Luteal and follicular populations in the ovary of the opossum (*Didelphis virginiana*) after ovulation*

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Summary. The maximum diameters of all luteal and antral follicular structures were measured in opossum ovaries on Days 3, 7 and 11 after oestrus, and follicles were classed as developing or atretic. Ovarian weights and luteal diameters were equivalent in comparisons of pregnant and non-pregnant animals on each day. The number of CL (range 45–85) per animal per cycle indicated a very high ovulation rate for a mammal. Luteinized follicles (1–4 per ovary) were identified in all Day-3 ovaries. Ovarian weight, luteal diameter and follicular diameter were greater on Day 7 than on Days 3 or 11. More antral follicles occurred on Day 11 (120 ± 10.7, s.e.m.) than Day 3 (77.8 ± 8.8), although the percentage of atretic antral follicles also increased from 20% to 50 and 57% on Days 7 and 11. These increases were not accompanied by an increase in the number of developing antral follicles (58.1 ± 4.0), thus indicating a mid-luteal increase in the rate of follicular recruitment, of growth and of atresia in the opossum.

Introduction

Gestation usually entails modification of ovarian structure through copulatory- or conceptus-induced alterations in the secretion of luteotrophic and/or luteolytic agents. However, many marsupials complete gestation within the time span of a single luteal phase, apparently without alteration of maternal physiology, e.g. there is no effect of mating and gestation on uterine fluid composition and reproductive cyclicity in the Virginia opossum (*Didelphis virginiana*) (Renfree, 1975; Fleming & Harder, 1981a, b) or on peripheral blood concentrations of ovarian steroids (Harder & Fleming, 1981). Luteolysis appears to be independent of uterine (Hartman, 1925a) and, perhaps, oestrogenic factors (Cook, Karsch, Graber & Nalbandov, 1977). Despite the potential of this species as the simplest mammalian model for ovarian regulation (Cook & Nalbandov, 1968), normal ovarian events have not been documented beyond the general histological descriptions of Hartman (1923) and Martinez-Esteve (1942). The present study was designed to assess the patterns of luteal and follicular abundance and growth at intervals during the luteal phase of the oestrous cycle or pregnancy.

The Virginia opossum is polyoestrous, breeding from January to June throughout most of its range (Hartman, 1923; Jurgelski & Porter, 1974). Litters of 6–12 young are born after a 13-day gestation; spontaneous ovulations occur at 29-day intervals if young are prevented from sucking (Hartman, 1928; Reynolds, 1952; Fleming & Harder, 1981b). After ovulation, granulosa cells quickly luteinize to form corpora lutea (CL); by parturition, all CL are regressing, characterized by infiltration of leucocytes and the presence of numerous connective tissue cells (Hartman, 1925b;}

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Martinez-Esteve, 1942). The ovaries of the opossum are not necessary for gestation, although parturition does not occur in their absence (Hartman, 1925b; Renfree, 1974).

**Materials and Methods**

Adult opossums were maintained in the semi-enclosed Department of Zoology Opossum Facility on a diet of dogfood, fish, and water. Oestrous cycles were monitored daily by examination of vaginal smear cytology (Hartman, 1923; Jurgelski & Porter, 1974); reproductive tracts were excised from pregnant or cyclic females on Day 3 (N = 5), Day 7 (N = 8) and Day 11 (N = 9) after oestrus (oestrus = Day 0).

Ovaries were weighed, and ovarian pairs were either fixed together in Bouin’s solution (N = 8) or fixed separately, one in Bouin’s solution for paraffin-wax embedding and the other ovary in buffered formalin for sectioning on a cryostat (N = 14). Ovaries processed routinely for paraffin-wax histology were sectioned (8 µm) serially, and every 10th section was stained with Harris’ haematoxylin and eosin. After serial cryostat sectioning (15 µm) every 5th section was stained for lipids with haematoxylin and oil red O to provide a qualitative index of luteal activity. Alterations due to formalin fixation and freezing limited the quantitative data from cryostat sections to the number of CL in each ovary.

Serial sections from paraffin-wax embedded ovaries were examined at ×10 magnification for the location of the largest cross-section of each CL and each antral follicle; the average of the two maximum right-angle diameters for each of these cross-sections was obtained with an ocular micrometer at ×40 magnification. A random subset of 5 ovaries from each of the 3 days was examined at ×400 magnification for atretic antral follicles, identified by at least two of the following criteria: loss of homogeneity of the granulosa layer, presence of leucocytes within the antrum, pycnosis of granulosa cell nuclei, or lack of mitotic divisions in the granulosa layer (Brand & de Jong, 1973; Harder & Moorhead, 1980). All other follicles were classified as developing antral follicles.

Differences among means for each measurement were determined by one-way analysis of variance, and differences between means were identified by least significant range (Sokal & Rohlf, 1969).

**Results**

No effect of gestation on luteal or follicular populations was found by comparisons of ovarian weights and of diameters of CL (Table 1). Therefore, all data were combined and analysed irrespective of breeding status.

<table>
<thead>
<tr>
<th>Table 1. Ovarian weights and size of corpora lutea (CL) from pregnant and non-pregnant opossums</th>
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<tbody>
<tr>
<td><strong>Days after oestrus</strong></td>
</tr>
<tr>
<td>Day 3</td>
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<tr>
<td>-------</td>
</tr>
<tr>
<td>No. of animals</td>
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<td>--------</td>
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<tr>
<td>Ovarian wt (mg)</td>
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<td>Maximum CL diam. (mm)</td>
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Values are mean ± s.e.m. for the number of observations in parentheses.

Means with the same superscript are significantly different (P < 0.05).
Fig. 1. Cross-section of a Day-3 opossum ovary with several corpora lutea (CL) and follicles (F) and with 2 luteinized follicles (LF). Note ovum (arrow) present in one of the luteinized follicles. Haematoxylin and eosin, × 36.

Fig. 2. Juxtaposed walls of a late atretic follicle and a developing follicle. The membrana granulosa in the atretic follicle was cuboidal and single layered; the antrum (A) was infiltrated with leucocytes. Several mitotic figures (arrows) are present in the healthy follicle. Haematoxylin and eosin, × 240.

(Facing p. 30)
Mean ovarian weight, mean diameter of CL and mean diameter of antral follicles were greatest in ovaries collected on Day 7 (Tables 1 & 2). Numbers of CL (30.6 ± 1.1 per ovary; range 15–58/ovary) were similar on all days. However, each Day-3 ovary also contained 1–4 luteinized follicles (Pl. 1, Fig. 1). In contrast, no oocytes were found within luteinized structures in ovaries from Days 7 or 11, indicating that oocytes might have degenerated after Day 3. Incorrect identification of luteinized follicles as primary CL on Days 7 and 11 might have increased the average number of CL above the actual ovulation rate in these animals, but the increase would have been <10% (based on the occurrence of 1–4 luteinized follicles on Day 3). Among ovaries on all days, CL stained faintly and uniformly for lipids, indicating similar intra-ovarian lipid metabolism or inadequate sensitivity of this staining index.

### Table 2. Number and size of antral follicles and number of CL from opossums after ovulation

<table>
<thead>
<tr>
<th>Days after oestrus</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 11</th>
</tr>
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<tr>
<td>No. of developing antral follicles/ovary</td>
<td>62.4 ± 9.1 (5)</td>
<td>52.6 ± 7.5 (7)</td>
<td>59.4 ± 4.2 (11)</td>
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<tr>
<td>Total no. of antral follicles/ovary</td>
<td>77.8 ± 8.8* (5)</td>
<td>103.9 ± 7.5 (7)</td>
<td>120.4 ± 10.7* (11)</td>
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<tr>
<td>Maximum diam. of all antral follicles (mm)</td>
<td>0.83 ± 0.01b (387)</td>
<td>0.96 ± 0.01b (723)</td>
<td>0.80 ± 0.01b (1327)</td>
</tr>
<tr>
<td>No. of CL/ovary</td>
<td>30.6 ± 2.4 (12)</td>
<td>32.6 ± 2.2 (15)</td>
<td>29.7 ± 2.0 (17)</td>
</tr>
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</table>

*Values are mean ± s.e.m. for the number of observations in parentheses.

*Means with the same superscript are significantly different (P < 0.05).

Follicles in early stages of atresia (i.e. few leucocytes and an intact granulosa layer) usually contained a membrana granulosa of 2 layers, a basal cuboidal layer and an antral squamosal layer. In contrast, those follicles that were not considered to be atretic had columnar basal granulosa cells and were multilayered in the smaller follicles (Pl. 1, Fig. 2).

A progressive increase in the proportion of atretic follicles occurred throughout the luteal phase (Text-fig. 1). However, the population of developing antral follicles was maintained at a similar number on all days (58.1 ± 4.0 per ovary) (Table 2). At 3 days after oestrus, few atretic antral follicles (20%) occurred within the ovary. These atretic follicles were generally below the 1.0 mm size class (Text-fig. 1). By Day 7, 50% of the follicles were atretic although developing and atretic follicles frequently had a diameter of >1.0 mm. In the Day-11 ovary, the total percentage of atretic follicles (57%) remained similar to that on Day 7, but the distribution and total number were altered. Most of the large follicles (>1.0 mm) were atretic on Day 11 (62%), and small follicles composed a greater proportion of the total population.

### Discussion

The maximum size of CL in our opossums was attained after Day 3, contrary to the results of Hartman (1919) and Martínez-Esteve (1942) who did not monitor cyclicity on a daily basis but relied, in part, on gross characteristics of the reproductive tract. Furthermore, direct luteal measurements were not obtained in their investigations which proposed that luteal regression occurred after Day 3. CL in our study were significantly larger on Day 7 and showed no characteristics of
regression such as small luteal cells, leucocytes, or abundant connective tissue cells. In addition, the size of CL closely parallels the peripheral levels of progesterone in the opossum (Harder & Fleming, 1981). Similar associations between progesterone concentration and CL size have been reported in other marsupials, including the tammar wallaby (Macropus eugenii) (Renfree, Green & Young, 1979) and the brush possum (Trichosurus vulpecula) (Shorey & Hughes, 1973).

The absence of large atretic follicles on Day 3 and the abundance of CL suggested that most large, antral follicles ovulated at oestrus. The mean numbers of ova or embryos collected from opossum reproductive tracts, 22 (N = 19), 23 (N = 87), 44 (N = 200) and 16 (N = 86) (Hartman, 1916, 1919, 1925a; Rafferty-Machlis & Hartman, 1953; respectively) were 27–73% of our predicted ovulation rate based on number of CL. However, these ova were not collected systematically immediately after oestrus to ensure total recovery and thereby might represent an underestimate. Our counts of CL indicated that the opossum has one of the highest ovulation rates of all mammals (Mossman & Duke, 1973). This high ovulation rate might include large numbers of infertile degenerating ova from follicles in early stages of atresia or might compensate for high mortality in utero or post partum, when neonates crawl into the pouch. Alternatively, because ova become impenetrable to spermatozoa after deposition of a cortical layer of keratin (Hughes, 1977), a sequential release of ova throughout the night of oestrus might ensure a maximum litter size regardless of the exact time of mating.

Changes in the abundance of antral follicles during the luteal phase reflected alterations in the atretic, but not the developing, populations. A developing class of antral follicles was maintained throughout the luteal phase at a number twice that of the ovulation rate in our opossums. The post-
Opossum ovary after ovulation

ovulatory increase in the size of follicles, and in the proportion of atretic follicles, accomplished primarily between Days 3 and 7, indicated a transitory increase in the recruitment and growth of antral follicles with a concomitant increase in the atresia rate.

Consonant with proposed eutherian models (Greenwald, 1974), follicles in our animals after oestrus eventually became atretic before attaining a large size (1.70 mm) perhaps due to lack of gonadotrophin support. In addition, variation in follicular recruitment and atresia might have been responsible for fluctuating peripheral oestradiol concentrations that occurred in the post-ovulatory opossum (Harder & Fleming, 1981). A more precise definition of these processes awaits development of gonadotrophin assays for the Virginia opossum. Furthermore, our findings complement the morphometric analysis of Lintern-Moore, Moore, Tyndale-Biscoe & Poole (1976) which found consistent growth patterns of oocytes and follicles in several marsupials as compared to eutherians.

In conclusion, our observations reveal the growth of a mid-luteal phase population of follicles beyond the 1-0 mm-size class, possibly resulting from a peri-ovulatory gonadotrophin release, and this follicular population had mostly undergone atresia by Day 11.

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