BIOLOGY OF REPRODUCTION **67,** 1405–1413 (2002)
DOI 10.1095/biolreprod.102.004226

Effects of Prenatal Treatment with Antiandrogens on Luteinizing Hormone Secretion and Sex Steroid Concentrations in Adult Spotted Hyenas, *Crocuta crocuta*[1]

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ABSTRACT

Prenatal androgen treatment can alter LH secretion in female offspring, often with adverse effects on ovulatory function. However, female spotted hyenas (*Crocuta crocuta*), renowned for their highly masculinized genitalia, are naturally exposed to high androgen levels in utero. To determine whether LH secretion in spotted hyenas is affected by prenatal androgens, we treated pregnant hyenas with antiandrogens (flutamide and finasteride). Later, adult offspring of the antiandrogen-treated (AA) mothers underwent a GnRH challenge to identify sex differences in the LH response and to assess the effects of prenatal antiandrogen treatment. We further considered the effects of blocking prenatal androgens on plasma sex steroid concentrations. To account for potential differences in the reproductive state of females, we suppressed endogenous hormone levels with a long-acting GnRH agonist (GnRHa) and then measured plasma androgens after an hCG challenge. Plasma concentrations of LH were sexually dimorphic in spotted hyenas, with females displaying higher levels than males. Prenatal antiandrogen treatment also significantly altered the LH response to GnRH. Plasma estradiol concentration was higher in AA-females, whereas testosterone and androstenedione levels tended to be lower. This trend toward lower androgen levels disappeared after GnRHa suppression and hCG challenge. In males, prenatal antiandrogen treatment had long-lasting effects on circulating androgens: AA-males had lower T levels than control males. The sex differences and effects of prenatal antiandrogens on LH secretion suggest that the anterior pituitary gland of the female spotted hyena is partially masculinized by the high androgen levels that normally occur during development, without adverse effects on ovulatory function.

anterior pituitary, early development, luteinizing hormone, neuroendocrinology, testosterone

INTRODUCTION

The external genitalia of the female spotted hyena (*Crocuta crocuta*) is highly masculinized, as evident by the absence of an external vagina and the presence of a penis-like clitoris through which the female copulates, voids, and gives birth [1–4]. This virilization has been associated with the peculiar hormonal milieu of these animals during sexual differentiation (i.e., high levels of androgens during gestation [5]). High testosterone (T) levels result from placental conversion of androstenedione (A_4), which is secreted by the maternal ovary in significant amounts [6]. Masculinization of the external genitalia carries considerable reproductive costs; nulliparous spotted hyenas experience high rates of stillbirth and maternal mortality [7]. However, exposure to prenatal androgens has the potential to alter the reproductive anatomy and physiology of female spotted hyenas beyond effects on the external genitalia. For example, regulation of gonadotropin secretion may also be affected, because the organizational effects of androgens on LH secretion are often sexually dimorphic in other mammals [8].

Exposure to elevated levels of T during sexual differentiation has been associated with irreversible changes in the secretion of LH in female rats [9], guinea pigs, [10], sheep [11], and rhesus monkeys [12]. These T-induced changes in LH secretion may, in turn, affect ovarian function. For example, ovulation may never occur in androgenized female rats, even when the administration of T is limited to a single injection during the neonatal period [13]. Whereas pseudohermaphroditic macaques sometimes ovulate, they are more likely to become anovulatory with time [14]. In one of the few studies in which prenatal androgens were given to a mammalian carnivore, Beach et al. [15] documented ovulatory function in androgenized beagles despite masculinization of the external genitalia. However, Beach et al. did not assess whether ovulatory function was gradually lost in these masculinized bitches.

In human females, prenatal androgen exposure has also been associated with changes in LH secretion and ovulatory dysfunction. Women with congenital adrenal hyperplasia (CAH) have an exaggerated LH response to a GnRH challenge, and a high incidence of anovulation is associated with the polycystic ovarian syndrome (PCOS), even when the adrenal source of androgen excess was corrected at birth [16]. Interestingly, Wynn and Amoroso [17] described the ovaries of the spotted hyena as being histologically similar to those of women with PCOS, specifically ''with regard to the hyperthecosis and rarity of follicles.'' The ovaries in reproductively active spotted hyenas have a striking paucity of follicles and an unusual abundance of androgen-secreting theca-interstitial tissue [1]. However, female spotted hyenas show no evidence of losing their capacity to ovulate, either in the wild or in captivity (unpublished observations), despite their exposure to naturally high levels of T during gestation.

[1]Supported by a National Research Service Award (NRSA) HD-08729 from the NICHD/NIH (to N.J.P.), by NRSA HD-07684 from the NIMH/NIH (to C.M.D.), by grant IBN99-06445 from the NSF (to K.E.H.), and by grant MH-39917 from the NIMH/NIH (to S.E.G.).
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Received: 25 March 2002.
First decision: 16 April 2002.
Accepted: 5 June 2002.

ISSN: 0006-3363. http://www.biolreprod.org

TABLE 1. Prenatal antiandrogen treatment regimens for four spotted hyena pregnancies (six fetuses)[a]

Animal ID	Sex	Present age (yr)	Drug	Mean dose (mg kg^{-1} day^{-1})	Duration (days)
55[b]	F	7	Flutamide	22.8	75
			Finasteride	0.56	48
56[b]	F	7	Flutamide	22.8	75
			Finasteride	0.56	48
57[a]	M	7	Flutamide	18.0	78
58[a]	M	7	Flutamide	18.0	78
59	M	7	Flutamide	23.1	72
			Finasteride	0.60	72
61	F	6	Flutamide	23.6	89
			Finasteride	0.61	89

[a] Gestation length is approximately 110 days, and treatments were continued until the day of parturition. Twins from the same pregnancy are identified with matching superscripts.

Understanding the effects of prenatal androgens on the hypothalamic-pituitary-ovarian (HPO) axis of spotted hyenas required an alternate approach from that adopted with other animal models, both because placental conversion of A_4 to T begins early in gestation and because maternal androgen levels are naturally high during gestation [5]. In rats, guinea pigs, sheep, and monkeys, the effects of androgens on differentiation of the HPO axis have been investigated by administering androgens to mothers and/or offspring during development and, later, by measuring the effects at various levels of the HPO axis [9–12]. In the present study, we did the obverse and investigated the effects of prenatal androgens on pituitary-gonadal function in spotted hyenas by administering androgen-blocking agents through most of gestation. We also compared LH and steroid secretion patterns in response to a GnRH challenge in adult hyenas. Furthermore, we compared plasma levels of LH before and after the GnRH challenge in untreated male and female spotted hyenas to determine if LH secretion is sexually dimorphic in a species in which females are highly masculinized. Finally, to determine whether prenatal administration of androgen-blocking agents specifically had an intraovarian effect on steroidogenic function, we administered a gonadotropin challenge to females after we suppressed endogenous secretion of LH and sex steroids with a long-acting GnRH agonist (GnRHa).

MATERIALS AND METHODS

Animals

Twelve adult spotted hyenas maintained at the Field Station for the Study of Behavior, Ecology, and Reproduction of the University of California, Berkeley, were studied. Half the animals, three males and three females, were treated prenatally with antiandrogens (AA). Six control animals, three males (C-males) and three females (C-females), did not receive AA at any time. Flutamide, an androgen-receptor blocker (Schering Corporation, Kenilworth, NJ), either alone or in combination with finasteride, a 5α-reductase inhibitor (Merck and Co., Inc., West Point, PA), was administered orally to pregnant females on a daily basis (see Drea et al. [18] for details, refer to Table 1 for dose and duration). These studies were initially done to examine the effects of prenatal AA treatment on genital morphology. Flutamide was used alone in an early attempt to block male-pattern sexual development in spotted hyenas; a similar regimen had worked well in dogs [19, 20]. A single triplet pregnancy was treated with flutamide alone, with two AA-males surviving to adulthood. However, Licht et al. [5] found that 5α-dihydrotestosterone (DHT) levels steadily increase in spotted hyenas throughout gestation, such that maternal concentrations sometimes exceed those measured in adult males. Finasteride was added to the regimen in an attempt to maximally block the actions of androgens on development of the external genitalia. Seven pregnant females were treated with this combination of flutamide and finasteride: One male and three female offspring survived to adulthood, whereas seven fetuses were stillborn or died as neonates [18].

Female spotted hyenas reach sexual maturity at approximately 3 yr of age and remain reproductively active for more than a decade [7]; males reach sexual maturity at approximately 2 yr of age [21]. All females in the present study were sexually mature and of an age to have undergone multiple estrous cycles. None were pregnant or lactating at the time of the study. With regards to fertility and fecundity, the AA-females appeared to suffer no sequelae of the AA treatment; all these females have proven to be successful breeders. Conversely, because of the developmental changes of the phallus, AA-males may have been rendered incapable of intromission [18]. None of the AA-males had bred successfully, but two had been observed mounting females. Two C-males were proven breeders, having sired offspring within the preceding year. The third C-male was always paired with receptive females in the company of other males but was thought to have sired offspring.

GnRH Challenge

Animals were immobilized with ketamine and xylazine administered by blow dart and anesthetized with isoflorane inhalant via mask. A blood sample for baseline hormone determination was obtained from either the jugular or cephalic vein several minutes before a single i.v. injection of GnRH (1 μg/kg of LHRH, L-7134; Sigma Chemical Co., St. Louis, MO). Additional blood samples were obtained from the cephalic vein 10, 20, 30, 60, 90, and 120 min following the GnRH injection. Blood was centrifuged at 1000 × *g* for 10 min, with plasma drawn and stored at −80°C until RIA. We measured plasma levels of LH, T, Δ^4-androstenedione (i.e., A_4), and estradiol (E_2).

GnRHa and hCG Challenge

Immediately after collecting the last blood sample from the GnRH challenge, we treated each female with an s.c. implant of a long-acting GnRHa, goserelin acetate (Zoladex, 3.6 mg; Astra-Zeneca, Wilmington, DE). We thought ovarian steroidogenesis might be influenced by an individual female's reproductive history and stage of estrus. Additionally, potential differences in the LH response because of prenatal AA treatment could also influence the ovarian steroidogenic response to the GnRH challenge. To account for these differences, we suppressed baseline gonadotropin secretion and ovarian steroidogenesis with GnRHa, then reassessed ovarian responsiveness to gonadotropins via an hCG challenge 3 wk later. After immobilization and anesthesia as described above, we measured plasma levels of LH and androgens from blood samples taken 20, 10, and 1 min before injection of hCG to demonstrate suppression by the GnRHa. Plasma levels of T and A_4 were also measured in samples obtained 10, 20, 30, 60, 90, and 120 min following the hCG injection. All GnRH and hCG challenges began between 0904 and 1120 Pacific Standard Time and were completed 2 h later.

Hormone RIAs

We measured plasma levels of LH by a heterologous RIA using reagents provided by Dr. A.F. Parlow at the National Hormone and Peptide Program (NHPP). The NHPP reagents were from a kit for measuring LH in rats. Serial dilutions of spotted hyena plasma showed parallelism with LH standards from the NHHP rat kit; attempts to demonstrate parallelism using reagents from other NHPP kits specific for human, canine, ovine, and bovine LH proved to be unsatisfactory. To further validate the LH assay in a biological setting, 54 free-ranging spotted hyenas inhabiting the Masai Mara National Reserve (Kenya) were given a GnRH challenge, whereas an additional eight hyenas received a saline injection and served as controls. Following immobilization with an i.m. injection of Telazol (2.5 mg/kg; Wildlife Pharmaceuticals, Fort Collins, CO), administered via dart delivered by a CO_2-powered rifle, initial blood samples were obtained at 5-min intervals for 45 min. An i.v. injection of GnRH (1 μg/kg) was followed by serial blood samples every 5 min for 2 h. The saline-injected hyenas showed no change in mean plasma LH levels, whereas the GnRH-injected animals experienced a significant increase in LH (Fig. 1), with a peak occurring approximately 10- to 30-min postinjection [22].

Plasma samples from the present study were assayed for LH in duplicate using 250 μl of unknown per tube. Standards (150 μl/tube) and unknowns were diluted to 300 μl with 0.10 M sodium phosphate buffer (pH 7.0) containing 0.14 M NaCl, 0.1% (w/v) pigskin gelatin, and 0.01% (w/v) thimerosol (0.1% [w/v] PBS-gel). Fifty microliters of primary antibody (anti-rat LH, NIADDK-Anti-rLH-S11, lot no. AFPC 697071P, diluted 1:30 000 in PBS containing 0.05 M EDTA and 3% normal rabbit serum)

were then added to each tube, followed by a 24-h incubation at 4°C. Fifty microliters of ^{125}I LH, diluted to generate approximately 10 000 counts per minute/tube, were then added, and after another 24-h incubation at 4°C, 50 μl of second antibody (anti-rabbit gamma globulin raised in sheep, Pel Freeze lot no. 1306) were added to precipitate antibody-bound LH. The assay was terminated after a final 24-h incubation at 4°C by addition of 3 ml of ice-cold PBS, and each tube was then centrifuged at 3500 rpm for 30 min. The supernatant was decanted, and tubes were drained for 3 min. The amount of ^{125}I precipitated was then determined in a gamma counter. All samples were run in a single assay, and seven samples with LH levels above the maximum detectable limit were reassayed after further dilution. The minimal detectable limit for LH was 0.06 ng ml^{-1}, and the intra- and interassay variations were 7.4% and 9.0% respectively.

Radioimmunoassay of steroids was performed as described by Licht et al. [5, 23]. Briefly, plasma samples were extracted in diethyl ether (Mallinkrodt, Boston, MA), dried under N_2, and reconstituted in PBS with gelatin. All extracted samples were separated into aliquots for assays to measure T and A_4, whereas only samples from females following the GnRH challenge were assayed for E_2. Samples were incubated for 1 h at 37°C with the appropriate antiserum and ^3H-labeled steroid (New England Nuclear, Boston, MA). Cross-reaction of the T antibody (no. T3-135; Endocrine Sciences, Calabasas Hills, CA) with DHT was 44%; cross-reaction of the A_4 antibody (no. AN6-22; Endocrine Sciences) for T, DHT, and E_2 was <2%; and cross-reaction of the E_2 antibody (no. E26-47; Endocrine Sciences) with other estrogens was ≤1.3%. Minimal detectable limits were 0.15 ng ml^{-1} for T, 0.27 ng ml^{-1} for A_4, and 0.07 ng ml^{-1} for E_2. When we determined that the E_2 levels of several samples were below the detectable limit of our assay, we had samples run by Esoterix Endocrinology (Calabasas Hills, CA) using their highly sensitive RIA (minimal detectable limit = 0.005 ng/ml). Because the GnRH challenge did not induce a change in E_2 levels, the highly sensitive E_2 assay was only used for pre-GnRH samples from untreated and AA-females. Intra- and interassay variations were <10% and <21% for all steroid assays. To control for interassay variation, all samples from the GnRH or hCG challenges for a control animal and an AA-animal of the same sex were assayed side-by-side within each assay.

Statistics

Plasma hormone data from the GnRH and hCG challenges were analyzed with a commercial statistical program (Super Anova 1.1; Abacus Concepts, Berkeley, CA) using analysis of variance for repeated measures (rANOVA). When testing for the effects of prenatal AA treatment on hormone secretion patterns, we analyzed the challenge responses of males and females independently. We limited our investigation of sex differences in hormone secretion to the LH response of C-males and C-females. Data were log-transformed as needed before analysis to achieve normality of distributions and homogeneity of variances and were back-transformed for plotting in figures. When the rANOVA revealed a statistically significant main effect of treatment or an interaction effect of treatment × time, we made pair-wise comparisons between groups within each sampling time using unpaired *t*-tests. Estradiol data were analyzed using the nonparametric Mann-Whitney *U*-test, and hormone data both pre- and post-GnRHa suppression were analyzed using the nonparametric Wilcoxon signed rank test (Statview 5.0; SAS Institute, Cary, NC). Pearson product-moment was used to determine if peak LH levels after GnRH injection correlated with the plasma E_2 concentration of individual females. Samples with undetectable hormone levels were assigned the minimal detectable level of the assay. Differences at $P < 0.05$ were considered to be significant.

RESULTS

Effects of Prenatal AA Treatment on Genital Development

Prenatal treatment with flutamide, either alone or in combination with finasteride, ''feminized'' the external genitalia of both sexes. Testes in AA-males were fully descended, and hypospadias was not observed. The phallus in AA-males shared morphological features with the hyena clitoris in that the penis was shorter and broader than in C-males. The diameter and elasticity of the urethral meatus in AA-males was greater than in C-males and similar to that in C-females. The increase in both diameter and elasticity of the urethral meatus was further exaggerated in AA-females. However, AA-females lacked an external vaginal opening, and the single urogenital canal traversed the clitoris to its tip, as is the norm in this species.

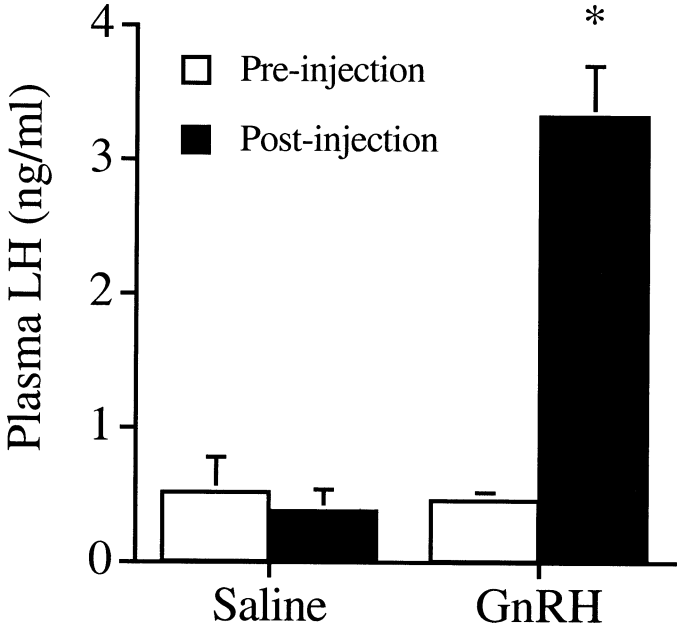

FIG. 1. Mean (±SEM) plasma LH concentrations in free-ranging spotted hyenas before and after injection with saline (n = 8) or GnRH (n = 54). Values represent mean plasma concentrations obtained from individuals sampled at 5-min intervals during the 45-min periods before and after the injection. *Plasma LH levels increased significantly after injection of GnRH ($P < 0.05$) but were unchanged following injection of saline.

Plasma LH in Control Animals: Baseline and GnRH Challenge

Plasma levels of LH in spotted hyenas were sexually dimorphic. Baseline and post-GnRH levels of LH were significantly higher in C-females than in C-males (Fig. 2) ($F_{1,4} = 16.984$, $P < 0.05$). The overall pattern of secretion appeared to be similar, with no significant interaction effect of sex and time postinjection ($F_{6,24} = 0.609$, $P = 0.72$). Concentrations of LH were generally more variable in females than in males (*F*-test for variance, $P < 0.05$ in both control and AA groups).

Effects of Prenatal AA Treatment on LH Response to GnRH Challenge

Prenatal AA treatment altered the secretion patterns of LH following the GnRH challenge in both sexes but had no significant effect on baseline LH levels. The pattern of LH secretion in AA-males was significantly different from that in C-males ($F_{6,24} = 4.047$, $P < 0.01$), with AA-males achieving peak LH levels more than 2.5-fold higher than those in C-males. The time of the LH peak relative to the GnRH injection was 10 min earlier in the AA-males, and their LH levels declined more rapidly. Interestingly, the level and timing of the LH peak was similar in AA-males and C-females (Fig. 3). In females, the AA-treated hyenas had higher peak LH levels, and the peaks occurred later than in C-females, yielding strikingly different patterns of secretion ($F_{6,24} = 8.370$, $P < 0.001$). Mean LH levels of C-females increased rapidly and then began to decline within 1 h of the GnRH injection, whereas LH levels of AA-females continued to rise throughout the 2-h study. Consequently, the LH levels of individual C- and AA-females showed no

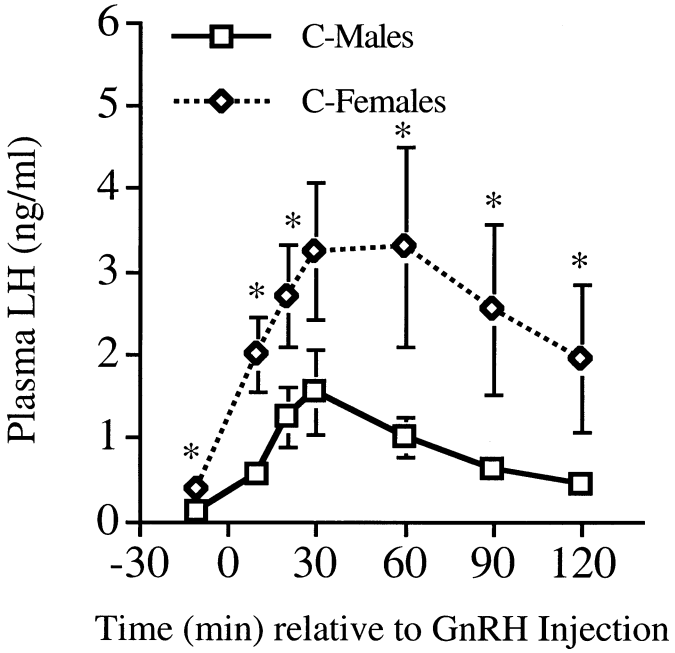

FIG. 2. Mean (±SEM) plasma LH concentrations in untreated male and female spotted hyenas. Baseline LH levels were measured in samples obtained 10 min before the GnRH challenge, which was administered at time zero (n = 3 per group). Plasma LH levels were significantly greater before and after the GnRH challenge in C-females than in C-males ($F_{1,4} = 16.984$, $P < 0.05$), with no significant interaction effect of sex × time ($F_{6,24} = 0.608$, $P = 0.72$). The LH levels in all post-GnRH samples were significantly different ($P < 0.05$) than preinjection LH levels in both sexes. *Concentrations of LH differ between males and females at the time points indicated ($P < 0.05$).

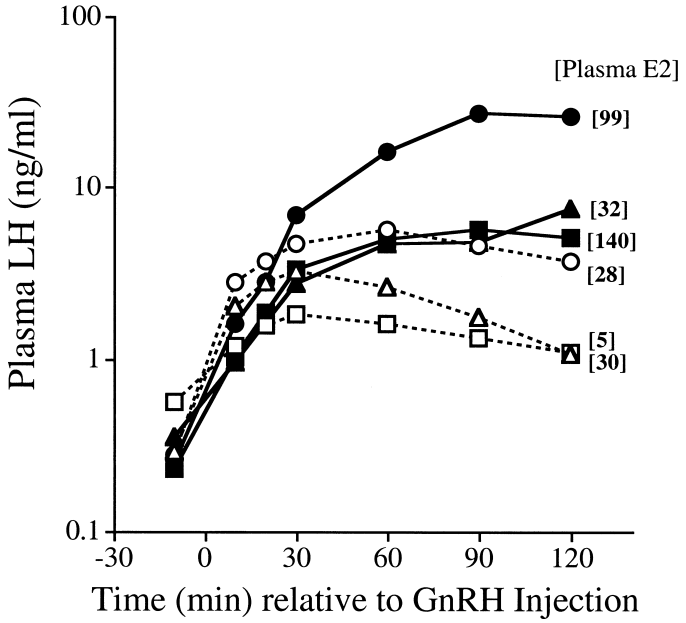

FIG. 4. Plasma LH concentrations, plotted on a $\log_{10}$ scale, in individual AA-treated (filled symbols) and untreated (open symbols) female spotted hyenas before and after GnRH challenge. The corresponding baseline E_2 concentration (pg/ml) of each female is given in the right margin, adjacent to the line representing LH levels. Pearson product-moment correlation is not significant ($P > 0.05$) for peak LH and E_2 concentrations.

overlap at 120 min post-GnRH injection (Fig. 4). One of the AA-females (no. 56) had very high LH levels in response to the GnRH challenge (Fig. 4), thus explaining the large error bars in Figure 3B. However, when data from this female were excluded from the analysis, the patterns persisted, and statistical significance was maintained ($F_{6,18} = 5.691$, $P < 0.01$).

Effects of Prenatal AA Treatment on Secretion of Sex Steroids

Plasma androgen (T and A_4) levels increased significantly ($P < 0.05$) in response to the GnRH challenges in all groups of both sexes (Fig. 5). Secretion of T during the 2 h following the GnRH challenge differed significantly between groups of males ($F_{6,24} = 31.198$, $P < 0.001$), with C-males maintaining T levels that were twofold higher than those of AA-males (Fig. 5, A and B). A similar secretion pattern was observed with A_4. However, with A_4, statistical significance was not achieved, because the A_4 levels of individual males from each group overlapped ($F_{6,24} = 1.163$, $P = 0.36$). Despite the greater LH response in AA-females, their plasma androgen levels were not significantly different from those of C-females, though the trend was toward lower levels in the AA-females (Fig. 5, C and D). Conversely, the baseline E_2 level of AA-females (90.3 ± 54.5 pg/ml) was significantly higher than that of C-females (21.0 ± 13.9 pg/ml; Mann-Whitney *U*-test, $P < 0.05$). No significant correlation ($P > 0.05$) between the plasma E_2 level of in-

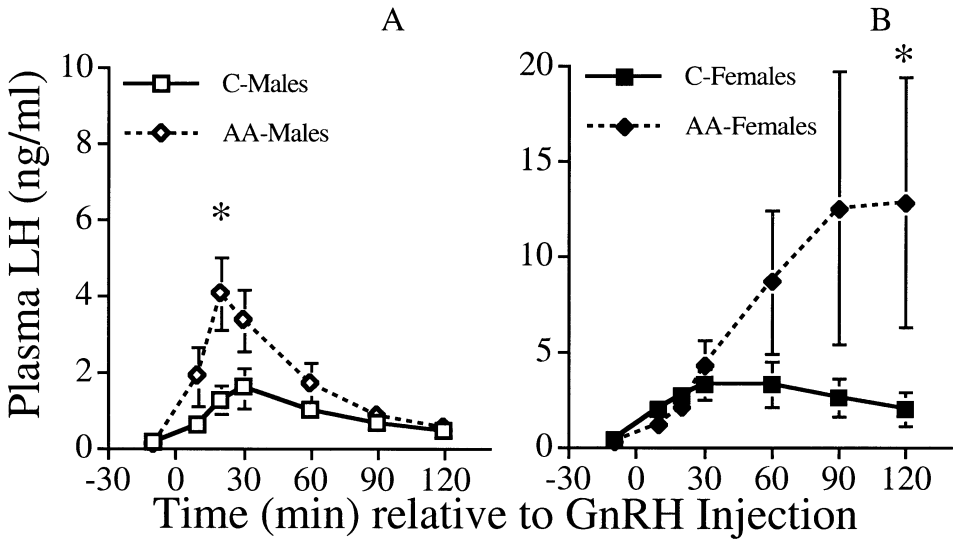

FIG. 3. Mean (±SEM) plasma LH concentrations in **A**) untreated male and prenatally AA-treated male spotted hyenas and in **B**) untreated female and prenatally AA-treated female spotted hyenas (n = 3 per group). Note the difference in scales of the y-axes in **A** and **B**. The main effect for treatment was not significant in either sex ($P > 0.05$), but the effect of prenatal AA treatment on the LH response to GnRH challenge (interaction of treatment × time) was statistically significant in male and female hyenas (males: $F_{6,24} = 4.047$, $P < 0.01$; females: $F_{6,24} = 8.370$, $P < 0.01$). *Concentrations of LH differ between AA-treated and control animals at the time points indicated ($P < 0.05$).

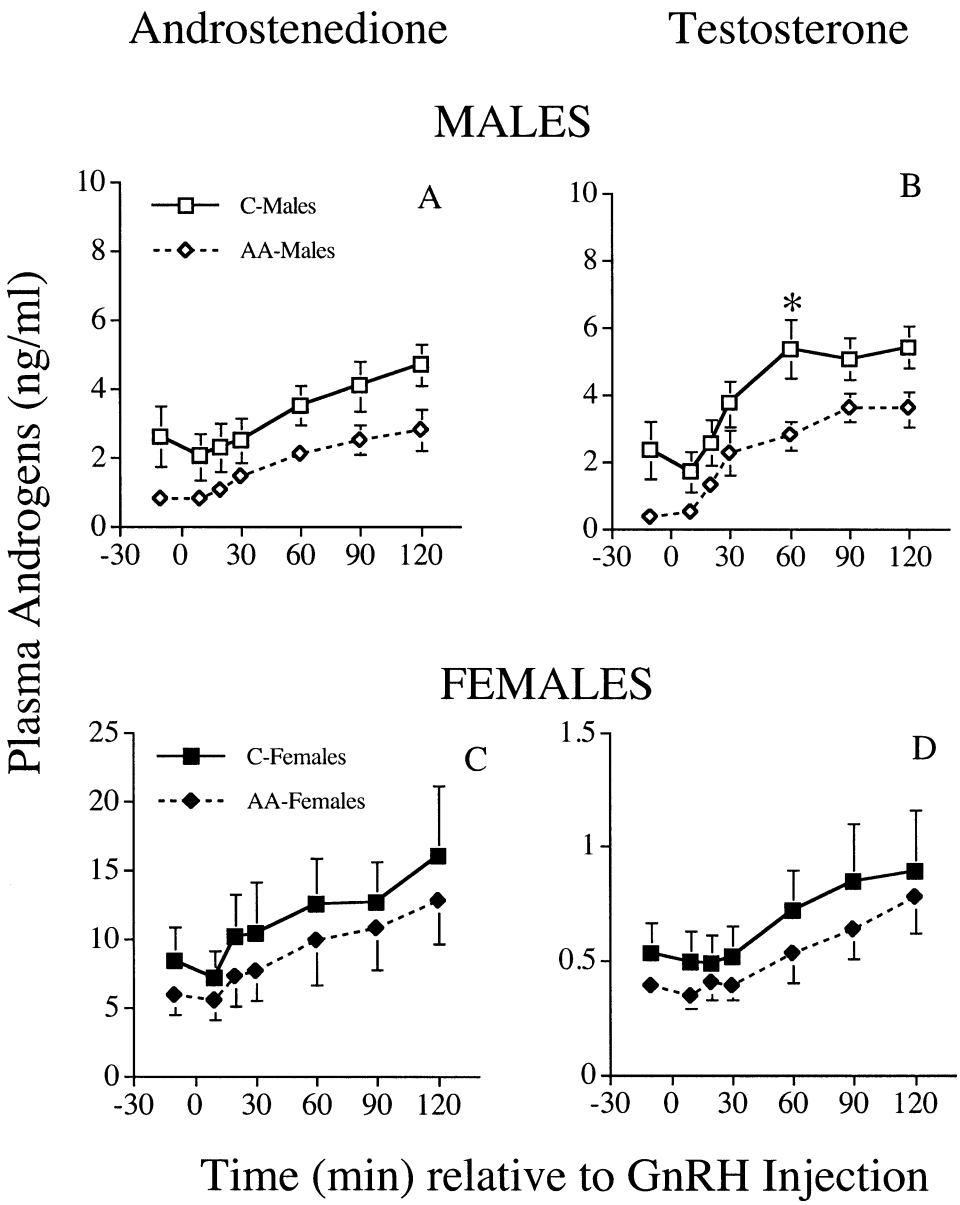

FIG. 5. Mean (±SEM) plasma A^4 (**A** and **C**) and T (**B** and **D**) concentrations in untreated and prenatally AA-treated male (top) and female (bottom) spotted hyenas. In male hyenas, differences in the A_4 levels did not achieve statistical significance (main effect: $F_{1,4} = 4.168$, $P = 0.11$; interaction effect: $F_{6,24} = 1.163$, $P = 0.36$). The main effect of treatment on T levels was not significant ($F_{1,4} = 5.991$, $P = 0.07$), but the effect of prenatal AA treatment on the T response to GnRH challenge (interaction of treatment × time) was statistically significant ($F_{6,24} = 31.198$, $P < 0.001$). Neither the main effect of treatment nor the interaction effect of treatment × time were significant for A_4 or T levels in female hyenas ($P > 0.05$). *Concentrations of T differ between AA-treated and control males at the 60-min time point.

dividual females and their baseline or peak LH levels was found (Fig. 4).

Androgen Levels in Females after hCG Challenge and GnRHa Pretreatment

Three weeks after treatment of female hyenas with the long-acting GnRHa, baseline plasma levels of LH, T, and A_4 were significantly decreased in the AA- and C-females (Fig. 6) (Wilcoxon signed rank test, $P < 0.05$ for all comparisons). Starting with nearly identical baseline levels of T and A_4 after treatment with the GnRHa, the androgen response to an hCG challenge was nearly identical in both groups (Fig. 7).

DISCUSSION

Treating spotted hyena fetuses with AA had long-lasting effects on the LH response of adults to a GnRH challenge. These findings complement the results of studies in which perinatal treatment with androgens permanently altered LH secretion in females of other mammalian species [9–12]. Moreover, the change in LH secretion of adult female spotted hyenas after prenatal AA treatment suggests that the naturally high levels of androgens to which female fetuses are exposed have a similar and lasting effect on gonadotropin secretion. However, the sex differences in baseline LH levels, the variability in the LH response to a GnRH challenge, and the effects of prenatal AA treatment point to a differential effect of androgens between the sexes. Although male and female fetuses are exposed to T deriving from the placenta, males would be exposed to additional T from the testes at a critical period during early development [24]. In addition, metabolism of maternally derived A_4 by placental 17β-hydroxysteroid dehydrogenase may produce T levels that are more variable in female fetuses, because maternal A_4 levels are themselves highly variable during gestation [5]. This, and the fact that female fetuses are not exposed to testis-derived T, may explain why the LH response to GnRH and the effects of prenatal AA treatment were more variable in females than in males. Alternatively, females may escape some of the effects of prenatal androgens on LH secretion by reducing the affinity or concentration of androgen receptors within the hypothalamic-hypophyseal unit [25].

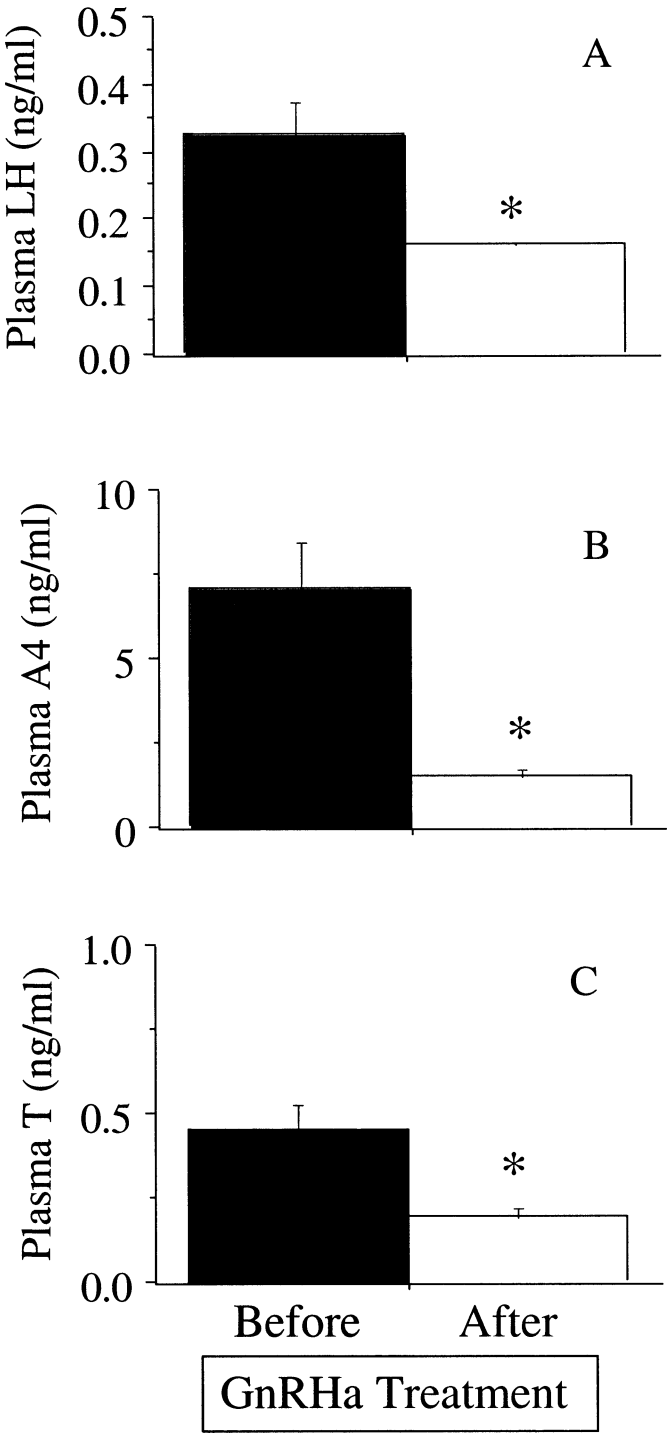

FIG. 6. Mean (±SEM) plasma concentrations of **A**) LH, **B**) A_4, and **C**) T in female spotted hyenas 2 h before and 3 wk after treatment with a long-acting GnRHa. *Plasma LH, A_4, and T levels were significantly lower after GnRHa treatment ($P < 0.05$).

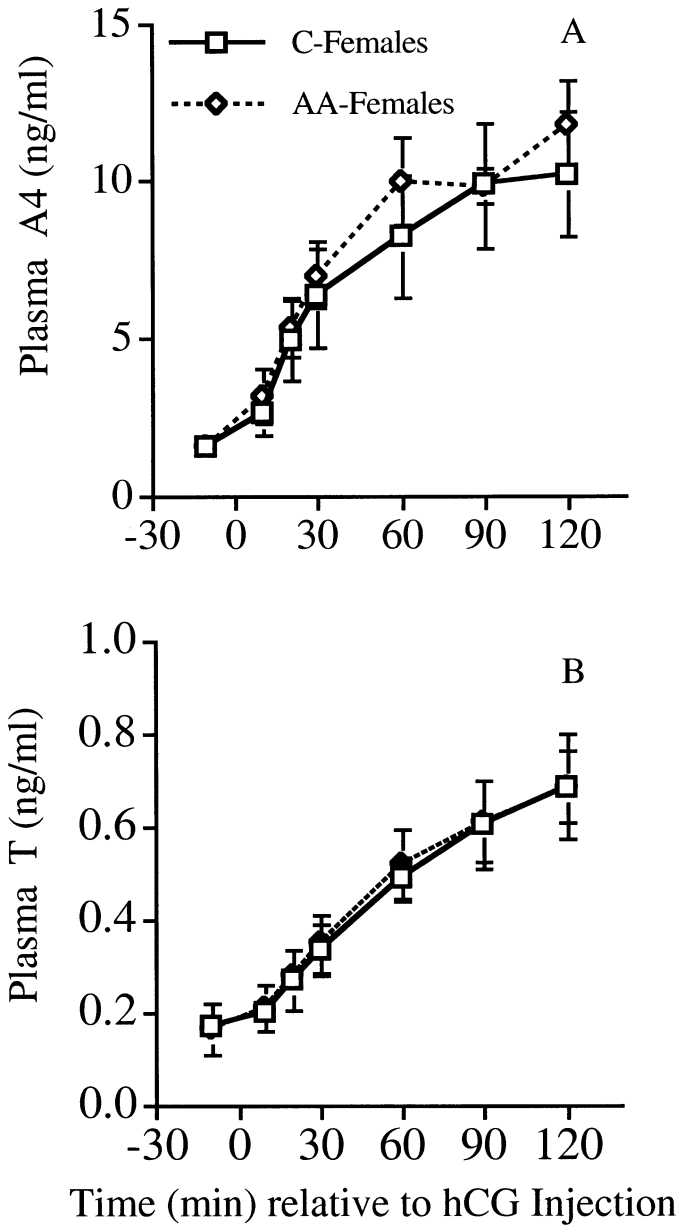

FIG. 7. Mean (±SEM) plasma A^4 (**A**) and T (**B**) concentrations in untreated and prenatally AA-treated female spotted hyenas in response to an hCG challenge. Baseline A_4 and T levels were suppressed by treatment with a long-acting GnRHa 3 wk before the hCG challenge (see Fig. 6). Neither the main effect of treatment ($P > 0.05$) nor the interaction effect of treatment × time were significant for A_4 or T levels ($P > 0.05$).

The effects that prenatal AA treatment had on the LH response to a GnRH challenge were qualitatively similar to the effects seen on development of the external genitalia in spotted hyenas [18]. Just as the penis of AA-males adopted some of the morphological features of the spotted hyena clitoris, the LH response has been similarly "feminized," with peak LH levels approximating those of untreated females. Additionally, AA treatment further exaggerated the subtle sex differences in phallic morphology in hyenas by increasing the diameter and elasticity of the clitoral meatus. The more protracted and robust LH response of the AA-females compared to C-females provides another example of further modification in the female direction [18]. Whether the alteration in the kinetics in the LH response of AA-females was the result of increased LH secretion or decreased clearance of LH awaits further in vitro incubation studies involving cultured gonadotropic cells.

In female spotted hyenas, prenatal AA treatment was generally associated with a later and higher peak in plasma LH levels following a GnRH injection. This effect was not necessarily expected, because the obverse studies in other species (i.e., perinatal exposure to T) demonstrated similar alterations in LH levels of androgenized females. For example, the maximal response to a GnRH challenge in an-

drogenized female rats occurred at 45-min postinjection, whereas untreated rats achieved maximal LH levels 30 min earlier [26]. Dumesic et al. [12] found that prenatal T exposure increased LH levels in female rhesus monkeys, whereas Steiner et al. [27] observed no differences in the LH response to an LHRH challenge in androgenized females of the same species. Similar to our study with hyenas, Dumesic et al. [12] studied gonadally intact control and experimental female monkeys, whereas Steiner et al. [27] gonadectomized their animals and attempted the GnRH challenge after pretreatment with a small dose of E_2. Steiner et al. further stated that the LH responses of all groups were too small to make meaningful interpretations regarding sex differences and the effects of prenatal androgens on pituitary function.

Women with CAH and PCOS have also been shown to have an elevated response to a GnRHa, even when the CAH is well controlled on glucocorticoid therapy [16, 28]. Why prenatal AA treatment had effects on female hyenas that were similar to those induced by prenatal androgens in other species remains a puzzle. Interestingly, Herman et al. [29] found that early prenatal treatment of female rhesus monkeys with either flutamide or testosterone enanthate (TE) resulted in higher LH levels in juvenile females. Neither flutamide nor TE had an effect on LH levels in rhesus monkeys when treatment was limited to late gestation. To our knowledge, studies involving prenatal treatment of female spotted hyenas with T have not been done, but these may prove useful in determining if flutamide might have androgen-like organizational effects on the hypothalamic-pituitary unit when administered at critical times during development. We now have animals in our hyena colony that were exposed to prenatal AA treatment during mid-gestation only. These animals have yet to achieve sexual maturity, but when they do, it will be of interest to see if AA treatment later in gestation is necessary to alter the LH response to GnRH.

Despite the elevated LH response in the AA-treated hyenas, plasma androgens were not increased in either sex. In fact, T levels of AA-males were actually lower than those of C-males, and a similar trend was seen in females. Baseline levels of T and A_4 were generally lower in AA-treated animals, and perhaps the deficit could not be overcome because of changes at the level of the gonad. However, the androgen response to an hCG challenge was identical in AA- and C-females after endogenous LH and sex steroids were suppressed by pretreatment with a GnRHa. This suggests that ovarian responsiveness to LH was unaltered in AA-females. However, during the first 6 mo of postnatal life, the same AA-females had reduced A_4 levels compared with age-matched controls [18]. These data were thought to support the epigenetic hypothesis proposed by Yalcinkaya et al. [6], which suggested that an AA-induced decrease in androgen levels in AA-females results from modifications in ovarian morphology and steroidogenic function. This could be achieved by a reduction in the number or function of theca-interstitial cells and/or an increase in the number or function of follicular granulosa cells. The higher E_2 levels and the tendency toward lower androgen levels in adult AA-females could simply reflect variations in hormone levels associated with the estrous cycle. Further testing of the epigenetic hypothesis should involve studies that quantify morphological and steroidogenic differences between the ovaries of treated and untreated hyenas.

Pretreatment with the GnRHa was limited to females, because we expected greater potential differences in the reproductive state of females than in that of males. This approach may now prove useful in assessing whether AA-males have an androgenic dysfunction at the level of the testes. Assessing the effects of perinatal flutamide treatment on the steroidogenic function in other mammals has been difficult, because flutamide often blocks testicular descent [30, 31], which can affect testicular androgen production depending on the degree of crpytorchidism [32, 33]. However, all the AA-males have normally descended testes. Once our on-going study of reproductive behavior in AA-males has been completed, we plan on castrating these animals to determine if prenatal AA exposure impairs testicular steroidogenesis and spermatogenesis.

The feedback effects of sex steroids on the LH response to GnRH are difficult to assess, and they may contribute to the differences in responses among the different study groups. Associating the robust LH response of AA-females with the higher mean E_2 levels of this group is tempting. In anestrous sheep, pituitary responsiveness to GnRH increased when ewes were pretreated with E_2 benzoate [34]. In a related study, Reeves et al. [35] found that pituitary responsiveness of cycling ewes varied with the stage of estrus, with the greatest response occurring during a short period (8 ± 4 h) on Day 1 of the estrous cycle. Conversely, the LH response to a GnRH challenge in the ferret, an induced ovulator, was greater in anestrous than in estrous females [36]. Unfortunately, the estrous cycle of the spotted hyena has not been well studied; the absence of an external vaginal opening and the lack of consistent behavioral signs of estrus make it difficult to track the estrous cycle in this species. As such, we could not administer the GnRH challenges on a specific day of the cycle. However, it seems unlikely that all three AA-females were tested during a brief period when the pituitary was hyperresponsive and that none of the C-females was tested in a similar state. Moreover, plasma E_2 did not correlate with the LH peak or the general pattern of secretion. Additionally, the three females with a plasma E_2 concentration of approximately 30 pg/ml had remarkably different LH responses to the GnRH challenge, with only the AA-female showing a continued rise in LH at 120-min postinjection (Fig. 4). Finally, the finding that the LH response of both male and female hyenas was affected by prenatal AA treatment strongly suggests that this treatment had long-lasting effects on the anterior pituitary gland that are independent of circulating levels of sex steroids.

The effects of prenatal AA treatment on LH secretion in spotted hyenas as well as the sex differences described may be associated with differences in the expression of GnRH and sex steroid receptors within the anterior pituitary gland. Sex differences in estrogen-receptor (ER) expression have been described in the rat pituitary. Demay et al. [37] found that the female rat pituitary exhibited two isoforms of ER mRNA, whereas a single ER form was detected in the male rat pituitary. Sex differences and prenatal androgen effects on nuclear androgen receptor (ARn) content are not known for the pituitary, but Toyooka et al. [38] found more ARn in the medial-basal hypothalamus (MBH) in fetal male than in fetal female guinea pigs. In addition, prenatal treatment with T increased the amount of ARn in the MBH of fetal females but not of males [39]. Sex differences in the morphology of the hypothalamus have been reported in spotted hyenas [40], and studies are now underway to look for sex differences in androgen receptor (AR) and ER content. To determine if the findings from the present study are associated with changes in hormone-receptor expression, this

line of research should be extended to the anterior pituitary, and it may ultimately include studies involving tissues from AA-treated hyenas.

In addition to the direct effects that flutamide has on blocking AR binding and the inhibitory effect that finasteride has on the conversion of T to DHT, we should also consider the potential effects that prenatal AA treatment might have had on estrogen levels in the mother and the fetus. Whereas conversion of T to DHT is important for sexual differentiation of the external genitalia [41], conversion of T to E_2 by aromatase plays an important role in sexual differentiation of the mammalian brain [9]. Thus, prenatal AA treatment might have affected LH secretion in spotted hyenas by altering estrogen levels in the hypothalamo-pituitary region of the fetus. Such alterations may reflect modifications in fetal aromatase activity and/or changes in circulating estrogen levels in the mother. Female mice lacking 5α-reductase type 1 had elevated T, A_4, and E_2 levels during gestation, with the higher E_2 levels being associated with a high rate of fetal death [42]. As alluded to in *Materials and Methods*, treating pregnant hyenas with AA was associated with a significant number of stillbirths and neonatal deaths; thus, a hormone pattern similar to the 5α-reductase type 1-deficient mice might have been expected. However, in addition to having lower DHT levels than controls, pregnant hyenas treated with flutamide and finasteride also had lower T levels [18]. We have subsequently measured E_2 concentrations in AA-treated and untreated pregnant hyenas and found significantly lower E_2 levels in the AA-treated mothers (unpublished data). With sufficient sample sizes, we could determine whether prenatal AA treatment has its impact on LH secretion via a reduction in maternal E_2 levels by simply adding E_2 to the treatment regimen. Lacking such studies, it will still be interesting to determine if sex differences exist in brain aromatase activity in spotted hyenas and if prenatal AA treatment alters that activity in any way.

The logistical constraints of working with a large carnivore prevented the acquisition of data that could more fully account for the sex differences and the effects that plasma steroid levels and prenatal AA treatment had on the LH response in spotted hyenas. To collect blood, these animals must be darted and anesthetized. Studying LH pulse frequency and variation in amplitude would be desirable but can only be done under prolonged anesthesia, which may affect the outcome of this type of study. However, studies are now underway to determine if the positive-feedback effect of E_2 on LH secretion is sexually dimorphic in spotted hyenas, as it is in rodents. Alternatively, both sexes may respond to an E_2 challenge by releasing surge amounts of LH, as is the case in primates [8]. The effects of an E_2 challenge on LH in our AA-treated hyenas will also be of interest, because Connolly and Resko [43] found that female guinea pigs treated prenatally with a 5α-reductase inhibitor showed a greater LH response to E_2 treatment. Given the profound organizational effects that prenatal androgens have on the hypothalamic-pituitary unit in a diverse group of female mammals, how the highly virilized female spotted hyena escapes those effects remains an open and intriguing question.

ACKNOWLEDGMENTS

The authors would like to thank Laurence Frank for his role in establishing the spotted hyena colony at the University of California, Berkeley. We thank the veterinary staff of the Office of Laboratory Animal Care at the University of California, Berkeley; T.B. Hayes for use of his RIA lab; and Paul Licht for his insightful comments regarding the data. We thank the Office of the President of Kenya for permission to conduct the field research described in this paper. We also thank the Kenya Wildlife Service, the Narok County Council, and the Senior Warden of the Masai Mara National Reserve for their cooperation. The following individuals provided valuable assistance performing GnRH challenges in the field: E.E. Boydston, S.M. Dloniak, A. Engh, P. Garrett, T.H. Harty, K. Nelson, G. Ording, and M. Szykman.

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