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# The anti-androgen combination, flutamide plus finasteride, paradoxically suppressed LH and androgen concentrations in pregnant spotted hyenas, but not in males

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ABSTRACT

The androgen receptor blocker flutamide and the 5α-reductase inhibitor finasteride have been used in a variety of species to investigate the ontogeny of sexual dimorphisms by treating pregnant females or neonates at critical periods of sexual differentiation. Likewise, we have used these drugs to study the profound masculinization of the external genitalia in female spotted hyenas. However, a potential pitfall of administering flutamide, either alone or in combination with finasteride, is that it maintains or even raises plasma concentrations of luteinizing hormone (LH) and testosterone (T), because negative feedback of the hypothalamic–pituitary–gonadal axis is disrupted. Contrary to expectations, when pregnant spotted hyenas were treated with flutamide and finasteride (F&F), the concentrations of T during late gestation were suppressed relative to values in untreated dams. Herein, we further investigate the paradoxical effects of F&F treatment on a battery of sex hormones in spotted hyenas. Beyond the effects on T, we found plasma concentrations of LH, estradiol, progesterone and androstenedione (A4) were also significantly lower in F&F-treated pregnant hyenas than in controls. Flutamide and finasteride did not have similar effects on LH, T, and A4 concentrations in male hyenas. The paradoxical effect of F&F treatment on LH and T concentrations in the maternal circulation suggests that negative feedback control of gonadotropin and androgen secretion may be modified in spotted hyenas during pregnancy.



## 1. Introduction

The female spotted hyena, *Crocuta crocuta*, is renowned for having the most highly masculinized external genitalia among extant mammals. The spotted hyena clitoris approximates the size and length of the penis and is traversed by a single urogenital canal, through which the female receives the male during copulation, urinates, and gives birth. To test the hypothesis that prenatal androgens masculinize the external genitalia in female spotted hyenas, Drea et al. [4] treated dams with anti-androgen drugs from early gestation to parturition. The most commonly used regimen was a combination of an androgen receptor blocker (flutamide) and a 5α-reductase inhibitor (finasteride). Finasteride blocks the conversion of T to the more potent metabolite 5α-dihydrotestosterone (DHT), which is the androgen principally responsible for the masculinization of the genital tubercle in eutherian mammals [13,27]. One potential drawback of using flutamide and finasteride (F&F) to prevent the masculinization of the external genitalia is that F&F treatment could disrupt negative feedback of the hypothalamic–pituitary–gonadal (HPG) axis and consequently increase the concentration of T [5,14]. As a result, increased T could theoretically overcome the intended androgen blockade. When F&F-treated hyena dams failed to produce offspring with a diminutive phallus and a vaginal opening separate from the urethra [4], the disruption of negative feedback due to F&F treatment was considered a possible explanation. However, contrary to expectations, Drea et al. [4] found T levels were actually lower in three F&F-treated dams during late gestation than in the same dams during untreated pregnancies.

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Because the preliminary investigation by Drea et al. [4] found no evidence of the anticipated negative feedback of F&F treatment on maternal T, in this study we performed a comprehensive analysis of sex hormones using archived samples from the original F&F-treated dams. To more fully assess the effects of anti-androgens on the HPG axis in spotted hyenas, we analyzed plasma concentrations of luteinizing hormone (LH), estradiol (E2), androstenedione (A4), and progesterone (P4), in addition to measuring T. We also increased the sample size of subjects by collecting blood samples from two additional F&F-treated hyenas and nine untreated hyenas during late gestation. The hormone concentrations of F&F-treated and untreated dams were matched for gestational age.

In spotted hyenas, the concentrations of T in untreated pregnant females during late gestation were as high, if not higher, than in adult males [8,15]. Therefore, we included a modest number of male hyenas in this study as a preliminary investigation to determine if the hormonal changes in response to F&F treatment observed in pregnant females would be apparent in males. Moreover the inclusion of males provided an opportunity to evaluate the effects of administering flutamide and finasteride alone, as well as F&F combined, on circulating LH and androgen concentrations.

## 2. Materials and methods

All experiments were completed at the Field Station for the Study of Behavior, Ecology, and Reproduction at the University of California, Berkeley, where a captive colony of spotted hyenas has been studied since 1985. All studies received prior approval from the Animal Care and Use Committee of the University of California at Berkeley and conformed to principles described in the NIH guide for the use and care for laboratory animals. All animals were adults when blood samples were collected for hormone analyses. Animals were immobilized with ketamine (4–6 mg $kg^{-1}$) and xylazine (1 mg $kg^{-1}$) administered by blow dart. Blood was collected from the external jugular vein, centrifuged, and the drawn-off plasma was aliquotted and frozen at −80 °C until assayed.

### 2.1. Experiment 1: pregnant hyenas

A single blood sample was collected from each of five F&F-treated and nine untreated, pregnant hyenas during the third trimester of an 110-d gestation. F&F-treated dams were given oral flutamide (17–25 mg/kg/d, Schering Corporation, Kenilworth, NJ) and finasteride (0.55–1 mg/kg/d, Merck and Co. Inc., West Point, PA) twice daily, starting in the first or second trimester and continuing until parturition [4]. All pregnancies derived from timed matings and fetal viability and growth were documented by serial ultrasound examinations [22].

### 2.2. Experiment 2: treatment of males

The number of adult males that could be treated with anti-androgens was limited by the modest size of the Berkeley colony and the need to maintain stud males for breeding. Three adult male hyenas were sampled once before treatment and then again at the end of a four-week of treatment with flutamide alone (25 mg/kg/d) and then with finasteride alone (1 mg/kg/d), given orally twice daily. The hiatus between the flutamide and finasteride treatments was at least 3 months. These three males received no further treatment so that they could resume breeding. Two different adult males were treated with F&F for 4 weeks, at the doses given above. These latter males were also sampled once before and at the end of treatment.

### 2.3. Hormone assays

All hormones were analyzed by radioimmunoassay (RIA). Plasma concentrations of testosterone (T), androstenedione (A4), and luteinizing hormone (LH) were measured in nearly all hyenas studied, but the determination of progesterone (P4) and estradiol (E2) concentrations were limited to pregnant dams.

For measurement of T, A4, and E2 concentrations, plasma samples were analyzed using RIA's that have been validated for spotted hyenas and in use by the Berkeley Spotted Hyena Project for many years [4,15,21]. Intra- and inter-assay coefficients of variation (CV) were < 12 and 10%, respectively. The cross-reactivity of the T antiserum with DHT was 44%. Licht et al. (1992) found that a substantial proportion of "testosterone" measured in unchromatographed hyena samples was DHT (29.5% in males and 44.1% in pregnant females), and thus DHT accounts for some proportion of the T concentrations reported herein.

Plasma concentrations of LH were measured with a heterologous RIA validated for spotted hyenas [21] 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. Samples were run in two batches. The first run included samples from pregnant females and had a minimum detectable limit (MDL) of 0.06 ng/mL. The second run included samples from males and had an MDL of 0.09 ng/mL. The intra- and inter-assay CVs for the LH assay were 7.4% and 9.0%, respectively.

Plasma P4 concentration was measured by RIA as previously described Dahl et al. [3]. Briefly, P4 concentrations spanned a broad range and thus hyena plasma samples were variably diluted (1:10 to 1:600) in PBSG-gelatin (PBSG) to ensure that values fell on the standard curve (12.5–5000 pg/ml; P4 standards from Steraloids, Newport, RI). Diluted samples were extracted with diethyl ether and reconstituted in PBSG. Extracted samples were incubated overnight at 4 °C with $^{3}$H-P4 (New England Nuclear, Waltham, MA) and antiserum (#8939, Stebenfeldt, University of California, Davis). Bound and unbound P4 were separated with charcoal dextrin. Samples were incubated for 20 min at 4 °C, centrifuged at 4 °C (1160*g*), and the supernatant was poured into vials with 4 ml of scintillation cocktail for counting. A serial dilution of pooled hyena plasma was parallel to the standard curve. The intra- and inter-assay CVs for the P4 assay were 4.2% and 6.9%, respectively.

### 2.4. Statistics

Hormone data were analyzed with a commercial statistical program (JMP 8.0.1, SAS Institute, Cary, NC). All data were log-transformed to better approximate normal distributions and homogeneity of variances. Untransformed data were used for graphical purposes. Observed differences were considered statistically significant if $p < 0.05$, and these are reported as such regardless of the actual *p*-value.

In experiment 1, log-transformed hormone values from F&F-treated and untreated pregnant dams were compared using Student's *t*-test. In experiment 2, sample sizes for males were necessarily small because treatment of hyenas with anti-androgens removed them from the pool of breeders for other investigations and for maintenance of the colony. Therefore, statistical analyses were limited to situations when the sample sizes were at least three per group. Log-transformed hormone data pre- and post-anti-androgen treatments were analyzed using paired *t*-tests. Analysis of data from the two F&F-treated males was limited to descriptive representations. Note, however, that when the paradoxical effects of F&F treatment on T levels were originally detected in pregnant hyenas, the outcomes were pronounced and apparent in just three animals [4]. Therefore, we were looking for large effects that could be evident in a small number of males.