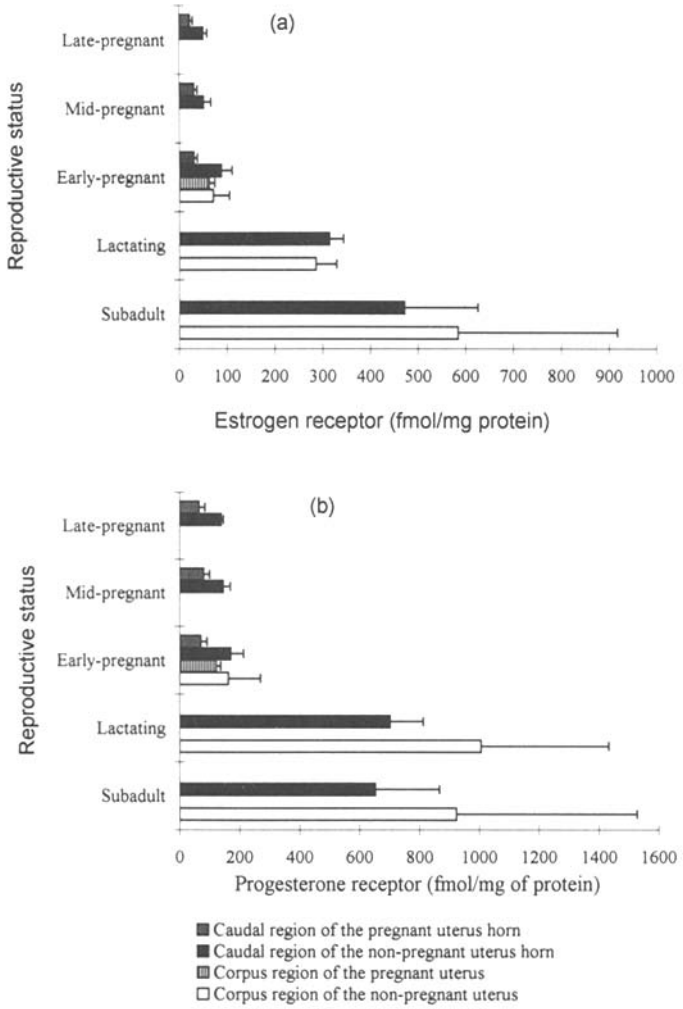

FIG. 1. Estrogen (**a**) and progesterone (**b**) receptor concentrations (fmol/mg protein ± SEM) for the various endometrial tissue types as a function of reproductive status.

plots were subjected to least-square regression analyses, and only data with correlation coefficients ≥ 0.9 were used in further statistical analyses.

RESULTS

Receptor concentrations were significantly affected by reproductive status for both the estrogen (H [4, n = 49] = 34.75, $p < 0.05$) and the progesterone receptors (H [4, n = 49] = 32.52, $p < 0.05$). However, tissues obtained from subadults and from lactating elephant cows did not differ significantly for either the estrogen (Z [U = 40.5] = −1.29, $p > 0.05$) or the progesterone receptors (Z [U = 35] = −1.64, $p > 0.05$). These values furthermore were significantly higher than those for the pregnant animals (Fig. 1).

Tissue biopsies taken from early-pregnant animals from the caudal region of the pregnant uterine horns had significantly lower receptor concentrations than those taken from the nonpregnant uterine horns for both the estrogen (Z [U = 2] = −2.34, $p < 0.05$) and the progesterone receptors (Z [U = 3] = −2.19, $p < 0.05$). This was also the case during late pregnancy for the estrogen (Z [U = 0] = −2.14, $p < 0.05$) and progesterone receptors (Z [U = 0] = −2.12, $p < 0.05$). Although both estrogen and progesterone receptor concentrations were lower in the pregnant uterine horn of the mid-pregnant animals, the difference was not significant when compared to the receptor concentrations of the other uterine horn (Fig. 1). Dextran-coated charcoal-stripped endometrial cytosols from the pregnant and nonpregnant uterine horns of a cow in her 7th month of pregnancy showed the same tendency of lower receptor concentrations in tissues from the pregnant side of the uterus. Similarly, no changes were observed in the receptor status of dextran-coated charcoal-treated endometrial cytosols of a subadult female. The lower receptor concentrations could therefore not be ascribed to the saturation of tissue receptors because of increased circulating levels of 5α-DHP.

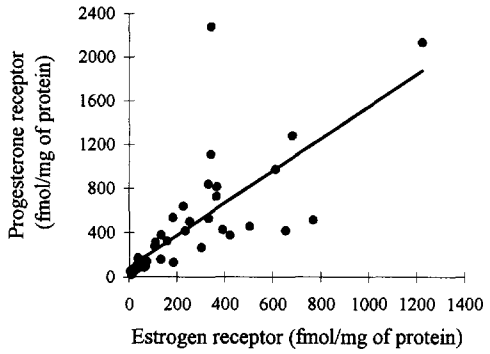

FIG. 2. The relationship between progesterone and estrogen receptor concentrations of African elephant cows (n = 49, $r = 0.75$, $p < 0.05$).

Estrogen and progesterone receptor concentrations were significantly linearly related ($r = 0.75$, n = 49, $p < 0.05$; Fig. 2). Circulating concentrations of 5α-DHP correlated positively with fetal age ($r = 0.70$, n = 13, $p < 0.05$). The concentrations of circulating progesterone were not significantly related to fetal age. Bivariate scatterplots (Fig. 3, a and b) illustrate the negative relationships that existed between progesterone receptor concentration and plasma 5α-DHP ($r = -0.93$, n = 12, $p < 0.05$; Fig. 3a) and progesterone ($r = -0.45$, n = 12, $p < 0.05$; Fig. 3b) concentrations, respectively. The slopes of the regression line (b) of plasma 5α-DHP and progesterone receptor concentrations differed significantly from zero ($t_{10} = -7.87$, $p < 0.05$). This was not found for the regression line depicting plasma progesterone and progesterone receptor concentrations ($t_{10} = -1.61$, $p > 0.05$). The slopes of the regression lines were, however, similar to each other ($t_{20} = 0.051$, $p > 0.05$).

Multiple regression analysis supported the strong relationships between the variables ($F_{2,9} = 30.76$, $R = 0.93$, n = 12, $p < 0.05$). The standardized regression coefficient (beta) for circulating 5α-DHP ($t_9 = -6.86$, beta = −1.004, $p < 0.05$) exceeded that of progesterone ($t_9 = 0.89$, beta = 0.131, $p > 0.05$) and illustrates the relatively strong contribution that circulating 5α-DHP made to changes in progesterone receptor concentrations. The relationship between circulating 5α-DHP and progesterone receptor concentrations was also significantly negative, and the partial correlation value (r) of 5α-DHP (−0.92) also exceeded that of circulating progesterone (0.29). There was no relationship between circulating estradiol-17β and the estrogen or progesterone receptor.

DISCUSSION

Receptor concentrations in estrogen and progesterone target tissues of mammals are known to change in a dynamic manner during the estrous cycle [17, 18] and gestation [19–21]. These published observations also illustrate

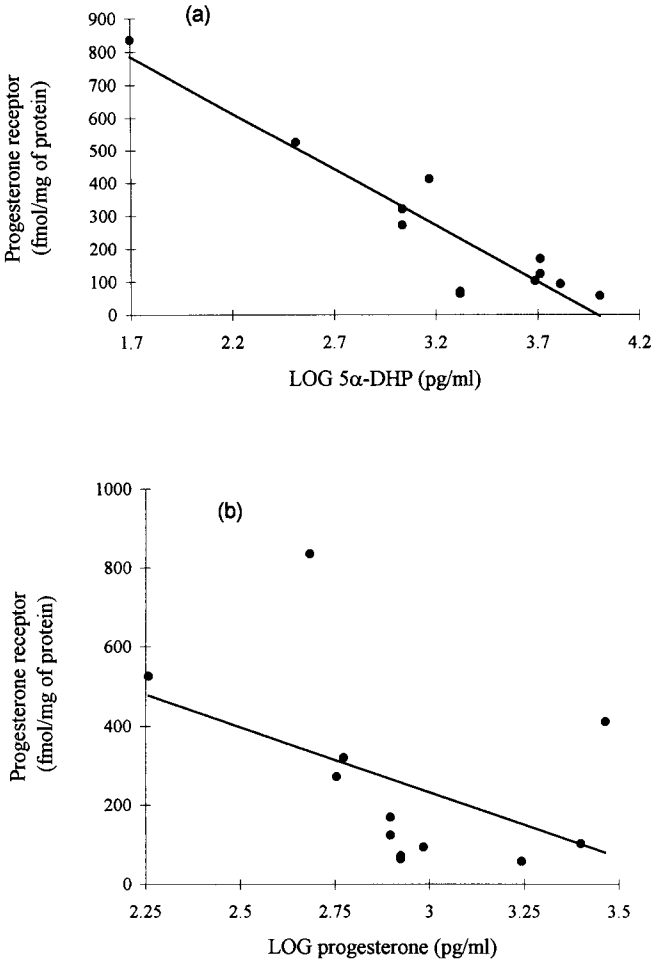

FIG. 3. The relationship between uterine progesterone receptor concentrations (fmol/mg of protein) and plasma concentrations (ng/ml) of (**a**) 5α-DHP and (**b**) progesterone.

an inversely proportional relationship between receptor concentrations in the target tissues and the concentration of estrogen and progesterone in the circulation. Padayachi et al. [19] reported that human, full-term-pregnant uterine tissue displayed no detectable receptors and that the removal of endogenous steroids by pre-assay incubation of the cytosol with dextran-coated charcoal did not affect the receptor status of these tissues.

Our results indicate that the uterine estrogen and progesterone receptor concentrations in elephant cows decreased as a function of gestational age. We also illustrated that both the estrogen and progesterone receptor concentrations in the pregnant horns of early-and late-pregnant elephant cows were significantly lower than in the nonpregnant uterine horns. Uterine tissues from mid-pregnant animals exhibited the same tendency, but results were not statistically significant, because of the few animals (n = 2) investigated. Receptor concentrations in tissues of the pregnant uterine horns of early- and late-pregnant elephant cows were approximately 50% lower than in those of the nonpregnant uterine horns. Treatment of the endometrial tissue cytosols of a pregnant cow with dextran-coated charcoal before incubation did not affect the receptor status of the tissues. These results clearly indicate that uterine receptors in the pregnant elephant cow are down-regulated by the steroid hormones. However, direct measurement of the uterine receptors by means of enzyme immunoassays, or of the steroid hormone receptor messenger RNAs by Northern analysis, will be necessary to verify the modulatory effects of steroids in the elephant cow [22, 23].

Endometrial progesterone receptor concentrations in the African elephant did not significantly correlate with plasma concentrations of estradiol-17β. In a variety of mammals, estrogen priming is known to lead to an increase of progesterone receptors in the uterus, oviduct, vagina, anterior pituitary, and hypothalamus [24]. Estrogen exposure is responsible for several signal production and signal response events, one of which is the increase in progesterone receptors in the gonadotroph [25]. Progestational compounds are often dependent on prior exposure to estrogen. Nyholm et al. [26] found that the tumor biochemical progesterone receptor content correlated positively with free estradiol serum concentrations. Kontula [27] also reported a significant positive relationship between estradiol and cytosol progestin receptor concentrations in human myometrium. Hodges et al. [28] suggested some time ago that estradiol-17β sulphate was probably the most abundant circulating estrogen during pregnancy in the African elephant and that estradiol-17β concentrations for nonpregnant and pregnant animals were similar. In our investigation, the concentrations of estradiol-17β were low, with considerable individual variation, so that the expected positive relationship between this hormone and the progesterone receptor may have emerged only if the conjugated form of the hormone (i.e., estradiol-17β sulphate) was also measured. Nevertheless, we could also illustrate an inverse proportional relationship between the uterine estrogen receptor concentrations and the gestation period of the elephant cow. Perrotapplanant et al. [29] managed to show that the absence or low concentration of estrogen receptor staining in the various cell types of the human endometrium during gestation concurred with the down-regulation of steroid hormones on the estrogen receptor mRNA and on receptor protein concentrations.

In the African elephant, progesterone receptor concentrations decreased with an increase in the concentrations of circulating 5α-DHP. Plasma concentrations of 5α-DHP also increased with fetal age. The apparent inverse relationship between progesterone receptor concentrations and 5α-DHP, but a lack thereof with progesterone, suggests that 5α-DHP, rather than progesterone, down-regulates the progesterone receptor in this species. 5α-DHP and other 5α-reduced metabolites may therefore be of biological importance in the African elephant [3, 5, 6]. Previous results published on the relationship between the plasma concentrations of 5α-DHP and gestational stage [3] seem to contradict the results reported in the present study. Although the antibody, raised against 5α-DHP and employed in the present study, was prepared in a way similar to that produced by Hamon et al. [13] and used in the first study [3], cross-reactivity of the two batches of antibodies differed. Furthermore, the measurement of steroid hormone receptor concentrations is more accurate than the determination of the gestational stage of a pregnant elephant cow, and this may explain the apparent discrepancy between this investigation and our earlier paper.

In the present study, the considerable cross-reactivity of the antibody for 5α-DHP with progesterone (56%) may be responsible for the lack of significant differences between the slopes of the regression lines describing the relationship between progesterone, 5α-DHP, and receptor concentrations. The lack of a significant relationship between progesterone receptor concentrations and circulating progesterone concentrations may also be due to the lack of a de-

finable trend in progesterone concentrations during pregnancy [3–5]. The importance of both progesterone and 5α-DHP to the down-regulation of receptor concentrations can therefore not be disregarded. Down-regulation of the progesterone receptor as a function of increasing circulating progesterone levels has been reported for several other species [19, 29–32].

With this study, we came to the conclusion that down-regulation of progesterone receptors occurs with progressing gestation in the African elephant. The down-regulation appears to be linked to increasing concentrations of 5α-DHP in the plasma of pregnant animals. The biological importance of this metabolite is furthermore substantiated by the relatively high binding affinity of 5α-DHP for the progesterone receptor [6]. It is therefore possible to employ structural analogues of 5α-DHP or progesterone in the management of elephant fertility as an alternative to the unpopular practice of culling these majestic animals.

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