Assessment: Effects of Porcine Zona Pellucida Immunocontraception on Estrous Cyclicity in Feral Horses

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Assessment: Effects of Porcine Zona Pellucida Immu

noncontraception on Estrous Cyclicity in Feral Horses

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The study used noninvasive fecal hormone monitoring to assess whether contraception with porcine zona pellucida (PZP) had an impact on estrous cycle characteristics in a herd of feral horses living on Assateague Island, Maryland. The study assayed longitudinal fecal samples, collected over a 2-year interval, for total estrogens (estradiol and estrone) and progestagens. Follicular (6.4 ± 2.4 days) and luteal (11.2 ± 3.2 days) phase durations were within the range reported for domestic horses, with an average estrous cycle duration of 18.6 ± 2.5 days. There were no differences (p > .05) in phase durations between currently treated and currently untreated mares in either year of the study. Endocrine data suggested that ovulatory failure occurred frequently in Assateague mares; however, we could not link this phenomenon directly to contraception. Overall, no estrous cycle characteristics differed between currently treated and currently untreated mares. However, all mares in this study had been treated with PZP at some point in the previous 10 years. Because PZP has been shown to induce decreased ovulation rates in several mammalian taxa, including Assateague horses, we speculate that in addition to blocking sperm binding PZP may adversely impact ovarian function in mares.

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The zona pellucida (ZP) and its potential role in contraception have received substantial attention (Skinner, Timmons, Schwoebel, & Dunbar, 1990). Immunocontraception relies on the body’s production of antibodies that interfere with critical events in reproduction. To date, vaccines have been developed against neuropeptides, gonadotropins (Moudgal et al., 1986), steroid hormones (Al-Kawafi, Hopwood, Pineda, & Faulker, 1974), sperm (Primikoff, Lathrop, Woolman, Cowan, & Myles, 1988), and ova (Sacco, 1987). The most promising vaccines are those directed against the ZP (Aitken, Richardson, & Hulme, 1984; Sacco & Yurewicz, 1989). Injection of foreign ZP into a host stimulates the production of antibodies that prevent sperm binding to ZP sperm receptors (Florman & Wassarman, 1985; Hasegawa, Koyama, Inoue, Takemura, & Isojima, 1992). The structure of these glycoprotein receptors has been so highly conserved among mammalian species that porcine ZP (PZP) can be used to immunize various taxa such as nonhuman primates (Sacco, 1987), rabbits (Wood, Liu, & Dunbar, 1981), dogs (Mahi-Brown, Yanagimachi, Hoffman, & Huang, 1985), and ungulates (Kirkpatrick, Turner, Liu, & Fayrer-Hosken, 1996). Subsequent contraception is achieved when host-produced antibodies physically block fertilization by binding to ZP sperm receptors.

PZP-induced immunocontraception is an attractive option for controlling wildlife populations because, in theory, this form of contraception does not alter hormone secretion and, therefore, is presumed to leave the treated individual behaviorally and physiologically intact. However, many studies of laboratory and free-ranging animals have shown that PZP treatment can cause abnormalities in ovarian steroid production and histology (Bamezai, Das, & Talwar, 1986; Gulyas, Gwatkin, & Yuan, 1983; Hasegawa et al., 1992; Kirkpatrick, Liu, Turner, Naugle, & Keiper, 1992; Kirkpatrick, Naugle, Liu, Bernoco, & Turner, 1995; Mahi-Brown et al., 1985; Sacco, Pierce, Subramanian, Yurewicz, & Dukelow, 1987; Skinner, Mills, Kirchick, & Dunbar, 1984; Wood et al., 1981). Among horses, no abnormalities in ovarian histology were documented after 1 year of treatment with PZP (Liu, Bernoco, & Feldman, 1989); however, feral mares on PZP for 3 consecutive years demonstrated diminished urinary estrogen conjugate levels and lower ovulation rates than untreated mares (Kirkpatrick et al., 1992). The decline in estrogen excretion and ovulation rate continued in mares that had been on PZP for up to 7 years (Kirkpatrick et al., 1995). These data suggest that, in addition to preventing sperm binding, PZP immunocontraception also may have an adverse impact on ovarian follicular development or function, or both.

In this study, estrous cycle characteristics were analyzed in a population of Assateague horses who were part of a long-term contraceptive field trial (Kirkpatrick et al., 1992, 1995). Young mares (age 2 years) are given two injections of PZP 2 to 4 weeks apart to begin contraception (Kirkpatrick et al., 1992). Liu et al. (1989) determined that PZP immunocontraception lasts for 1 year in mares; thus, on Assateague contraception is prolonged by administering an annual booster of vac-
Noninvasive fecal hormone monitoring was used to facilitate consistent sampling in these feral, free-ranging horses. Fecal sampling also permitted behavioral monitoring to be conducted without animal disturbance. Cyclic fluctuations in fecal estrogen and progesterone metabolites are known to reflect ovarian follicular and luteal dynamics, respectively, in a diversity of wildlife species, including horses (Brown, Wasser, Wildt, Graham, & Monfort, 1997). Specific comparisons between currently treated and currently untreated mares in this study focused on examining differences in follicular and luteal phase lengths and ovulation rates. We assumed that mares who did not receive an annual booster prior to the onset of our study during the 1997 breeding season no longer were effectively contracepted. We tested the hypothesis that estrous cycle parameters would differ in currently treated and currently untreated mares because PZP is an immunocontraceptive that should not interfere with estrous cycles or ovarian hormone production. Thus, any effects that PZP may have while a mare is treated in the 12-month period following initial or booster injection should not be evident once treatment has expired—more than 12 months after initial or booster injection.

**METHOD**

**Sample Collection**

The study site and population was described previously (Powell, 1999). Fresh fecal samples were collected from individually identified mares (see Table 1) every 2 to 5 days during the following intervals: July 3 to September 4, 1997 ($n = 13$ mares); and June 9 to July 23, 1998 ($n = 12$ mares). The terms *currently treated* and *currently untreated* refer to mares that did or did not receive an inoculation with PZP for contraception during the breeding season.

Fecal samples were collected off the ground within 1 to 3 min after animals defecated. A composite sample of each fecal pile was collected to ensure a homogeneous sample, and samples were collected only from animals who could be positively identified. No samples were taken from fecal piles on which the stallion urinated. Feces were stored in polypropylene tubes at $-20 \degree C$ or $-80 \degree C$ until assay.

**Fecal Extraction and RIA**

We conducted fecal hormone metabolite assays according to the methods of Wasser, Monfort, Southers, & Wildt (1994). Briefly, $\sim 0.025$ g of dried, pulverized feces were boiled in 100% ethanol for 20 min and centrifuged for 18 min at 1500 revolutions per minute. The supernatant was decanted into a clean tube, evaporated to dryness, reconstituted in 1 ml methanol, and then diluted 1:10 with phosphate-buffered saline for the progestagen radioimmunoassay (RIA; Brown, Wasser, Wildt, & Gra-
ham, 1994) and diluted 1:10 with steroid diluent buffer for the total estrogens RIA (see following). Tritiated estradiol (~6,000 d.p.m.) and 14C-labeled progesterone (~1,700 d.p.m.) were added to each fecal sample before extraction to monitor procedural losses. The 125I-progestagen RIA employed a broad-spectrum monoclonal antisem that cross-reacts with progesterone, 100%; 5α-pregnane-3β-ol-20-one, 96%; 5α-pregnane-3α-ol-20-one, 36%; 17β-hydroxyprogesterone, 15%; pregnenolone, 13%; 5β-pregnane-3α-ol-20-one, 7%; 5β-pregnane-3α, 17α-diol, 20α-one, 5% (Brown, Wasser, et al., 1994; Wasser et al., 1994). Fecal extracts were assayed for total estrogens (unconjugated estradiol-17β [E2] and estrone [E1] using a commercially available, double antibody 125I kit (ICN Pharmaceuticals, Inc., Costa Mesa, CA) that cross-reacts with estradiol-17β, 100%; estrone, 100%; estriol, 9%; estradiol-17α, 7% and equilin, 2.5%. Assays for fecal progestagens and total estrogens were validated using standard techniques to confirm specificity, sensitivity, accuracy, and precision.

**Estrous Cycle Duration**

We calculated follicular and luteal phase duration estimates using an iterative process described by Brown, Citino, Shaw, & Miller (1994) in which high values for total estrogens or progestagens (considered separately) for each mare

<table>
<thead>
<tr>
<th>ID</th>
<th>Age</th>
<th>Foaled Previously</th>
<th>Year Sampled</th>
<th>On PZP During Sampling</th>
</tr>
</thead>
</table>

Note. PZP = zona pellucida.
within a sampling year (1997 or 1998) were excluded if they exceeded the mean hormone concentration for all fecal samples plus 2 standard deviations. After exclusion of these values, calculations were iterated until no further outliers (i.e., values that exceeded baseline) were identified. The remaining values were used to approximate baseline estrogen or progestagen concentrations. Follicular and luteal phase duration estimates were obtained by regressing second-order polynomial curves through each outlying point and the adjacent baseline points on either side. The resulting equation was solved for \( x \) (day) when \( y \) (hormone level) exceeded a 95% confidence interval for the mean baseline value for that hormone. The values obtained were measured to the nearest 0.1 day. The regressions proved to be a good fit for the data (\( r = .98 \) for estrogens and .89 for progestagens) and provided more precise estimates for phase lengths than could be obtained by using the fecal samples alone. Phase length estimates are presented as mean ± standard deviation.

Behavioral Data Collection

All occurrences of sexual behavior were recorded using an ethogram modified from Asa, Goldfoot, & Ginther (1979) as part of a larger study of social behavior in this population of horses. For each instance of sexual behavior, we recorded the actor, behavior(s), recipient(s), and any responses. When fecal samples were collected from a group, behavioral data were not formally collected because sampling sometimes produced disturbance in the group (e.g., moving away from the observer); however, ad lib notes were taken on any sexual behavior observed, and a mare was characterized as “in heat” if she demonstrated frequent urination, presenting, or if she allowed the stallion to mount.

Statistical Analysis

Estrous cycle characteristics were compared between years using \( t \) tests or Mann–Whitney \( U \) tests when the assumption of normality could not be met.

RESULTS

Fecal Extraction and RIA

**Fecal progestagens RIA.** Serial dilutions of pooled fecal extracts (neat to 1:16,000) yielded displacement curves parallel to those obtained with standard preparations of progesterone. Recovery of unlabeled progesterone (3.75–480 pg/tube) from pooled fecal extracts was 102.0% ± 3.7%. Linear regression analysis of the amount of progesterone measured compared to the amount added, after subtraction of endogenous hormone, yielded a correlation coefficient of 0.98 for the fe-
cal extract \((y = -3.2 + 1.3x)\). Assay sensitivity was 1.8 pg/tube, whereas interassay coefficients of variation for high- (~30% binding) and low- (~60% binding) mass internal controls were 9.8% and 11.1\((n = 9)\), respectively. Intraassay coefficients of variation were less than 10%. Hormone concentrations were expressed as mass equivalents of hormone per gram of dry feces.

RIA of fecal extracts after high performance liquid chromatography (HPLC) revealed at least nine immunoreactivity peaks. One peak (40.7% of total immunoreactivity) coeluted with progesterone, whereas each of the other minor peaks constituted 4.4% to 10.8% of total immunoreactivity.

**Fecal total estrogens RIA.** Serial dilutions of pooled fecal extracts (neat to 1:16,000) yielded displacement curves parallel to those obtained with standard preparations of estradiol (E2). Recovery of unlabeled E2 (1.25–100 pg/tube) from pooled fecal extracts was 106.0% ± 4.5%. Linear regression analysis of the amount of E2 measured compared to the amount added, after subtraction of endogenous hormone, yielded a correlation coefficient of 0.99 for the fecal extract \((y = 0.16 + 0.97x)\). Assay sensitivity was 0.63 pg/tube, whereas interassay coefficients of variation for high (~25% binding) and low (~67% binding) mass internal controls were 8.5% and 16.8\((n = 5)\), respectively. Intraassay coefficients of variation were less than 10%.

RIA of HPLC-separated fractions of fecal total estrogens showed immunoreactive peaks at fractions 37 to 40 that coeluted with the \(^3\)H-E2 tracer, and this peak accounted for 91.3% of the total immunoreactivity in the fecal pool.

**Estrous Cycle Characteristics**

In 1997, mean estrous cycle duration was 17.9 ± 2.1 days \((n = 6\) mares showing a follicular phase and subsequent luteal phase) compared to 19.7 ± 3.3 days in 1998 \((n = 3)\), giving a composite mean of 18.6 ± 2.5 days. Mean follicular phase duration in 1997 was 6.1 ± 2.3 days versus 6.6 ± 2.5 days in 1998. Mean luteal phase estimates were 11.2 ± 3.3 and 10.5 ± 4.4 days in 1997 and 1998, respectively. A typical estrous cycle is shown in Figure 1.

There was no significant difference in follicular phase durations between currently treated and currently untreated mares in 1997 or 1998: 1997, \(t(11) = -0.68, p = .51\); 1998, Mann–Whitney \(U = 28, n = 5, 8, p = .35\) (see Figure 2). Luteal phase durations between currently treated and currently untreated mares were not different in either year: 1997, \(t(10) = -1.89, p = .09\); 1998, \(t(5) = 0.27\).

The occurrence of estrous behavior generally coincided with periods of high total estrogens or low progesteragens, or both (see Figure 1). The majority \(80%, n = 15\) of mounts and observations of estrus occurred within 1 to 3 days of an estrogen peak or progesteragen nadir. All observations of low-intensity sexual receptivity (approaching male, sniffing, proximity) also occurred during these periods. Of the
FIGURE 1  A typical pattern of fecal total estrogen and progestagen excretion in an Assateague, Maryland mare treated with porcine zona pellucida for 7 consecutive years. Black arrows indicate observations of estrous behavior.

FIGURE 2  Follicular and luteal phase durations in porcine zona pellucida treated and currently untreated Assateague, Maryland mares in the 1997 (A) and 1998 (B) breeding seasons.
15 mares sampled, 7 (47%) showed signs of high-intensity sexual receptivity (frequent urination, presenting, allowed mounting) during the fecal sampling period. Using a logistic regression model, Powell (2000) demonstrated that contraception has no effect on performance of sexual behavior.

**Apparent ovulatory failure.** Overall, endocrine data from 11 (52%) of the 21 hormone profiles examined (1997 and 1998) suggested ovulatory failure. Presumed anovulatory cycles were characterized by a significant \( p < .05 \) increase in total estrogen excretion that was not followed by a corresponding increase in luteal progestagen excretion. The incidence of ovulatory failure was 41.7% in 1997 (5/12): 66.6% (2/3) of currently treated mares and 33.3% (3/9) of currently untreated mares (Fisher exact \( p = .60 \)). Additionally, there was no significant difference in the number of years on PZP treatment between mares that demonstrated ovulatory failure and mares that did not (Mann–Whitney \( U = 37.5, n = 5, 7, p = .43 \)).

The ovulatory failure rate in 1998 (66.6%, 6/9) was not significantly different from the rate observed in 1997 (Fisher exact \( p = .71 \)). Because there was a 7-day gap in fecal sampling in 1998, cases in which the peri-ovulatory estrogen peak immediately preceded the sampling gap were not included because a portion of a luteal phase could have been missed. In 1998, the incidence of ovulatory failure was 60% (3/5) for currently treated mares versus 75% (3/4) for currently untreated mares (Fisher exact \( p = 1.0 \)). As in 1997, there were no significant differences in the number of years on PZP treatment between mares who demonstrated ovulatory failure and mares who did not (Mann–Whitney \( U = 16, n = 3, 6, p = .91 \)).

One mare, N6BP, experienced ovulatory failure in both years, and although luteal activity appeared to precede an anovulatory cycle in 1997, there was no evidence of a luteal progestagen excretion in 1998 (see Figure 3). This mare had been treated for 3 consecutive years but was not treated in 1997. No other females, whether they were treated or not, experienced ovulatory failure in both years.

Of the 11 profiles indicating ovulatory failure, 6 (55%) of these profiles showed significant rises in progesterone at some other point during the sampling period within that year. The 5 remaining profiles were obtained from five different mares, and each of these mares demonstrated normal cycles during the other of the two sampling periods (1997 or 1998). None of the mares in our samples, treated or not, gave birth during the study period.

**DISCUSSION**

These results and those of Barkuff, Carpenter, & Kirkpatrick (1993) demonstrate that fecal hormone monitoring is useful for tracking ovarian cyclicity and identifying follicular and luteal phases of the estrous cycle in feral horses. Fecal steroid analysis has also been used to track ovarian function in a variety of mam-
malian taxa, including primates, perissodactyls, artiodactyls, and carnivores (Schwarzenberger, Mostl, Palme, & Bamberg, 1996). Assay of fecal progestagens and total estrogens in this population of horses produced patterns of hormone excretion that were similar to those reported for equids using blood or urine (or both) hormone analyses (Ginther, 1992). Lag time between hormone secretion in blood circulation and excretion in the feces was not determined in this study; however, analyses of digesta passage in equines have found an average retention time of 23 hr (Uden, Rounsaville, Wiggans, & Van Soest, 1982); therefore a minimum lag time of approximately 1 day might be expected.

Progesterone accounted for only a small proportion of the total immunoreactive progestagens in feral horse feces, and, based on HPLC results, at least eight other metabolites were detected by our antibody. Schwarzenberger et al. (1996) found that unmetabolized progesterone was virtually absent in feces and generally is metabolized before excretion to several $5\alpha$- and $5\beta$-reduced pregnanes with the $20\alpha$-, $20\beta$-, or $20\beta$-hydroxylated group. In scimitar-horned oryx (Oryx dammah) feces, $5\alpha$- and $5\beta$-reduced pregnanes were measured, and unmetabolized progesterone contributed a small proportion of total progestagen

![Figure 3](image-url)
immunoreactivity (Morrow & Monfort, 1998). In contrast to progesterone, estradiol and estrone accounted for nearly all of the immunoreactivity in horse feces, and this is in agreement with studies conducted in numerous mammalian species (Schwarzenberger et al., 1996; Brown et al., 1997).

Average estrous cycle lengths reported in this study were generally shorter than those reported in studies of domestic horses, although they were within the range of reported variability (Ginther, 1992). Follicular phase durations for the Assateague mares were very similar to those of domestic mares; however, luteal phase durations were at the lower end of the range reported for domestic mares. These discrepancies may be related to variability introduced by less than daily fecal sampling. Among-individual variability in phase durations in Assateague mares was similar to variation observed in domestic mares (Ginther, 1992). There were no significant differences in phase durations between years or between currently treated and currently untreated mares, although there was a nonsignificant trend toward longer luteal phases in currently untreated mares.

The Assateague mares were contracepted with PZP for 1 to 7 years consecutively, and all showed some evidence of ovarian cyclicity during the 2-year study. Assateague mares appeared to experience a high incidence of ovulatory failure. However, the sample size precluded making a direct link between contraceptive treatment and ovulatory failure in this study. It appears that the apparent ovulatory failure seen in some mares in this study is episodic. Our data show that all mares who demonstrated ovulatory failure did have significant luteal increases in progesterone or normal estrous cycles (or both) at some time during the 2-year sampling period; however, none of the sampled mares foaled. Longer term and more frequent sampling would be required to determine conclusively the rate of occurrence of apparent ovulatory failure in these mares.

Kirkpatrick et al. (1992) found that Assateague mares treated with PZP for 3 years excreted reduced concentrations of urinary estrone conjugates compared to currently untreated mares, and the absence of a luteal phase increase in progesterone metabolites suggested that some treated females were anovulatory. In a later study, Kirkpatrick et al. (1995) found that the percentage of mares excreting normal range concentrations of urinary estrone conjugates declined from 80% for mares treated with PZP for 1 year to 0% for mares treated for 7 years. Hormonal assessments of mares conducted after 1, 2, 3, 6, and 7 years of annual PZP administration revealed that 50% of the treated mares excreted reduced concentrations of urinary estrone conjugates, although most females continued to cycle at least intermittently (Kirkpatrick et al., 1995). Here we present data from a sample of mares assessed in 2 consecutive years. We have demonstrated that the apparently anovulatory condition of some mares is episodic and that the duration of these episodes is variable. Some mares showing ovulatory failure did show evidence of a normal cycle in the same breeding season. Other mares did not show a normal cycle during one breeding season but did so in the other. Contrary to Kirkpatrick et al. (1995), we found no association between the number of years of PZP treatment.
and the incidence of ovulatory failure. We also have documented one case of a mare showing ovulatory failure in 2 consecutive years.

In summary, ovarian activity in PZP-treated mares (for 1 to 7 consecutive years) ranged from anovulation to intermittent cycles to completely normal estrous cycles (Kirkpatrick et al., 1995; Liu et al., 1989). To date, all mares who have been treated with PZP for 3 years and then taken off contraception have produced healthy foals within 3 years posttreatment (unpublished National Parks Service raw data). However, our data suggest that PZP immunocontraception may not be reversible in all mares after only 1 year. In addition, our data indicate that ovulatory failure appears to be episodic as opposed to chronic in these mares because all mares showed evidence of a luteal phase at some point during the 2-year study.

Ovulatory failure, defined as an increase in estrogen not followed by a sustained increase in progesterone secretion, has been documented in several other mammalian species after treatment with PZP (Bamezai et al., 1986; Gulyas et al., 1983; Mahi-Brown et al., 1985; McShea et al., 1997; Skinner & Dunbar, 1984; Skinner et al., 1984). In the only study to examine PZP-mediated ovarian pathology in mares, Liu et al. (1989) found no histological abnormalities in females ovariectomized 1 year after PZP immunization. However, significant ovarian pathology has been associated with PZP treatment in several other species (Gulyas et al., 1983; Hasegawa et al., 1992; Mahi-Brown et al., 1985; Sacco et al., 1987; Sacco, Subramanian, Yurewicz, DeMayo, & Dukelow, 1983; Skinner & Dunbar, 1984; Skinner et al., 1984; Wood et al., 1981). Histopathological findings range from decreased numbers of oocytes or various stage follicles (or both) to decreased ovarian size and the appearance of abnormal cellular masses. The degree and range of these abnormalities was variable across individuals and species (Sacco et al., 1987), and this may be related to individual or species-specific (or both) differences in immune responsiveness to PZP administration (Liu et al., 1989; Mahi-Brown et al., 1985).

The immune-mediated mechanisms whereby PZP has an impact on ovarian function are not well understood; however, it has been suggested that PZP may interfere with communication between developing oocytes and surrounding follicular cells—dogs (Mahi-Brown, Huang, & Yanagimachi, 1982) and rabbits (Skinner et al., 1990)—or that antibodies to ZP attack the developing oocyte when certain ZP glycoproteins are secreted early during oocyte development, causing oocyte death: guinea pigs (Hasegawa et al., 1992), dogs (Mahi-Brown et al., 1985), nonhuman primates (Sacco et al., 1987), and rabbits (Skinner et al., 1984; Wood et al., 1981).

A review of the literature suggests that, compared to other mammalian species, horses may be relatively insensitive to the adverse effects of PZP on ovarian function. We are unable to conclude that ovulatory failure in Assateague mares was directly related to PZP contraception because untreated controls were not available when this study was conducted. It is possible that antibody titres were still high enough in our currently untreated mares to affect ovarian func-
tion, making them indistinguishable from treated mares in terms of fecal hormone metabolite excretion. Ovulatory failure is relatively common in domestic horses and may be related to a variety of factors (Ginther, 1992). How common this phenomenon is in feral horses not exposed to PZP remains unknown. However, substantial evidence in other mammalian species provides incentive for conducting additional controlled studies in equids to determine the mechanism of action of PZP and its potential for inducing adverse side effects (ovarian pathology). Without such evidence, it is impossible to evaluate the actual potential of PZP for managing reproduction in wildlife species.

Immuocontraception is an attractive option for population management. Vaccines appear to be long acting and effective at stopping reproduction (Kirkpatrick et al., 1996). PZP does not appear to affect activity budgets of mares, their levels of aggression and sexual behavior, or their spatial relationships with the stallion (Powell, 1999, 2000). Despite these benefits, more research is required before PZP immunocontraception can be recommended for widespread use in wildlife. Long-term (up to 7 years) effects of PZP have only been assessed in equids, and these have been based on hormonal analyses only and not on an examination of ovarian histopathology. Behavioral effects have been assessed in a few studies, but these are restricted to ungulates: moose (Heilmann, Garrot, Cadwell, & Tiller, 1998), white-tailed deer (McShea et al., 1997), and feral horses (Powell, 1999, 2000). Clearly, more integrative research in horses is needed to addresses putative changes in reproductive–endocrine patterns, behavior, ovarian histology, and anatomy.

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