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# Developmental Programming: Postnatal Estradiol Amplifies Ovarian Follicular Defects Induced by Fetal Exposure to Excess Testosterone and Dihydrotestosterone in Sheep

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**Abstract**

Excess of prenatal testosterone (T) induces reproductive defects including follicular persistence. Comparative studies with T and dihydrotestosterone (DHT) have suggested that follicular persistence is programmed via estrogenic actions of T. This study addresses the androgenic and estrogenic contributions in programming follicular persistence. Because humans are exposed to estrogenic environmental steroids from various sources throughout their life span and postnatal insults may also induce organizational and/or activational changes, we tested whether continuous postnatal exposure to estradiol (E) will amplify effects of prenatal steroids on ovarian function. Pregnant sheep were treated with T, DHT, E, or ED (E and DHT) from days 30 to 90 of gestation. Postnatally, a subset of the vehicle (C), T, and DHT females received an E implant. Transrectal ultrasonography was performed in the first breeding season during a synchronized cycle to monitor ovarian follicular dynamics. As expected, number of $\geq$8 mm follicles was higher in the T versus C group. Postnatal E reduced the number of 4 to 8 mm follicles in the DHT group. Percentage of females bearing luteinized follicles and the number of luteinized follicles differed among prenatal groups. Postnatal E increased the incidence of subluteal cycles in the prenatal T-treated females. Findings from this study confirm previous findings of divergences in programming effects of prenatal androgens and estrogens. They also indicate that some aspects of follicular dynamics are subject to postnatal modulation as well as support the existence of an extended organizational period or the need for a second insult to uncover the previously programmed event.

**Keywords**

infertility, follicular dynamics, polycystic ovary syndrome

## Introduction

In sheep, a precocial species, the establishment of the ovarian reserve and folliculogenesis up to the antral stage occurs in utero, as in humans.[1] Later in life, several causes, including irradiation or exposure to endocrine disruptors, may enhance follicular depletion and reduce follicular reserve.[2,3] Experimental manipulation of the prenatal steroid environment during in utero life has provided a resource to determine how follicular reserve, depletion, and persistence are established prenatally. Comparative studies of sheep treated prenatally with testosterone (T), an aromatizable androgen, and dihydrotestosterone (DHT), a nonaromatizable androgen, have helped dissect out the programming effects of androgen and estrogen in establishing rate of postnatal follicular recruitment and degree of follicular persistence.[4] Extensive ovarian phenotyping across the reproductive life span of sheep has revealed that prenatal T treatment increases ovarian follicular recruitment[5,6] and follicular persistence[7] with both these events contributing to a multifollicular ovarian morphology.[8] Studies comparing the effects of T versus DHT suggest that follicular recruitment is programmed via androgenic actions of T[5] and follicular persistence via estrogenic actions.[9] Confirmation

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of the programming effects via estrogens would require prenatal estradiol (E) treatment. However, exposure to excess estrogens, such as diethylstilbestrol, during early pregnancy in sheep has been reported to induce abortion, thus posing constraints relative to the amount of E that could be delivered to pregnant sheep.

The detrimental effects of exposure to excess steroids during fetal life raises concerns relative to the exposure from endocrine-disrupting chemicals with steroidogenic potential, which are present ubiquitously in nature.[10,11] An aspect to consider is whether detrimental effects of steroids are limited to the prenatal period when ovarian differentiation occurs, or can continue, postnatally. Postnatal effects may involve organizational effects at the ovarian level if follicular differentiation is not complete or serve as amplifiers or decoders of programmed events. The double-hit hypothesis proposes that an early-life environmental insult sets up a predisposition to a pathologic state, which may then emerge in the presence of a subsequent insult or the so called "second hit" in later life.[12] For instance, in the prenatal T-treated sheep, the severity of the reproductive defects is enhanced by postnatal overfeeding.[13] The progressive loss of cyclicity that occurs in prenatal T-treated sheep may also be the consequence of postnatal endocrine changes ("second hit"), namely, increased exposure to continuous high E levels[14] or reduced exposure to progesterone due to oligo- or anovulation ($P_4$).[7,14] Similarly, the shift in the rate of follicular depletion observed after puberty in prenatal T-treated sheep, by the slowing of depletion rate and stockpiling of growing follicles,[5] may relate to the altered steroid milieu. If so, this has enormous implications since humans are ubiquitously exposed to endocrine-disrupting chemicals throughout their life span. For example, exposure to estrogenic compounds postnatally, beyond the critical period of ovarian development in utero, has been found to reduce conception rates, increase embryonic loss, and impair ovarian function.[15]

The aims of the current study were to (1) distinguish between the roles of prenatal E and DHT (estrogenic vs androgenic), (2) determine whether a combined treatment with E and DHT would recapitulate the effects of prenatal T, the aromatizable androgen, and (3) examine whether continuous postnatal exposure to E ("second hit") will amplify prenatal T and DHT effects on ovarian follicular and luteal dynamics.

## Materials and Methods

### Animals and Prenatal Treatment

*Prenatal treatments.* All procedures were approved by the Institutional Animal Care and Use Committee of the University of Michigan and were consistent with the National Institutes of Health Guide for Use and Care of Animals.

The study was conducted at the University of Michigan Sheep Research Facility (Ann Arbor, Michigan; 42° 18′N). Suffolk sheep used in this study were treated prenatally with vehicle (C), T, the nonaromatizable androgen DHT, E, or ED (E and DHT; n = 14, 14, 13, 6, and 7, respectively (see Figure 1 regarding allocation of animals to experimental groups). Husbandry and nutrition of maternal sheep and newborn lambs and details of prenatal treatments with T and DHT have been published.[16,17] Briefly, pregnant sheep were injected (intramuscularly [im]) twice weekly with either 100 mg T propionate (Sigma Chemical Co, St Louis, Missouri) or 100 mg DHT propionate (Steraloids, Inc, Newport, Rhode Island) suspended in 2 mL cottonseed oil from 30 to 90 days of gestation. Prenatal E-treated animals were generated by treating pregnant sheep for the same duration with a 30-mm SILASTIC implant (Dow Corning Corp, Midland, Michigan) filled with crystalline E. Implants were placed subcutaneously in the axillary region as described previously[18] and designed to produce plasma levels of 1 to 3 pg of E/mL.[18] The ED group received E implants and DHT injections for the same length. Control animals did not receive the vehicle, cottonseed oil, since no differences in reproductive attributes were found between vehicle-treated and nontreated controls in a previous study.[14] Lambs were weaned at ~8 weeks of age. Lambs were fed a pelleted diet (Shur-Gain, Elma, New York) consisting of 3.6 MCal/kg digestible energy and 18% crude protein. At 8 weeks of age, animals were weaned and maintained outdoors at the University of Michigan Sheep Research Facility. They were fed ad libitum until they attained 40 kg of body weight, at which point they were switched to a diet with 15% crude protein with a feed ration consisting of 2.3 MCal/kg digestible energy and 11.3% crude protein.

*Postnatal treatments.* Nine weeks after birth, approximately one-half the number of C, T, and DHT female lambs were inserted with 10 mm E implants, which produces circulating levels of <1 pg/mL E,[19] levels comparable to that found during the follicular phase. The final sample size of prenatal treatment groups was 7, 7, 7, 6, and 7, for C, T, DHT, E, and ED groups, respectively. Sample size of postnatal E-treated groups was 7, 7, and 6, for C+E, T + E, and DHT + E groups, respectively (see Figure 1 regarding allocation of animals to experimental groups). There were insufficient numbers of E and ED females born for developing postnatal E groups.

### Experimental Design

The study was conducted during the first breeding season. All females were synchronized with 2 prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 5 mg/mL; Lutalyse, Pfizer Animal Health, Michigan) injections, im, 11 days apart. Beginning with the second $PGF_{2\alpha}$ injection (0 h), blood samples were obtained daily until day 21 to monitor plasma $P_4$ concentrations that were used to confirm the presence of a corpus luteum (CL) and its function. Plasma samples were stored at −20°C until assayed.

### Ultrasonographic Assessment of Ovarian Status

Starting on the day of the second $PGF_{2\alpha}$ injection, daily ultrasonographic examinations of the ovaries were carried out for 21 days to monitor follicular dynamics. Ultrasongraphy was carried out using a scanner (SSD 500; Aloka, Wallingford,

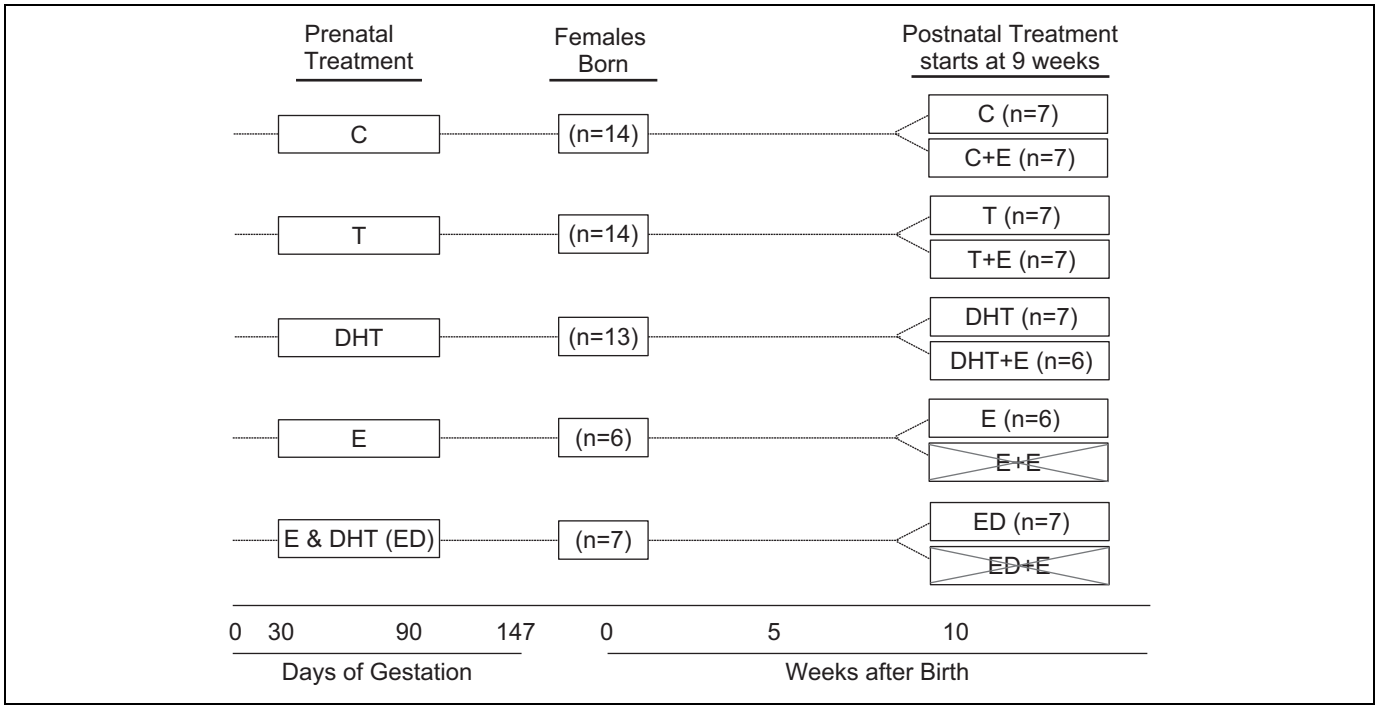


**Figure 1.** Schematic illustration of the experimental design of the study. Five prenatal groups (C, T, DHT, E, and ED) were generated following specific treatments from days 30 to 90 of gestation. Three postnatal treatment groups (C + E, T + E, and DHT + E) were generated which involved postnatal exposure to estradiol (see Methods section for detailed description). The small number of females born of the prenatal E and ED groups precluded generation of corresponding postnatal groups. C indicates vehicle; DHT, dihydrotestosterone; E, estradiol; ED, E and DHT; T, testosterone.

Connecticut) fitted to a 7.5-MHz linear array transducer adapted for transrectal examinations. Details of ultrasound scanning have been previously described.[7,9] In brief, sheep were placed in dorsal recumbence, and the probe inserted in the rectum with the transducer orientated perpendicular to the abdomen wall. After passing the urinary bladder and locating the uterine horns, the probe was rotated laterally 90° clockwise and 180° counterclockwise to locate both the ovaries. A coating of carboxymethyl cellulose (Sigma-Aldrich Corp) as a 3.5% gel was applied to the probe to enhance the ultrasound transmission.

All follicles with antral diameter of ≥2 mm and CL were identified and measured. The sizes and relative positions of the follicles and CL were sketched on ovarian charts and were used to assess daily changes in follicular dynamics. Digital video images of the ovarian scans were obtained to document follicular and luteal changes. The same 2 investigators were involved in the performance of ultrasonography throughout the study to avoid subjectivity of measures. Follicles were tracked across successive days using the CL and/or the largest follicles as landmarks. Luteinized follicles were defined as a follicular structure with a thick and highly echogenic wall suggestive of luteinization.

## Hormone Assays

Plasma $P_4$ concentrations were measured in daily plasma samples using a solid phase radioimmunoassay kit (Coat-A-Count P Diagnostic Products Corp., Los Angeles, California), as previously described.[20] All the samples were assayed in duplicate 100-μL aliquots. The assay sensitivity was 0.017 ± 0.002 ng/mL (mean ± standard error of the mean) and intra-assay coefficient of variations (CVs), based on the 2 quality control pools measuring 1.9 ± 0.1 and 14.8 ± 0.1, were 3.1% ± 0.6% and 3.0% ± 0.5%, respectively. The interassay CVs for the same quality control pools were 10.7% and 4.6%, respectively.

## Statistical Analysis

*Overall analysis.* Two main comparisons were carried out. The first analysis involved the comparison of all prenatal-treated groups relative to controls (C vs T, C vs DHT, C vs E, and C vs ED). In the second analysis, each prenatal group was compared to its postnatal E-treated counterpart (C vs C + E, T vs T + E, and DHT vs DHT + E).

*Number and diameter of follicles.* Follicles were classed by size as 2, ≥3 to 4, >4 to 8, and ≥8 mm in diameter. Size delineation is based on previously established functional attributes, namely, gonadotropin responsive (2 mm), gonadotropin dependent (≥3-4 mm), ovulatory-sized follicles (>4-8 mm), and beyond (≥8 mm).[21-23] Follicles were considered persistent if they were present on the ovary for ≥12 days.[24] This analysis was carried out in 2 ways; first, including the luteinized follicles in the analysis and second, excluding the luteinized follicles. Analysis of luteinized follicles was performed

separately (see section on luteinized follicles subsequently). Analyses were performed on all females regardless of whether they had responded or not to the $PGF_{2\alpha}$ synchronization. It needs to be recognized that the neuroendocrine environment in responder compared to nonresponder females is likely to be different and therefore have the potential to influence follicular population. However, this is an inherent caveat of the studies where one of the groups is oligo- or anovulatory, such as prenatal T-treated females.[9] To test the difference in follicular counts between prenatal and postnatal groups, analysis of variance (ANOVA) was used for all the size categories, except the size category $\geq$8 mm, for which Wilcoxon test was used. A generalized Fisher exact test was used to compare follicle size distribution between the groups. Bonferroni adjustment was used to adjust for multiple comparisons.

*Duration of follicles.* Follicular survival was determined in follicles that grew to $\geq$3 mm in diameter and were present on the ovary for $\geq$2 days, as previously reported.[7,9] Follicles <3 mm were included only in the calculation of the total number of follicles. For follicle survival analysis, duration was calculated as the interval between the first day observed at $\geq$3 mm size until largest size was achieved and then back to the 3 mm size. First detection of some follicles did not correspond to their actual day of appearance (ie, first scanning day); therefore, for a $\geq$3-mm follicle, we assumed duration was longer than the time it was observed and set value as right censored. The estimated follicle duration was based on the assumption that follicles grow and regress at a constant rate of approximately 1 mm/d in control females.[25] A linear mixed effect model was used to test the differences in the duration of follicles.

*Ovulatory follicles analysis.* The proportion of females that ovulated and the diameter and duration of the ovulatory follicles were calculated. As described previously, first detection of ovulatory follicles did not correspond to their actual day of appearance (right censored data on the first scanning day). The product-limit survival estimates for the duration of ovulating follicles in each treatment group were computed, and these survival curves were compared using a log-rank test. Duration of ovulatory follicles was calculated from the time they were $\geq$3 mm until ovulation, which was confirmed by presence of a new CL with a corresponding increase in plasma concentrations of $P_4$.

*Luteinized follicles.* Differences in number of luteinized follicles were assessed using the exact Kruskal-Wallis test and post hoc analysis using exact Wilcoxon test adjusting for multiple comparisons using the Bonferroni method. Differences in the proportion of females that had luteinized follicles were assessed using the generalized Fisher exact test. The ANOVA was used to test the difference in duration of luteinized follicles between groups. Since luteinized follicles were not present in the C group, comparisons were made among the other prenatal groups (T, DHT, E, and ED).

*Corpora lutea and $P_4$ analysis.* Corpora lutea data were collected from 3 consecutive cycles, presence of CL when scanning began (regressing CLs), CL formed from ovulatory follicles during the first follicular phase tracked by ultrasonography (first cycle), and CL detected a week after daily ultrasonography had stopped (second cycle). Information of the entire luteal phase was only available in first cycle CLs. The proportion of ewes that had regressing first-cycle and second-cycle CLs was calculated and compared using Fisher exact test. Poisson regression was used to compare counts of CLs in all 3 CLs types (regressing, first, and second cycle) assessed. The differences in size and duration of the CL in the first cycle between the groups were tested using Kruskal-Wallis test. Circulating $P_4$ levels were used to classify the progestogenic responses into 3 abnormal categories; subluteal cycles ($P_4$ levels below 2 ng/mL), short cycles ($P_4$ levels lasting less than 10 days), and nonregressing cycles ($P_4$ levels remained elevated over 0.5 ng/mL $\geq$ 15 days). Pearson correlation between circulating $P_4$ levels and CL size for each group was calculated. Fisher *z* statistics was used to compare whether the correlation between $P_4$ level and CL differed among groups. For this correlation, $P_4$ data from all the females in the group were used irrespective of whether a CL was present or not. Follicular wave analyses were not carried out since some of the prenatally treated females did not ovulate, and therefore no alignment to the day of ovulation was possible. Significance was defined as $P < .05$. All analyses were performed using SAS for Windows (release 9.1.3; SAS Institute Inc, Cary, North Carolina).

## Results

Mean number of follicles per day categorized by size are shown in Figure 2; and representative follicular profiles of all nonovulatory follicles, ovulatory follicles, luteinized follicles, and $P_4$ profiles throughout the scanning period in both prenatal (C, T, DHT, ED, and E) and postnatal (C, C + E, T, T + E, DHT, and DHT + E) groups are shown in Figure 3.

### Follicular Number: Prenatal Comparisons

Mean number of follicles of prenatal groups categorized by size are shown in Figure 4 (left panels). Analysis of the mean number of follicles revealed no differences in the number of follicles of any category except for the number of $\geq$8 mm follicles, which was higher in the T group (2.0 $\pm$ 0.2) versus the C group (0.3 $\pm$ 0.3; $P < .05$). No differences were observed in the mean number or duration of ovulatory follicles (data not shown). Duration of nonovulatory follicles was similar among the prenatal groups (C: 3.3 $\pm$ 0.2, T: 3.3 $\pm$ 0.3, DHT: 3.2 $\pm$ 0.2, ED: 3.3 $\pm$ 0.3, and E: 3.1 $\pm$ 0.2 days; ns).

### Follicular Number: Postnatal Comparisons

Analysis of the mean number of all classes of follicles (Figure 4, right panels) found no effect of postnatal E (C vs C + E, T vs T + E, DHT vs DHT + E; ns) on follicle classes $\leq$2 mm, 3 to $\leq$4 mm, and $\geq$8 mm. Postnatal E treatment reduced the number of 4 to 8 mm follicles relative to prenatal DHT-treated group (DHT: 7.0 $\pm$ 0.7 vs DHT + E: 3.7 $\pm$ 0.9, respectively; $P <$ .05). No differences were observed in the mean number or

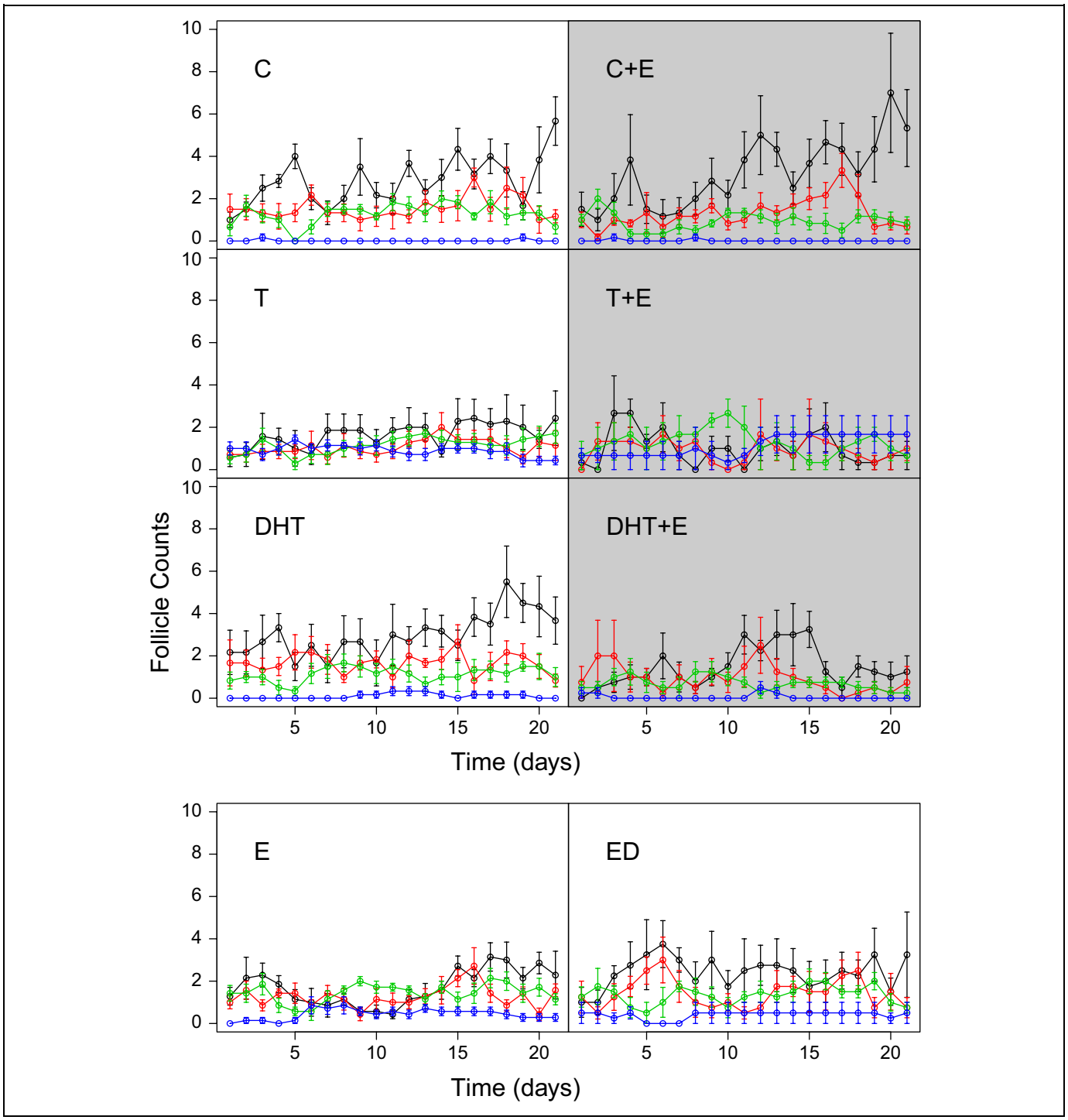


**Figure 2.** Mean (±standard error of the mean) of ≤2 mm (*black*), ≥3 to 4 mm (*red*), >4 to 8 mm (*green*), and ≥8 mm (*blue*) nonovulatory follicles throughout the scanning period (21 days) relative to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) administration in C, T, DHT (*top left panels*), C + E, T + E, DHT + E (*top right panels*), E (*bottom left panels*), and ED (*bottom right panels*). Postnatal E-treated groups (C + E, T + E, and DHT + E) are represented by a shaded area. C indicates vehicle; DHT, dihydrotestosterone; E, estradiol; ED, E and DHT; T, testosterone.

duration of ovulatory follicles (data not shown). Postnatal E did not have an effect on the duration of nonovulatory follicles (C: 3.3 ± 0.2 vs C + E: 2.5 ± 0.2 days; ns, T: 3.3 ± 0.3 vs T + E: 3.2 ± 0.6 days; ns, and DHT: 3.2 ± 0.2 vs DHT + E: 2.8 ± 0.3 days; ns).

### *Luteinized Follicles: Prenatal Comparisons*

Percentage of females bearing luteinized follicles was significantly different among groups ($P < .001$). Higher percentage of prenatal T- (100%; $P < .01$) and E-treated (71.4%; ns) females had

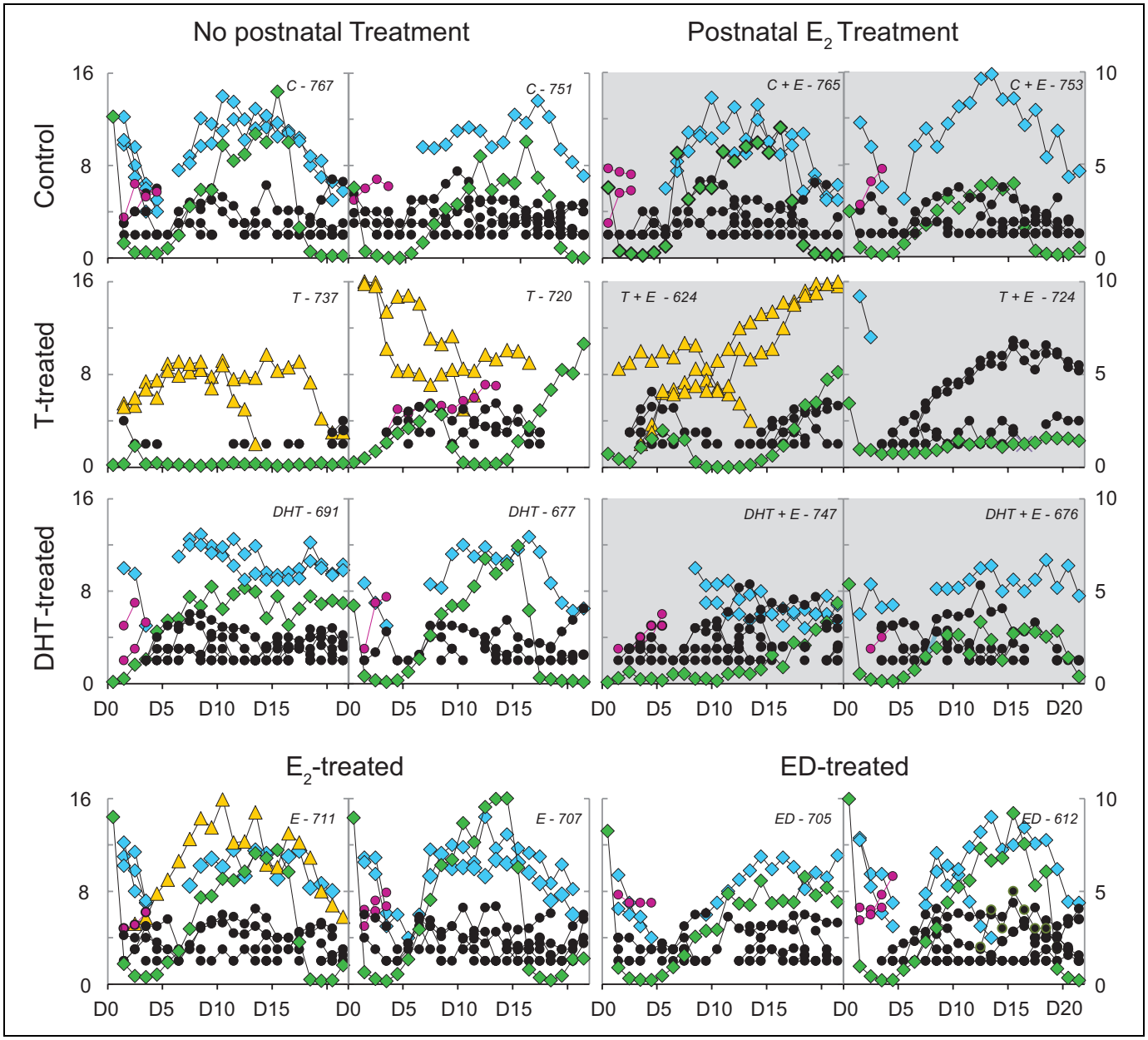


**Figure 3.** Changes in nonovulatory follicles (≥2 mm; *black*), ovulatory follicles (*magenta*), luteinized follicles (*yellow*), corpora lutea (*blue*), and circulating $P_4$ levels (*green*) throughout the scanning period (21 days) relative to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) administration in 2 representative females of each of the treatment groups studied: C, T, DHT (*top left panels*), C + E, T + E, DHT + E (*top right panels*), E (*bottom left panels*), and ED (*bottom right panels*). Postnatal E-treated groups (C + E, T + E, and DHT + E) are represented by a shaded area. Left axis used for follicular and corpora lutea data (mm) and right axis used for $P_4$ levels (ng/mL). C indicates vehicle; DHT, dihydrotestosterone; E, estradiol; ED, E and DHT; T, testosterone.

luteinized follicles compared to the control group (0; Figure 5). The number of luteinized follicles (Figure 5, left panels) differed among prenatal treatment groups ($P = .001$). Post hoc analysis revealed that both prenatal T- ($1.4 \pm 0.2$; $P < .01$) and E-treated ($1.0 \pm 0.4$; $P < .05$) groups had more luteinized follicles compared to the C group (0). Since no luteinized follicles were present in the control group it was not included in the luteinized follicles survival analysis. The survival analysis of the luteinized follicles found that duration was longer in the T- ($27.2 \pm 2.8$ days) and ED-treated ($32.5 \pm 1.5$ days) groups compared to the DHT-treated group ($15.0 \pm 3.1$ days; $P < .05$). Maximum size analysis revealed that luteinized follicles in the DHT group were smaller ($8.4 \pm 1.05$ mm) when compared to both T and ED groups ($15.9 \pm 0.44$ and $18.6 \pm 0.52$ mm, respectively; $P < .05$).

### *Luteinized Follicles: Postnatal Comparisons*

Postnatal E did not have an effect on the percentage of females bearing luteinized follicles, number, maximum size, or duration of luteinized follicles (Figure 5, right panels).

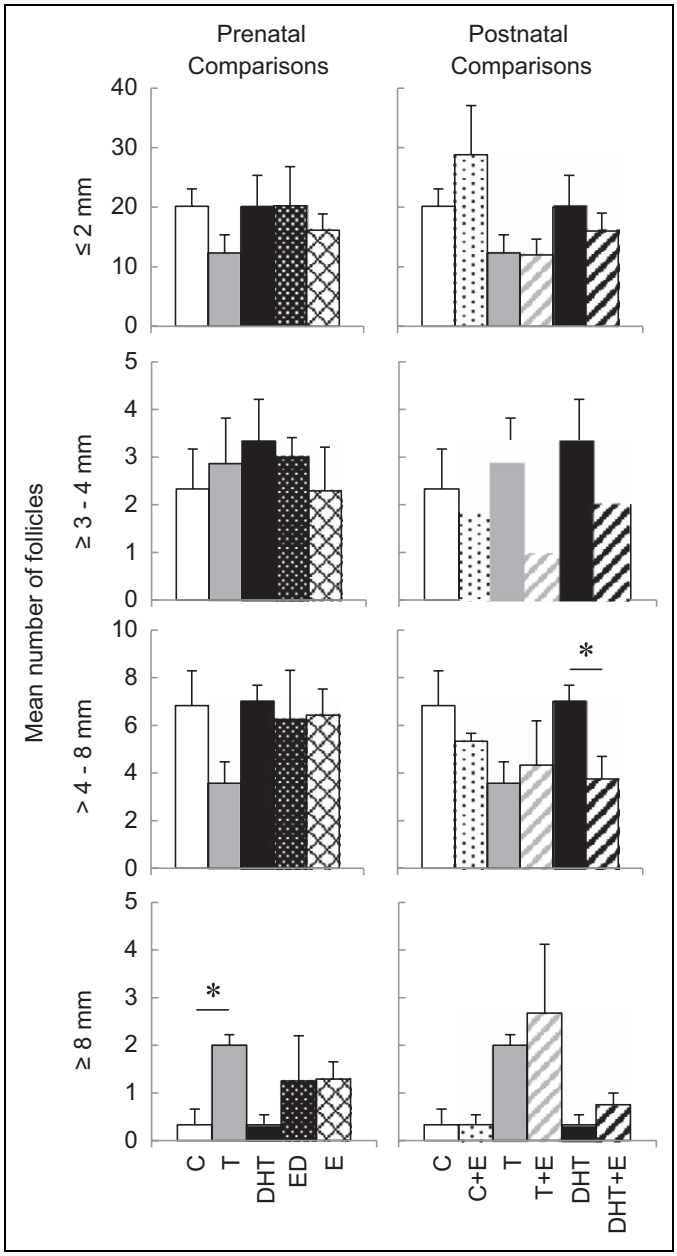


**Figure 4.** Mean (± standard error of the mean) number of follicles by size classes of ≤2 mm (gonadotropin responsive), ≥3 to 4 mm (gonadotropin-dependent recruited follicles), >4 to 8 mm follicles (selected to become ovulatory sized), and ≥8 mm (larger than ovulatory sized follicles) detected by ultrasonography in ovaries of prenatal groups (C, T, DHT, ED, and E; *left panels*) and postnatal groups (C, C + E, T, T + E, DHT, and DHT + E; *right panels*). Asterisks represent significant differences ($P < .05$). C indicates vehicle; DHT, dihydrotestosterone; E, estradiol; ED, E and DHT; T, testosterone.

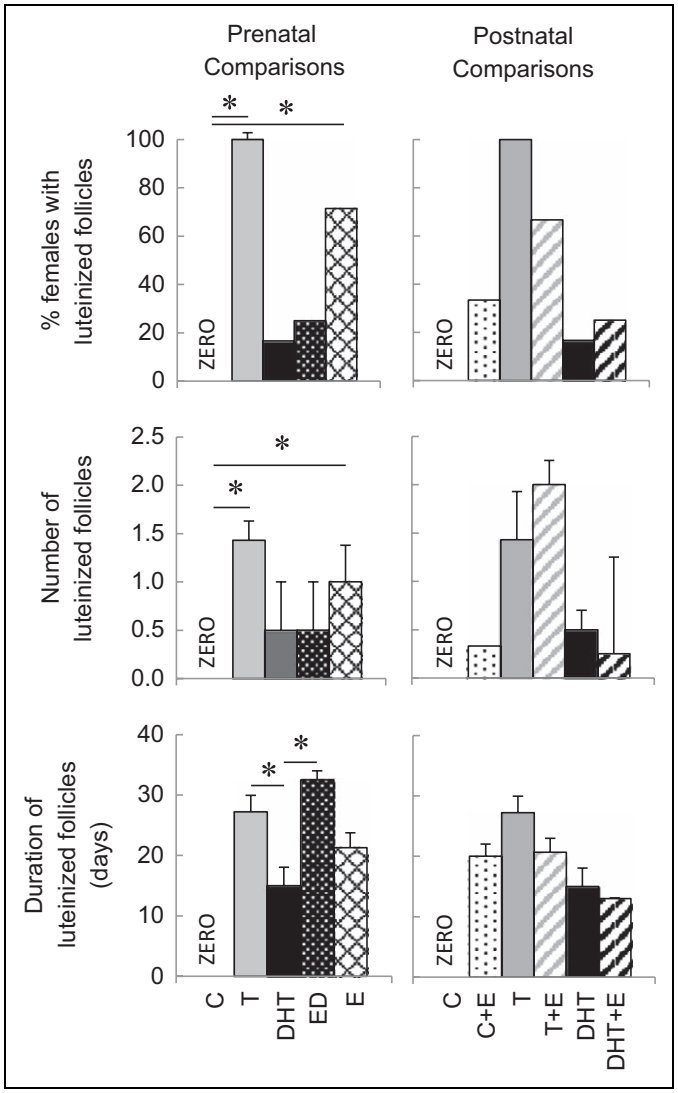


**Figure 5.** Percentage of females bearing 1 or more luteinized follicles (*top panels*) in experimental groups treated only during prenatal period (C, T, DHT, ED, and E; *left panels*) and groups treated both during prenatal and postnatal periods (C, C + E, T, T + E, DHT, and DHT + E; *right panels*). Mean (± standard error of the mean) number (*middle panels*) and duration (days; *bottom panels*) of luteinized follicles detected by ultrasonography in ovaries of experimental groups treated only during prenatal period (C, T, DHT, ED, and E; *left panels*) and prenatal plus postnatal-treated groups (C, C + E, T, T + E, DHT, and DHT + E; *right panels*). Asterisks represent significant differences ($P < .05$). C indicates vehicle; DHT, dihydrotestosterone; E, estradiol; ED, E and DHT; T, testosterone.

## *Corpus Luteum Function: Prenatal Comparisons*

All C females synchronized in response to the $PGF_{2\alpha}$ administration. The percentage of females that synchronized did not differ among the prenatal groups (Figure 6, left panels). The number of CLs after $PGF_{2\alpha}$ administration was also not significantly different among the prenatal groups, although only 28.6% of T females and 50% of DHT females had a CL after $PGF_{2\alpha}$ synchronization. The size and duration of the CLs were similar among all prenatal groups. In all prenatal groups, $P_4$ levels correlated with the size of the corpora lutea (Pearson range 0.51-0.66; $P < .0001$) and were similar among prenatal groups. The lag between $P_4$ increase (above 0.5 ng/mL) and CL detection by ultrasonography was not statistically different between the groups. However, compared to C females (0.83 ± 0.4) most prenatally treated groups had a numerically longer $P_4$

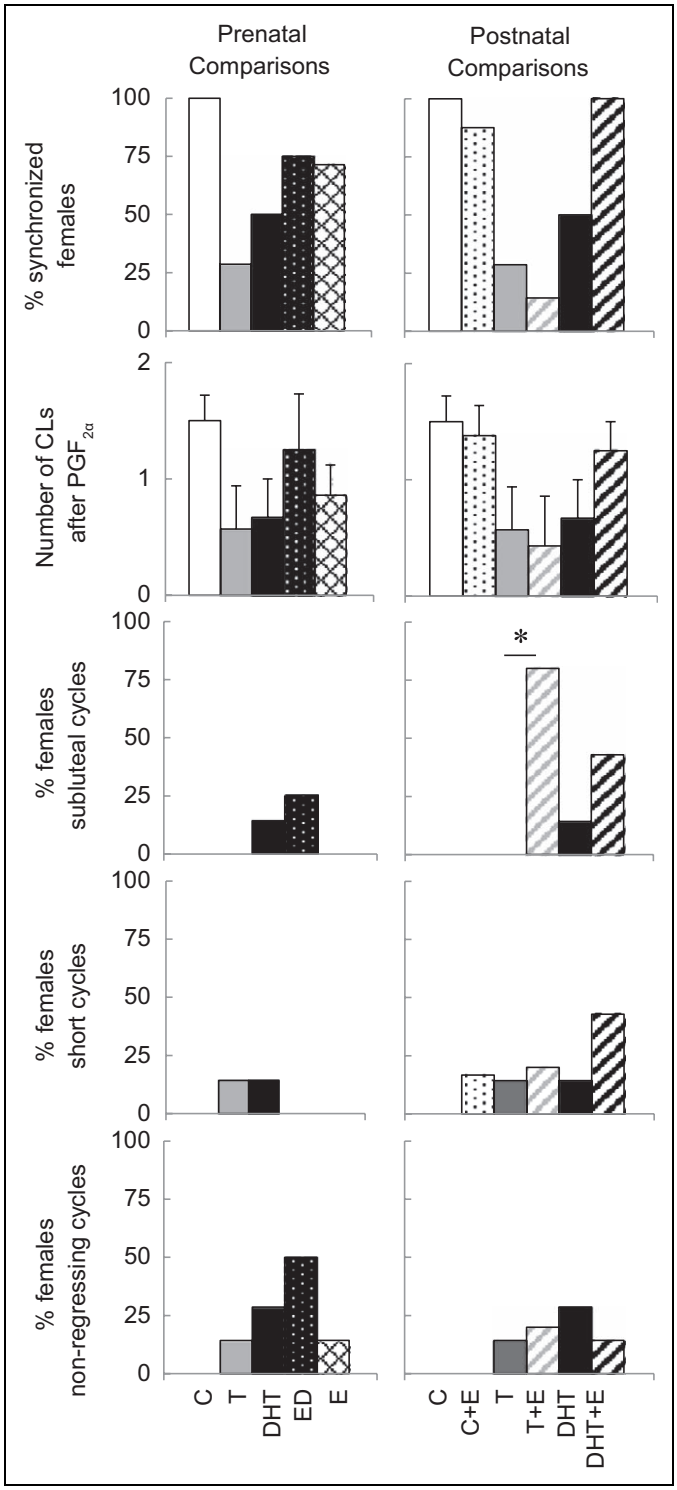


**Figure 6.** Percentage of ewes that synchronized (*top panels*) and had abnormal progestogenic cycles (subluteal: $P_4 \leq 2$ ng/mL; short: $P_4 \leq 10$ days, or nonregressing $P_4$ cycles: $P_4$ elevated $\geq 15$ days; *3 bottom panels*). Number of corpus luteums (CLs) after prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) administration in experimental groups treated only during prenatal period (C, T, DHT, ED, and E; *left panels*) and treated both during prenatal and postnatal period (C, C + E, T, T + E, DHT, and DHT + E; *right panels*). Asterisks represent significant differences ($P < .05$). C indicates vehicle; DHT, dihydrotestosterone; E, estradiol; ED, E and DHT; T, testosterone.

to CL lag [T, DHT, E, and ED: 2.5 (n = 2), 2.4 ± 0.7, 1.8 ± 0.4, and 2.7 ± 1.2, respectively].

Although several luteal disruptions (Figure 6, left panels) were evident among the prenatal-treated groups, including subluteal cycles, short cycles, and nonregressing cycles (see Methods for details), no statistical differences were found in the percentage of females displaying such disruptions compared to the control group.

### *Corpus Luteum Function: Postnatal Comparisons*

Postnatal E did not have a significant effect on the percentage of females that synchronized in response to the $PGF_{2\alpha}$ administration (Figure 6, right panels). Postnatal E neither affected the number of CLs after $PGF_{2\alpha}$ administration nor the size and duration of the CLs. In all postnatal groups, $P_4$ levels correlated with the size of the corpora lutea (Pearson range 0.45-0.72; $P < .0001$), with no postnatal E effect. The lag between $P_4$ increase (above 0.5 ng/mL) and CL detection by ultrasonography, although not statistically different, was numerically lower in C and C + E females (0.83 ± 0.4 and 0.33 ± 0.3) compared to the rest of the groups [T, DHT, and DHT + E: 2.5 (n = 2), 2.4 ± 0.7, and 2.25 ± 1.3, respectively].

Similar to the prenatal groups, several luteal disruptions (Figure 6, right panels) were observed among the postnatal-treated groups. Postnatal E increased the incidence of subluteal cycles in the T + E versus T group (T + E: 80% vs T: 0%; $P < .05$) and had a numerical increase in DHT + E versus DHT group (DHT: 14.3% vs DHT + E: 42.9%) but did not significantly affect the incidence of females displaying short or nonregressing cycles.

## Discussion

This study is the first to examine the interaction of prenatal steroid excess and postnatal E excess on ovarian follicular dynamics in female sheep. Findings from this study provide support for previous findings of divergences in programming effects of prenatal androgens and estrogens on ovarian follicular dynamics. In addition, results indicate that some aspects of follicular dynamics are subject to differential postnatal organizational or activational modulation or may need a second insult (such as E in this study) to uncover a previously programmed event, consistent with the "second hit" hypothesis.[12] Contributions from prenatal and postnatal programming to ovarian follicular differentiation are discussed subsequently.

### *Prenatal Effects of Follicular Dynamics*

Consistent with previous findings,[7] increased number of large follicles (≥8 mm) contributing to the development of polycystic ovarian morphology was evident in prenatal T-treated females. Because comparative studies with prenatal T- (aromatizable androgen) and DHT (nonaromatizable androgen)-treated females had suggested that the increase in the number of antral follicles is likely programmed via estrogenic actions of T,[9] we predicted that both prenatal E and ED treatment

groups would induce an increase in antral follicular accumulation while prenatal DHT treatment would not. Although the findings from prenatal DHT treatment group were consistent with this premise, findings from the prenatal E and ED treatment groups failed to provide support for estrogenic mediation of increased antral follicle presence. One possibility is that levels of E achieved via E implant in the prenatal E- and ED-treated groups was lower than the levels of E achieved via aromatization of T and endogenous E contribution in the prenatal T-treated group. Alternatively, the failure to replicate the prenatal T phenotype may stem from the inability to reproduce the androgen/estrogen milieu of the prenatal T-treated group. A previous study involving prenatal exposure to the estrogenic endocrine disruptor, octylphenol,[26] from day 70 of gestation through weaning, also failed to produce follicular disruption. In Wright et al's study,[26] the treatment began after completion of gonadal differentiation as opposed to the current study where treatment was initiated prior to start of gonadal differentiation at day 30 of gestation.[1]

The increase in the number of large follicles in the present study was largely driven by the presence of large luteinized follicles, an occurrence previously reported in prenatal T-treated females.[9] The presence of luteinized follicles was recorded in 60% and 100% of prenatal E- and T-treated females, respectively, suggestive of estrogenic programming of this trait. Because this trait was predicted to be programmed via estrogenic actions, the expectation was that ED-treated females would have a similar phenotype as prenatal T- or E-treated females. The fact that an increase in luteinized follicles was not observed in ED-treated females may be reflective of increased androgenic potency of DHT used, the pharmacokinetics of DHT and its metabolites, and/or the ratio of androgen to estrogen achieved.

Luteinized follicles observed in this study were similar in structure to those described as naturally occurring hemorrhagic anovulatory follicles in humans[27,28] and baboons[29] or luteinized unruptured follicles (LUFs) in mares.[30] The LUFs have also been induced in other animal models, including sheep,[31] when the prostaglandin inhibitor indomethacin was administered intrafollicularly. Interestingly, the duration of the indomethacin-induced LUFs in sheep is similar to that of naturally occurring corpora lutea and, therefore, likely controlled by the same luteolytic factors.[31] Some, but not all, of the luteinized follicles observed in the current study followed this pattern of follicular regression. The similarity of this trait between what is observed in this study and the spontaneous occurrence of LUFs in humans and mares,[28,30] as well as pharmacologically induced LUFs in humans and sheep,[27,31] may be suggestive of a common pathway, although not the same etiology. Because the incidence of LUFs in mares is associated with synchronization of cycles with $PGF_{2\alpha}$ occurring a week after $PGF_{2\alpha}$ administration but is not associated with human chorionic gonadotropin administration, they have been hypothesized to originate from early antral follicles.[32,33] Importantly, elevated circulating luteinizing hormone (LH) levels have been found to be associated with an increased prevalence of LUFs in the mare[34] and humans.[28] As such, the LH excess seen in prenatal T-treated female, the result of reduced sensitivity to steroid-negative feedback,[14,35] and/or increased pituitary sensitivity to GnRH[36] may have contributed to the development of these luteinized follicles.

Alternatively, luteinization may be the result of a specific defect in the granulosa cells of large antral follicles. Granulosa cells of prenatal T-treated females have been found to have several disruptions at the level of steroid receptors and members of the insulin signaling and apoptosis pathways.[37-39] Premature luteinization and a concomitant upregulation of Cyp11a1 in granulosa cells are features of Smad-4 knockout mice.[40] An upregulation Cyp11a1 expression is also evident in prenatal T-treated sheep fetuses,[41] although it remains to be determined whether a similar disruption persists into adulthood.

## Postnatal Effects on Follicular Dynamics

Because 4 to 8 mm size follicles represent the selected pool of follicles in a given follicular wave in sheep,[42] the reduced number of 4 to 8 mm follicles seen in prenatal DHT-treated females following postnatal E treatment may be indicative of compromised selection process or failure to transition into dominance, which in sheep occurs when follicles reach a 6-mm diameter.[42] The lack of ovulatory defects in DHT + E females, in the face of reduced number of 4 to 8 mm follicles, indicates that those follicles that overcome this compromise in the selection process are able to transition to dominance and ovulate, although the conditions required for the selection process may be more rigorous in DHT + E females. The fact that prenatal T-treated females are not as sensitive to the postnatal E insult as prenatal DHT-treated females suggests differences in the follicular developmental trajectory resulting from differences in estrogen exposure (T can be aromatized to E, while DHT, is a nonaromatizable[43]). Because both groups of females respond similarly to E-negative feedback,[44] the differential ovarian outcomes should stem from differential ovarian sensitivities and involve an intrinsic defect at the follicular level. In support of this, prenatal T-treated females have increased expression of the androgen receptor in granulosa cells of preantral and antral follicles.[37] A reduction in androgen receptor expression has been described to be critical during follicular maturation.[45] Considering that androgens are known inducers of follicular atresia and apoptosis,[46,47] the divergent response of prenatal T- and DHT-treated females to postnatal E may relate to differences in androgenic potency (DHT is more potent than T[43]).

The finding of lack of differences in other follicular categories may relate to composite analyses of all follicles observed throughout the course of study. The inability to do this analysis by follicular wave stems from the fact that not all females synchronized and ovulated in response to the $PGF_{2\alpha}$ administration; as such, alignment to the day of ovulation was not possible. If the LH excess seen in prenatal T-treated females[35,36] is the trigger for occurrence of luteinized follicles, absence of postnatal E effects on luteinized follicles suggests

that levels of E achieved with E implant postnatally may not be of sufficient magnitude to impact LH secretion, in the face of reduced sensitivity to the E-negative feedback.[35] Although postnatal E treatment had no effect on the number and size of ovulatory follicles, its impact on other aspects of ovarian function cannot be ruled out. For instance, low doses of diethylstilbestrol and the xenoestrogen, bisphenol A, administered postnatally reduce the follicular recruitment in female lambs.[48]

### *Postnatal Effects on Luteal Function*

Because postnatal E treatment disrupted ovarian cyclicity in control females, despite the presence of a functional preovulatory LH surge mechanism,[49] we expected postnatal E treatment to also disrupt luteal function. Absence of luteal defects in the C + E group in this study may relate to lower levels of E achieved (10 mm implant for current study vs 30 mm implant in the previous study[49]) or the timing of initiation of E exposure (2 weeks in the previous study[49] compared to 9 weeks in this study). In contrast, postnatal E treatment increased occurrence of subluteal cycles in prenatal T-treated females. It is not uncommon to find subluteal cycles in prenatal T females.[14] Considering that luteinization process is triggered by LH,[50] subluteal cycles are likely the result of the dampened LH surges observed in prenatal T-treated females.[14] Alternatively, although the reduced LH surge may be adequate to induce luteinization, the granulosa cells of prenatal T-treated females may have a reduced sensitivity to LH. Increased occurrence of subluteal cycles in prenatal T-treated females following postnatal E treatment may be the result of changes in expression of genes regulated by E,[51] which are critical for early CL formation. Alternatively, because prenatal T-treated females manifest compromised E-positive feedback,[52] postnatal E may further compromise the neuroendocrine trigger.

## Conclusions

The findings from this study indicate that luteal and follicular responses to postnatal E differ between prenatal T- and DHT-treated females. As such, the prevailing steroid milieu during critical periods of differentiation may establish varying set points leading to differential responses to the same postnatal insult. This is of relevance, since humans and animals are exposed ubiquitously to endocrine-disrupting chemicals with steroidogenic properties throughout their development.[53] Developmental exposure to such disruptors has the potential to reprogram tissue susceptibility during postnatal life and trigger differential activational effects in different individuals based on the history of their prenatal exposure.

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