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A COMPARISON OF THE REPRODUCTIVE PHYSIOLOGY OF THE
ESTROUS CYCLE AND GESTATION IN THE VIRGINIA OPOSSUM
(DIDELPHIS VIRGINIANA)

The Ohio State University

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A COMPARISON OF THE REPRODUCTIVE PHYSIOLOGY OF THE ESTROUS CYCLE AND GESTATION IN THE VIRGINIA OPOSSUM

(DIDELPHIS VIRGINIANA)

DISSERTATION

Presented In Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Michael William Fleming, B.S., M.S.

******

The Ohio State University

1980

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ACKNOWLEDGMENTS

In many dissertations, this section regrettably resembles a litany of token platitudes that were inserted hastily in the final draft. By calling the reader's attention to this trend, my intent is to avoid that disservice to those who have aided and abetted this research, for each shares in the authorship, and to each I am indebted.

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INTRODUCTION

Alternative strategies for viviparity are the basis for dividing the subclass Theria into two infraclasses, Metatheria and Eutheria. The metatherians, or marsupials, produce relatively embryonic neonates that attach via oral closure to a mammary gland for prolonged lactational nourishment. Gestation periods of shorter length than an estrous cycle and prolonged extra-uterine development, inclusive of major stages of organogenesis, characterize marsupialism. Time, energy, and risk components of the reproductive effort in metatherians are allocated largely to lactation rather than gestation. Pregnancy involves no alteration in reproductive cyclicity, the interval between recurrent ovulations irrespective of breeding status. Consequently, maternal physiology might remain completely autonomous regardless of reproductive status or the presence of the conceptus. In this study, this concept is termed the equivalence hypothesis of marsupial reproduction. The equivalence hypothesis is based on temporal coincidence of gestation and the estrous cycle and requires that all morphological and physiological processes occurring within the estrous cycle are adequate to complete gestation and that no embryonically-induced changes occur. The objective of this study was to test systematically the equivalence hypothesis.
The metatherian strategy clearly contrasts with eutherianism in which the expense of reproduction is more equally distributed between gestation and lactation. For example, the Virginia opossum (Didelphis virginiana), a marsupial, produces after 13 days of gestation a litter of 8 to 12 young each weighing 0.1 g (Hartman, 1928). A comparably sized eutherian, the raccoon (Procyon lotor), produces a litter of 4 young, 70 g each, after 63 days of gestation (Sanderson and Nalbandov, 1973). Despite this differential proportionment of gestation and lactation, total reproductive cost might be similar at weaning for both species. The physiological consequences of prolonged eutherian gestation on the mother are profound. Endocrinological, biochemical, and morphological alterations occur, thus terminating the estrous cycle and providing a uterine environment adequate for embryonic and fetal growth. Initial adjustments usually entail maintenance of progesterone production by corpora lutea and sensitization of the endometrium for implantation of the blastocyst. Among eutherians, copulation and/or the conceptus induce these and other unique physiological changes that lack estrous cycle correlates.

The evolutionary consequences of these dichotomous strategies of viviparity have been discussed in terms of maternal risk and energetic investment by Parker (1977), Kirsch (1977), and Low (1978). In essence, the decision to breed by the marsupial female does not entail an irrevocable or large investment of time or resources in gestation as it normally does for the eutherian. Ecological factors apparently selected for flexibility in early termination of reproductive processes in the ancestors of the metatherians, thereby reducing maternal
vulnerability during gestation. In contrast, a eutherian's investment in gestation is relatively insensitive to variable external conditions, and termination of the process is difficult. Therefore, parallel allopatric evolution of viviparity has resulted in the eutherian mode, production of young at the expense of the mother, and the metatherian mode, survival of the mother at the expense of the young.

This fundamental aspect of marsupial reproduction, summarized in the equivalence hypothesis, reflects an evolutionary alternate strategy for viviparity, akin to eutherianism but clearly distinct. In addition, if confinement of gestation, not only temporally but also physiologically, to the luteal phase of the estrous cycle occurs, the opossum provides a highly simplified model for mammalian reproduction. Confounding and synergistic interactions of the maternal-fetal-placental system of eutherians might be reduced exclusively to maternal events in marsupials. The appropriate uterine environment is available for embryo-culture regardless of the presence of the conceptus. Therefore, the definition of the physiology of gestation in the opossum implicates both evolutionary and experimental consequences. Furthermore, a critical test of this hypothesis provides a basis for intrametatherian comparisons of reproductive diversity and a system for investigation of complex mammalian reproductive processes such as immunological tolerance, parturition, and maternal-embryonic transport.

The equivalence hypothesis was tested in the Virginia opossum by comparison of reproductive parameters potentially sensitive to the presence of the conceptus on equivalent days of the estrous cycle.
and gestation. These parameters were:

1) circulating levels of estradiol and progesterone;
2) protein, glucose, glycogen, and water composition of the uterus;
3) histomorphology of the uterus and ovary.

The opossum was selected as a model for the prototypic marsupial reproductive pattern because, within the context of mosaic evolution, a species retaining ancestral characteristics is considered primitive. The opossum resembles the late Cretaceous didelphids (Clemens, 1977) and exhibits the reproductively primitive features of high ovulation rate, gestation confined to the length of the luteal phase of the estrous cycle, noninvasive trophoblasts, no embryonic diapause, and minimal maternal investment in individual offspring.

This multiparameter analysis of the equivalence hypothesis offers a spectrum of potential results grading from no embryonically-induced effects to multiple, unique gestational alterations at the histological, biochemical, and endocrinological levels throughout pregnancy. Acceptance of the hypothesis based on these tests would indicate that maternal physiology is independent of reproductive status and that gestational requirements are satisfied during each estrous cycle. Conversely, rejection of the equivalence hypothesis would imply a maternal response to or interaction with the conceptus and would implicate mechanical and/or physiological mechanisms for recognition. The degree and nature of this alteration would define the complexity of this relationship and would help classify didelphid reproduction within the mammalian array.
Reproductive Biology of the Virginia Opossum

The opossum, a seasonal breeder, usually has produced two litters between January and June throughout most of its range (Hartman, 1923a; McCrady, 1938; Morgan, 1941; Lay, 1942; Reynolds, 1952; Jurgelski and Porter, 1974). The 12- to 13-day gestation was contained within a 28-day estrous cycle (Hartman, 1923a, 1928; Reynolds, 1952; Jurgelski and Porter, 1974) and breeding status did not affect reproductive cyclicity if the 90- to 110-day lactational anestrus (Hartman, 1928; Reynolds, 1952; Jurgelski, 1974) was prevented by removal of the neonates. That is, ovulation was induced 5 to 15 days after removal of pouch young during any phase of lactation (New and Mizell, 1972; Jurgelski and Porter, 1974; Renfree, 1974). However, these authors did not report values of central tendency variation, and sample size. Morgan (1947) and Farris (1950) induced reproductive activity in anestrous, captive adults by artificially increasing the light cycle. Changes in vaginal smear cytology provided an accurate monitor of the estrous cycle (Hartman, 1923a; Jurgelski and Porter, 1974).

Adults are intolerant of each other except for the duration of breeding activity, and most investigators have contended that a large outdoor enclosure was necessary to maintain a breeding colony (McCrady, 1938; Coghill, 1939; Moore and Bodian, 1940; Farris, 1950; Reynolds, 1952; Sherwood et al., 1969; Fritz, 1971). However, a laboratory breeding colony was established recently in a uniquely designed, semi-
enclosed facility by housing adult animals individually in small cages and pairing mates only near estrus (Jurgelski, 1974; Jurgelski et al., 1974; Jurgelski and Porter, 1974). Breeding activity was restricted to several hours during the night of estrus (Hartman, 1923a; Reynolds, 1952; McManus, 1967, 1970). According to these authors, preliminary male sexual behavior included clicking vocalizations, erections of the penis, persistent nuzzling of the female genital area, and attempts to mount. On estrus, the female permitted the male to mount her from the rear, grasp her on the nape region with his forefeet and by her hindlegs at knee level with his hindfeet. The male then shifted his weight laterally and caused the pair to topple onto their right side. Intromission and insemination occurred within the next 15 to 30 minutes. The female was receptive only for a single copulation.

The marsupial, or didelphid, reproductive tract consists of paired ovaries, oviducts, uteri, cervixes, and lateral vaginal canals (Fig. 1) (Nelson and Maxwell, 1942; McCrady, 1938; Tyndale-Biscoe, 1973). The distal ends of these canals terminate in a single urogenital sinus. During parturition in the opossum, a temporary pseudovaginal canal formed through a connective tissue strand between the anterior vaginal cul de sac and the urogenital sinus (Hartman, 1920; Risman, 1947). The force of uterine contractions on the hormonally loosened and edematous strand, while the lateral vaginal canals were occluded, presumably directed the expulsion of the embryos through this medial tract (Tyndale-Biscoe, 1966).

During anestrus, Hartman (1923a) found that the ovary consisted of an almost solid mass of follicles, mostly undergoing chromatolytic
Fig. 1. Diagram of the didelphid female reproductive tract.

B = bladder; K = kidney; LVC = lateral vaginal canal; 
O = ovary; PVC = pseudovaginal canal; USG = urogenital sinus; VCS = vaginal cul de sac.
and lipolytic atresia, always degenerating before much antral fluid accumulated. As graafian follicles formed during breeding season, the granulosa cells contained abundant cytoplasmic RNA (Guraya, 1968). The cumulus in these follicles was only one or two layers thick and the zona pellucida was extremely thin (Martínez-Esteve, 1942). Hartman (1925b) and Martínez-Esteve (1942) examined serial histological sections of ovaries and concluded that, after extrusion of the first polar body, spontaneous, multiple ovulations occurred nearly simultaneously in the ovaries, and the granulosa cells quickly luteinized to form corpora lutea that encompassed almost the entire ovary. Because follicles also frequently luteinized without ovulation (Martínez-Esteve, 1942), the number of corpora lutea always exceeded the number of eggs shed (Hartman, 1925c).

The corpus luteum consisted of a solid mass of luteinized granulosa cells with a small connective tissue core, reached a maximum size 3 days post-ovulation, and began to decline 4 to 5 days later (Hartman, 1923a). Luteal development was characterized histochemically by the occurrence of diffuse lipoproteins throughout the cytoplasm (Guraya, 1968). At parturition, regressing corpora lutea were distinguished by the presence of infiltrated leucocytes and numerous connective tissue cells, marked accumulation of fat, and somewhat smaller luteal cells (Martínez-Esteve, 1942). Both Hartman (1919, 1923a) and Martínez-Esteve (1942) claimed that the corpora lutea of pregnancy tended to be more persistent than the corpora lutea of the estrous cycle; the latter author maintained that the 3-day difference (13 days versus 10 days) in the life of the corpora lutea was correlated with the 3-day difference
In the condition of the genital tract and mammary glands during the final days of gestation and the luteal phase of the estrous cycle. However, the conditions of the tract and glands during these 3 days were not specified or examined by Martínez-Esteve (1942).

The opossum ovary was also noteworthy, compared to other mammals, for the frequent occurrence of primary tubules, the presumptive seminiferous tubules, (Hartman and League, 1925; Morgan, 1943; Appendix B), polynuclear ova, polyovular follicles (Hartman, 1926), and anovulatory follicles (League and Hartman, 1925).

The functional role of the ovary in the reproductive process has been investigated by various surgical manipulations. Hartman's (1923b, 1925b) original excision experiments, from which he concluded that the ovaries were essential for pregnancy, were re-examined and repeated by Renfree (1974). She found that embryogenesis usually proceeded in the absence of the ovaries but parturition never occurred after ovariectomy. Similarly, double hysterectomy (N = 9) was without effect on the involution of the corpora lutea and the duration of the estrous cycle (Hartman, 1925a), thus negating the role of a uterine luteolytic factor. After irradiation of ovaries to destroy endogenous follicular sources of steroids, exogenous estrogen treatment was not instrumental in the luteolytic process (Cook et al., 1977a). Compensatory hypertrophy and increased ovulation rate occurred in the intact ovary after unilateral ovariectomy (Hartman, 1925c).

Hormonal treatments have been utilized to manipulate ovulation. Nelson and White (1941) induced ovulation in the anestrous adult by administration of follicle stimulating hormone (FSH) and a commercial
luteinizing factor, AntultrIn-S. Moore and Morgan (1943) produced only large cystic follicles in the ovaries after injection of PMSG. However, human chorionic gonadotrophin (HCG), either alone or with PMSG, produced luteal cells. Fritz (1971) designed alternative regimes to induce ovulation in anestrous and reproductively active animals:
anestrus, Day -6,-4, and -2 (75 i.u. PMSG); Day 0 (100 i.u. HCG);
active, Day -8,-6,-4, and -2 (100 i.u. PMSG); Day 0 (125 - 150 i.u. HCG).
Cook and Nalbandov (1968) utilized the following treatment to initiate ovulation in the opossum:
Day -3 (500 i.u. PMSG); Day 0 (250 i.u. HCG).
Plasma levels of progesterone after this treatment ranged from 22 to 83 ng/ml during the 6 days postovulation compared to a maximum of 12 ng/ml during the normal luteal phase (Cook et al., 1977b). Unlike corpora lutea from most mammals, opossum luteal cultures did not incorporate acetate-1-14C into progesterone even though they did convert cholesterol and pregnenolone to progesterone (Cook and Nalbandov, 1968). Exogenous gonadotrophin treatment of the culture resulted in a biphasic progesterone profile with peaks on Day 0 and Day 4, the probable result of initial gonadotrophin stimulation and maximal luteal mass, respectively (Cook et al., 1974, 1977b). Cook and Nalbandov (1968) proposed that the opossum might exhibit the simplest form of mammalian ovarian regulation, a single release of an ovulating hormone that triggers all mechanisms necessary to complete an estrous cycle. This hypothesis predicts that one pituitary hormone ovulates the follicle, luteinizes the granulosa cells, and provides the necessary luteotrophic and steroidogenic stimuli for the corpora lutea without the involvement of other gonadotrophins or luteotrophic agents.
After ovulation, the uterus underwent progressive morphological changes, growing larger, more vascularized, and increasingly turgid until the fifth or sixth day (Hartman, 1923a; Spurgeon and Brooks, 1916). If fertilized eggs were present, the internal pressure increased greatly (not measured by these authors), but only a slight further increase in size occurred prior to parturition. Hartman (1919) claimed that if fertilization did not occur, the uteri became dull, dark red, and flaccid 4 to 5 days after ovulation. However, my inspection of his daily accounts (Hartman, 1923a) refutes the predictive reliability of gross, uterine appearance. The size of postpartum uteri was reduced greatly and by 20 days postovulation had returned to an anestrous condition (Hartman, 1923a). These descriptive changes largely agree with Renfree's (1975) measurements of endometrial weights, although she found no differences between pregnant and nonpregnant animals up to Day 8. However, only pregnant and ovariectomized animals were sampled after this day and a maximum endometrial weight occurred on Day 10 of pregnancy. Lactating animals maintained the lowest endometrial weights.

Maternal serum and uterine exudate (fluid released after thawing frozen tissue) were similar in total protein content, 93.6 ± 21.4 mg/ml and 116.7 ± 24.9 mg/ml, respectively, from Days 3 to 12 irrespective of reproductive condition (N = 26) (Renfree, 1975). The electrophoretic patterns of these fluids were similar except that the uterine exudate contained an albumin with greater mobility as well as prealbumins.
The patterns of uterine proteins from nonpregnant, ovariectomized, and pregnant individuals were identical, thus indicating the absence of any unique gestational proteins.

Histology of the anestrous uterus revealed closely packed tissue with reduced endometrium and muscularis; uterine glands were straight and lined with low columnar or cuboidal epithelium (Hartman, 1923a). Uterine enlargement during proestrus resulted from increased vascularity and infiltration of ground substance, a type of connective tissue characterized by a paucity of stromal cells, which were almost always fibroblasts, and by extracellular components that included occasional collagen fibrils and a flocculent precipitate, probably a glycoprotein (Padykula and Taylor, 1976). During the follicular phase, the uterine epithelium was stratified (Morgan, 1946), and the mucosa began to differentiate into a dense glandular basal area and a central layer with more deposition of ground substance. The estrous uterus had greatly coiled glands, large, thin-walled blood vessels pervading the loose connective tissue, and more capillaries beneath the epithelium. After ovulation, glandular growth and ground substance accumulation continued in the hypertrophied mucosa.

Throughout midpregnancy and the midluteal phase of the estrous cycle (Days 7 and 8), Hartman (1923a) found the uteri to be microscopically and macroscopically indistinguishable. At this time the surface epithelium was thick and pseudostratified; by Day 10 it was simple columnar (Padykula and Taylor, 1971). Uteri of late pregnancy also were characterized by numerous maternal capillaries and venules in the stroma subjacent to this epithelium. However, during the corresponding period
of the estrous cycle, the vascularity and edema were less pronounced (Hartman, 1923a). By Day 12, the subepithelial region was infiltrated with masses of leucocytes, and glands were dilated.

The postpartum uterus had a collapsed mucosa reflecting withdrawal of ground substance and a lumen filled with cellular material, largely the result of macrophage migration through the glandular epithelium (Padykula and Taylor, 1976). These authors contended that cellular and tissue differentiation during the luteal expansion of the endometrium, as well as subsequent regression, were similar in both pregnancy and the luteal phase of the estrous cycle. These conclusions, however, were based on individuals dated only by ovarian histology and from a study focused primarily on postpartum changes; comparative data from both reproductive states were not presented to substantiate their conclusions.

Padykula and Taylor (1971) concluded that the ultrastructural features of the surface epithelium during late pregnancy indicated an absorptive function. The straight, regular microvilli, multivesicular bodies, dilation of the basal intracellular space containing a material of low density, and particularly, the rich supply of capillaries and aggregations of mitochondria suggested that active transport might occur (Padykula and Taylor, 1971). Thus, the surface epithelium might transfer compounds between embryonic and maternal compartment. The glandular epithelium had abundant endoplasmic reticulum and expanded Golgi complexes indicating secretory activity. Therefore, the surface transport cells and secretory cells presumably provide the embryo with hemotrophe and histiotrophe. McCrady (1938) also indicated that the uterine glands
secrete a clear, cell-free, lymph-like fluid into the uterine cavity for absorption by the embryonic vesicles.

No investigator has collected ova systematically to obtain an accurate ovulation rate, and the numbers of eggs, embryos, or corpora lutea that have been recorded vary widely (Table 1). These data were collected routinely from excised tracts and might not account for all tubal eggs, eggs deeply embedded in uterine mucosa, or eggs lost through the cervixes. In addition, corpora luteal counts might provide an inflated estimate of actual ovulation rates due to luteinization of unruptured follicles (Martínez-Esteve, 1942).

Although McCrady (1938) found tubal ova up to 36 hr postcoitus, eggs generally arrived in utero 12 to 36 hr after ovulation encased in a thick layer of mucopolysaccharide (albumin according to McCrady) and a thin, keratinous cortical layer produced in the oviduct (Hughes, 1977). These layers continued to thicken during the first days of gestation and the estrous cycle in fertilized and unfertilized eggs (Smith, 1925; Hartman, 1916, 1919, 1924). The term shell membrane, customarily applied to the cortical layer, is a misnomer because this layer is an acellular product of glands near the tubal-uterine junction. The blastocyst probably is not isolated from the uterine environment by the cortical layer. The mucopolysaccharide layer thickened during early gestation possibly due to continued deposition of material or water absorption (Hartman, 1919); the entire vesicle increased 200-fold in volume before breakdown of the cortical layer, and mucopolysaccharide reserves were depleted by Day 7 of gestation (McCrady, 1938), necessitating uterine provisions of nutrients. Embryos in early cleavage and
Table 1. Mean and range of ova and embryos recovered in a single reproductive cycle in the opossum. Ova and embryos were collected from excised reproductive tracts.

<table>
<thead>
<tr>
<th>Mean</th>
<th>Range</th>
<th>N</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>1 - 45</td>
<td>19</td>
<td></td>
<td>Hartman, 1916</td>
</tr>
<tr>
<td>25</td>
<td>1</td>
<td>1</td>
<td></td>
<td>Spurgeon and Brooks, 1916</td>
</tr>
<tr>
<td>23</td>
<td>1 - 22(^a)</td>
<td>87</td>
<td>33 % unfertilized or abnormal</td>
<td>Hartman, 1919</td>
</tr>
<tr>
<td>44</td>
<td>200</td>
<td></td>
<td></td>
<td>Hartman, 1925a</td>
</tr>
<tr>
<td>30</td>
<td>11</td>
<td></td>
<td>Ova or corpora lutea after unilateral ovarlectomy</td>
<td>Hartman, 1925c</td>
</tr>
<tr>
<td>16</td>
<td>1 - 60</td>
<td>86</td>
<td>38 % unfertilized or abnormal</td>
<td>Rafferty-Machills and Hartman, 1953</td>
</tr>
<tr>
<td>12 - 24</td>
<td></td>
<td>8- to 12-day embryos</td>
<td>New and Mizell, 1972</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Per ovary.
small blastocysts were found primarily near the caudal end of the
uterus often closely bunched (Hartman, 1919) but, by the time of chorio-
vitelline membrane elaboration, vesicles were distributed randomly
throughout the uterine lumen (McCrady, 1938).

Between Days 7 and 8, the cortical layer ruptured and the chorio-
vitelline membrane spread over the luminal surface. However, the
embryonic vesicles remained unattached and rolled about freely in the
uterine cavity until Day 10 (McCrady, 1938; New and Mizell, 1972).
At this time the animal pole rested against the uterine mucosa, and
the area vasculosa of the embryonic membrane adhered to the epithelium
(McCrady, 1938). Although fusion between embryonic and maternal tissues
did not occur, these membranes followed closely the deep contours of
the mucosal crypts (Hartman, 1928; McCrady, 1938; Padykula and Taylor,
1976).

A systematic examination of the equivalence hypothesis in the
Virginia opossum has not been performed. However, several of the
investigations in this review provided indirect evidence in support
of the similarity of maternal physiology during gestation and the
estrous cycle. First, reproductive cyclicity apparently remains unaltered
by gestation, although duration of cycles has not been examined statisti-
cally. Second, no author has reported consistent gross or
histological morphological differences in the reproductive tract due
to breeding although physical parameters have been quantified by only
one author (Renfree, 1975). This analysis of endometrial weights and
protein composition of uterine exudates was limited to a few
animals in several treatment groups. Animals from equivalent days,
particularly during the final trimester, were not compared specifically. Contentions by Hartman (1919, 1923a) and Martínez-Esteve (1942) that corpora lutea were more persistent during gestation were based on descriptive studies and not substantiated with measurements.
Reproductive Physiology of Other Marsupials

Detailed comparisons of equivalent stages of the estrous cycle and pregnancy have been limited largely to three Australian species, the tammar wallaby (*Macropus eugenii*), the brush-tailed possum (*Trichosurus vulpecula*), and the quokka (*Setonix brachyurus*), although miscellaneous observations have been repeated on a few other species (see review by Renfree, 1977). The examination of the reproductive physiology of these three species does not relate necessarily to their representative status of the marsupial strategy but probably relates to their availability, ease in handling and husbandry, and novel reproductive aspects such as embryonic diapause, lactational luteal activity, or monovulatory cycles. Therefore, comparative data might be indicative of unique, divergent reproductive adaptations rather than a representative, prototypic system.

Tammar Wallaby

The female tammar wallaby usually carries a single diapausing blastocyst for 10 months each year. During the first half of the year, the diapause was maintained by lactational anestrus, the second half by seasonal anestrus (Renfree and Tyndale-Biscoe, 1973a), probably terminated by reduced photoperiod (Sadlier and Tyndale-Biscoe, 1977). The mechanisms for this diapause appeared to involve the production of a uterine inhibitor substance that was terminated by hormonal activity of the corpus luteum (Smith and Sharman, 1969; Tyndale-Biscoe, 1970), which, in turn, was suppressed during the anestrous period by a pituitary factor (Hearn, 1974). According to Hughes' (1974)
classification, this species has slightly invasive trophoblasts, an intermediate type between the noninvasive trophoblasts in Didelphis and the highly invasive trophoblasts in bandicoots (Perameles sp.). Without the intervention of diapause, the 26-day gestation was accommodated within the 30-day estrous cycle.

Renfree (1972, 1973c) and Renfree and Tyndale-Biscoe (1973b) defined three unique effects of the conceptus on the pregnant uterus on the tammar wallaby; an increase in endometrial weight, and qualitative and quantitative changes in the uterine proteins, particularly the presence of a uterine specific β-globulin. These changes occurred after attachment of the yolk sac to the uterine epithelium and might be involved in the initiation and maintenance of growth of the embryo, resulting from an embryonically-induced hormonal or immunological response. Lemon (1972) found peripheral plasma progesterone concentrations in combined samples significantly higher during the last 10 days of pregnancy (N = 4) than during the equivalent period of the estrous cycle (N = 5). This analysis was based on samples pooled over the entire period; individual days, animals, or cycles were not analyzed. In addition, size of the corpus luteum, total luteal progesterone content, and peripheral plasma levels were correlated, suggesting that peripheral progesterone was derived predominantly from the corpus luteum (Renfree et al., 1979).

Functional differences occurred in the motility of the myometrium in nonpregnant and pregnant wallabys during the final 10 days of gestation (Young, 1977). The nonpregnant uterus (N = 4) was characterized by high, non-cyclic contractions, whereas the pregnant uterus maintained
low, cyclic activity. As parturition approached, the intensity of activity was variable but always cyclic. Finally, Merchant (1979) found a significant reduction in the length of a cycle solely due to the presence of the conceptus.

The mechanisms of embryonic involvement in the alteration of maternal physiology of the wallaby have not been defined, but Renfree and Heap (1977) found in vitro synthesis of progesterone from pregnenolone by the yolk-sac membranes, implicating a steroidal influence on the uterus by the conceptus. In addition, the nonvascularized region in these membranes apparently takes an active role in selective active transport of nutrients, particularly glutamic acid and glucose (Renfree, 1970, 1973b,c). Extra-embryonic metabolism was demonstrated in the yolk sac and in the vitelline fluid (Renfree, 1973a; Renfree and Tyndale-Biscoe, 1973a). These are the first physiological processes defined for the chorio-vitelline membrane in a marsupial and suggest dynamic interaction between maternal and embryonic compartments.

The role of the conceptus in the initiation of parturition has not been investigated in any marsupial. However, prepartum fetal plasma concentrations of corticosteroids and androgens were 1.2 and 0.4 ng/ml, respectively (N = 2) (Catling and Vison, 1976). Maternal contractions might be a response to initiation of fetal steroid production. Walker and Tyndale-Biscoe (1978) found sensitization to male histocompatibility antigens and repeated pregnancy to the same male had little effect
on fertility or the length of gestation, suggesting that the embryo is protected from maternally-produced, deleterious immunological effects.

Brush-tailed Possum

The 18-day gestation in this monovular species was contained within the 26-day estrous cycle; embryonic diapause did not occur (Pilto and Sharman, 1962). The trophoblast was noninvasive (Hughes, 1974). Ultrastructure of glandular and luminal epithelia indicated differential secretory activity. Surface glandular cells appeared functional in secretion of the cortical layer and basal glandular cells in secretion of uterine milk (Shorey and Hughes, 1973a).

No differences occurred between pregnant and nonpregnant brush-tailed possums in luteal morphology (Pilto and Sharman, 1962; Shorey and Hughes, 1973a), urinary pregnanediol (Pilto and Sharman, 1962), endometrial secretory activity (Shorey and Hughes, 1973a), or ovarian secretion rates of progesterone (Shorey and Hughes, 1973b). In addition, concentrations of peripheral plasma progesterone were similar in the estrous cycle (N = 15) and pregnancy (N = 5) although these measurements did not constitute serial samples or include comparative gestational samples during mid-pregnancy when the estrous cycle profile reached a maximal value of 4.5 ng/ml (Shorey and Hughes, 1973a). Thorburn et al. (1971) also concluded that circulating progesterone was similar in both reproductive states, reaching maximal levels 11 to 15 days after estrus (3.8 to 5.0 ng/ml) and returning to basal levels (<1 ng/ml) by Day 22. In nonpregnant animals the uterus that was ipsilateral to
the corpus luteum was always heavier (Pilton and Sharman, 1962), indicating a local effect by this gland. On Day 8, gravid uteri were slightly larger than the contralateral, nongravid uteri, and this difference increased throughout the remainder of gestation. In addition, the subepithelial capillary layer was more highly developed during late gestation in the pregnant uteri. Hysterectomy had no effect on the estrous cycle (Clark and Sharman, 1965).

In addition to these morphological changes, further evidence for the dynamic nature of the maternal-embryonic relationship was provided by the light and electron microscopic examination of the transfer of toluidine blue (mol. wt. 306), horseradish peroxidase (mol. wt. 40,000), and ferritin (mol. wt. 460,000) across the extra-embryonic ovum layers, i.e. the cortical layer, the mucoid coat, and the zona pellucida (Hughes and Shorey, 1973). Little regulation of the passage of embryonic nutrients or wastes to and from the vitellus apparently occurred based on the permeability of these substances. Hughes and Shorey (1973) concluded that the vitellus had virtually unrestricted access to almost all luminal enzymes, antibodies, and protein-containing hormones.

Sharan (1955a,b) reported a 27-day gestation in the quokka; estrus was observed every 28 days if not delayed by embryonic diapause during the 5-month lactational anestrus. Hughes (1974) classified this trophoblast as noninvasive.

Pregnant uteri in vitro were more responsive to oxytocin than were
nonpregnant uteri, but equivalent-day nonpregnant uteri were more responsive to arginine-vasopressin based on contractions (Heller, 1972, 1973). Replacement injections of progesterone or estrogen in ovariectomized animals indicated that these differences resulted from different progesterone levels or estrogen:progesterone ratios. The fact that the chorio-vitelline membrane converted pregnenolone to progesterone in vitro (Bradshaw et al., 1975), suggested involvement of a local mechanism.

Other Marsupials

The monovular rat kangaroo (Betongila cuniculus) had macroscopically and microscopically identical uteri throughout the first half of gestation. After midpregnancy, the nonpregnant uteri decreased slightly in size and the pregnant uteri increased somewhat, apparently due to the physical presence of the growing embryo according to Flynn (1930). The nonpregnant uteri showed some involution of the superficial portion of the uterine glands earlier in the cycle.

Parturition in the long-nosed bandicoot (Perameles nasuta), a species with an allantoic placenta and highly invasive placentation occurred with an apparently functional corpus luteum, suggesting to Hughes (1962) that the hormonal control of pregnancy might be different from the normal estrous cycle.

This complex of data from Australian species provides considerable and varied evidence for refutation of the equivalence hypothesis of marsupial reproduction. However, the majority of the observations were obtained from the tammar wallaby, a species in which limited erosion
of the uterine epithelium occurs. Uterine morphology, biochemistry, and contractility were altered in response to the wallaby conceptus. In addition, cycle lengths were decreased due to gestation. Uterine microscopic and microscopic changes due to pregnancy were reported in the brush-tailed possum and the rat kangaroo. This response of the uterus to the conceptus might be attributable to the physical presence of the conceptus and/or the contralateral corpus luteum in these monovular species. Uterine contractility was altered due to gestation in the quokka. These physiological alterations of the muscularis might be induced locally by the conceptus. The demonstration of embryonic production of steroids in vitro in two species and maternal peripheral steroid levels unaffected by reproductive state provided indirect evidence for embryonic control of parturition.
MATERIALS AND METHODS

Animals

Adult female opossums (2.38 ± 0.07 kg, \(X \pm S.E.M\).) were trapped with Tomahawk traps, which were baited with dog food and sardines, and set throughout Ohio (N = 9), at the Savannah River Ecology Laboratory, South Carolina (N = 6), and at North Carolina State University, Raleigh (N = 4). Adult females also were purchased (N = 34). If the female was lactating, pouch young were removed to permit the adult to return to estrus. Adult males (N = 8) were trapped in Ohio and Savannah River Ecology Laboratory. Upon arrival, each animal was isolated and treated routinely with Diryl\(^2\) for ectoparasites and Thiabenzadole\(^3\) for intestinal parasites.

Opossum Facility

The Department of Zoology Opossum Facility, organized and equipped in order to conduct this research, is a semi-enclosed kennel (7.5 X 5.9 m) with a metal roof and cinderblock (1 m)-chainlink fence (2 m) walls at The Ohio State University Research Center, Columbus (Fig. 2). Opaque, polyethylene curtains (6 mil) were lowered to cover the exterior chainlink fence during periods of heavy rains, snow, or subfreezing temperature. Ten individual pens (1 m\(^3\)) had cinderblock

\(^1\)Ryder Animal Supply, Rt#2, Box 515, Brooksville, FL 33512.
\(^3\)Merck Animal Health Division, Merck and Co., Inc., Rahway, NJ 07065.
Fig. 2. External view of the Department of Zoology Opossum Facility, The Ohio State University, Columbus. Polyethylene curtains covered the external fencing during inclement weather.
and cement floors and rear walls; the ceiling and other 3 walls were chainlink fence (Fig. 3). Each pen could be divided in half with a portable plywood partition to house individually 2 animals. Mated pairs were permitted access to an entire 1 m$^3$ pen. Standard stainless steel primate cages (0.2 m$^3$) also were used, particularly for isolation of new animals. Each pen was equipped with a wooden shelf 0.5 m above the floor and, during cold months, a wooden nest box. Shredded paper was supplied throughout the year. All concrete and wooden surfaces were coated with cement paint and polyurethane, respectively.

**Husbandry**

Water was provided daily in plastic bottles (500 ml) attached with metal springs to the outside of each cage and delivered to the animal by a plastic waterer.$^1$ Additional water was available in open containers during summer months. Commercial dry dog food, approximately 90 g/day, was supplemented frequently with whole fish. Exclusive use of these dry foods greatly facilitated the removal of wastes.

Estrous cycles of nonpregnant and nonlactating adults were monitored daily from February through July, 1978 and 1979, by examination to vaginal smear cytology. To obtain the smear, the opossum was grasped by the tail and placed on top of the cage. The hindlegs were raised off the floor by elevating the tail, and its head was directed away from the observer. The animal was permitted to grasp the cage during this procedure to reduce struggling. With the tail raised, anal gland secretions were wiped away and the urogenital sinus

$^1$ Ronson Farms, Columbus, OH 43212.
Fig. 3. An individual pen ($1 \text{ m}^3$) in the Department of Zoology Opossum Facility equipped with nest box, shredded newspaper, and shelf.
was flushed with tap water and aspirated with a medicine dropper. Smears were air-dried and stained with methylene blue. Diagnosis of estrous phase was based on Hartman (1923a) and Jurgelski and Porter (1974). Consistent breeding success occurred when animals were paired during proestrus, characterized by a vaginal smear of few leucocytes and of epithelial cells with light staining cytoplasm but visible, faint nuclei.

**Experimental Design**

Blood samples (3 - 10 ml), assayed for circulating levels of estradiol and progesterone, were collected by cardiac puncture with a 21-ga needle on the days of estrus (Day 0) and several days post-estrus, inclusive of Days 1 through 14, Day 18 and Day 24 of the estrous cycle and gestation (Appendix A). Individuals were restrained under light ether\(^1\) anesthesia for approximately 5 minutes during blood sampling and were returned to their pens immediately thereafter (0700 - 1100 hr). Full coordination returned within 15 to 40 minutes after the initial restraint. Blood was allowed to clot for 24 hr at 4° C; after centrifugation, serum was decanted and stored at -15° C until assay.

Twenty-five adult females were bled during 33 estrous cycles, and 22 females were bled during 28 pregnancies. Six animals provided samples for both an estrous cycle and a gestation. Most animals were not bled daily. However, 10 blood samples per cycle were obtained from many animals. In addition, 15 postlactational females were bled on alternate days after removal of pouch young until estrus.

\(^1\)Mallinckrodt, Inc., St. Louis, MO 63147.
Uteri were excised at 0800 hr on Day 3, 7, and 11 of the estrous cycle and gestation (Table 2) of animals that were starved for 24 hr. After anesthetization with ether, a midline, ventral incision through the pouch allowed occlusion of uterine blood vessels with hemostats and excision of the reproductive tract. One uterus was divided into approximate 1-g samples, placed in tared vials, frozen in a solid CO$_2$-acetone bath, weighed to the nearest 0.001 g, and stored at -15° C until biochemical assay. Pregnancy was confirmed by the characteristic size and features of embryonic vesicles (Figs. 4 and 5). The contra-lateral uterus was placed in physiologic saline to determine volume by displacement, fixed in Bouin's solution, and stored in 70% EtOH prior to paraffin histology. Ovaries were fixed and stored similarly. Ovaries were obtained from an additional eight animals that died after cardiac puncture on Days 3, 7, 11, 11, 14, and 18 of the estrous cycle and Days 4 and 5 of gestation.

Steroid Assays

 Estradiol and progesterone were extracted separately from 2.0 and 0.5 ml of serum, respectively, by vortexing with 4 volumes of fresh, diethyl ether in centrifuge tubes. In estradiol assays, 50 pg estradiol was added to each tube before mixing. After centrifuging (2000 RPM for 5 minutes) and snap-freezing the serum, the ether extract was drawn off and evaporated under N$_2$ at 38° C. Each sample was extracted twice. Phosphate-saline buffer and male opossum serum served as blanks.
Table 2. Number of reproductive tracts collected from adult female opossums during the luteal phase of the estrous cycle and gestation for biochemical assays and histological evaluation.

<table>
<thead>
<tr>
<th>Reproductive State</th>
<th>Days Postestrustr</th>
<th>3</th>
<th>7</th>
<th>11</th>
</tr>
</thead>
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<tr>
<td>Estrous Cycle</td>
<td></td>
<td>2</td>
<td>3</td>
<td>4</td>
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<tr>
<td>Gestation</td>
<td></td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>
Fig. 4. Three-day embryo (A) and three-day ovum (B) from an opossum uterus (stored in 70% EtOH; 10 X).
Fig. 5. Seven-day embryo (A) and seven-day ovum (B) from an opossum uterus (stored in 70 % EtOH; 10 X).
Recoveries were 88% (external estimate) and 84% (internal estimate) for the tracers\(^{1}\) \([2, 4, 6, 7]\)-\(^3\)H estradiol and \([1, 2]\)-\(^3\)H progesterone, respectively.

Extract residues were dissolved overnight in 1 ml of phosphate buffer. Duplicate aliquots (0.2 - 0.4 ml) were placed in culture tubes for assay. Antiserum (0.1 ml) and 100 - 200 pg of tracer (10,000 - 20,000 CPM in 0.1 ml of buffer) were added to each tube and the solutions were vortexed and incubated 12 hr at 4°C. Duplicate tubes containing 0, 5, 10, 20, 50, 100, and 250 pg authentic estradiol-17\(^\beta\) or 0, 0.05, 0.10, 0.20, 0.50, 1.00, 2.00, and 5.00 ng authentic progesterone were included in each assay. After incubation (4 - 18 hr) unbound steroid was removed by vortexing with dextran-coated charcoal suspension and centrifuging at 2500 RPM for 10 minutes (Appendix A). The supernatant containing antiserum with labelled and unlabelled steroid was decanted into 10 ml of commercial scintillation fluid.\(^2\) Tritium was measured on a Packard Tri-Carb Scintillation Spectrometer, Model 3320. A computer-based regression analysis translated CPM of tritium into concentration of steroid based on assay-specific standard reference curves (Rodbard et al., 1969).

The limits of assay sensitivity were 5 pg/tube for estradiol and 0.1 ng/tube for progesterone. Accuracy and interassay variation of the steroid assays were estimated from repeated measurements of pooled male opossum serum spiked with 100 pg/ml estradiol and 5 ng/ml progesterone. In consecutive assays, estradiol

\(^1\)New England Nuclear, Boston, MA 02118.

\(^2\)Budget-Solve, Research Products Internat. Corp., Elk Grove Village, IL 60007.
and progesterone averaged $101.9 \pm 3.1$ pg/ml ($N = 15$) and $4.8 \pm 0.2$ ng/ml ($N = 32$), respectively. Coefficients of variation on duplicate determinations of estradiol and progesterone averaged 7.6% and 5.4%, respectively.

Antisera were produced in the laboratory of Dr. Vernon C. Stevens, The Ohio State University. Estrogen antiserum was specific for estradiol (17α- and 17β-epimers), not binding at detectable levels to any other steroid except estrone. Cross reactivity with estrone was less than 1%. Progesterone antiserum cross-reacted less than 5% with 17α-hydroxyprogesterone, less than 0.1% with other progestins, and below detectable levels with other steroids (Powell and Stevens, 1973).

**Biochemical Assays**

Protein, glucose, and glycogen were isolated from separate uterine subsamples, measured spectrophotometrically, and compared with standards to obtain concentrations. Percent water was calculated gravimetrically after drying a sample at $44^\circ$ C for 48 hr and cooling 24 hr in a desiccator.

Protein was isolated by homogenization in 70% EtOH (10 ml) and centrifugation. The supernatant was centrifuged three times after treatment with 10% trichloroacetic acid (10 ml) at $80^\circ$ C. The resultant precipitate was assayed by the method of Lowry et al. (1951).

Glucose was extracted by rapid homogenization in 60% EtOH (5 ml) and centrifugation. The precipitate was rinsed with additional 60% EtOH (4 ml), and the supernates were combined and measured by glucose oxidase method.
Glycogen concentrations were determined by rapid homogenization of samples that were treated with 30% KOH (2 ml) and heated to 100°C for 15 minutes. Glycogen was precipitated with 95% EtOH (2.4 ml) and careful heating. After centrifugation, glycogen was measured by the method of Montgomery (1957).

**Histology**

Three cross-sectional segments were taken from the midportion of the uterus of each animal, one including the broad ligament and two others equidistant around the uterine circumference. Tissues were processed routinely for paraffin histology, sectioned (8 μm), and stained with Harris hematoxylin and eosin. Three sections per uterine segment, each separated by more than 500 μm from the others, were measured in 3 areas with an ocular micrometer to obtain a mean endometrial width, the maximum distance from myometrium to the uterine lumen. Relative abundance of glandular tissue was measured by photographically enlarging (40 X) each of these sections. Three transects from the myometrium to the lumen were placed randomly on the prints and total glandular tissue crossing each transect was measured to the nearest 1 mm. Some tissues were stained with alcian blue and Harris hematoxylin for identification and location of mucopolysaccharides.

One ovary (N = 23) or both ovaries (N = 8) from each animal were processed routinely for paraffin infiltration and staining. Entire ovaries were sectioned (8 μm), and every tenth section was stained. For each corpus luteum, the section with the largest diameter was located through a dissecting microscope, and the average was taken of two diameters set at right angles, measured to the nearest 0.01 mm with an
ocular micrometer on a binocular microscope at 40 X. Observations on luteal lipid accumulation were made on the contralateral ovary (N = 16), which was stored in buffered formalin, frozen, and sectioned (15 μm) on a cryostat. Every fifth section was stained with oil red 0 and Mayer's hematoxylin.

A CL mass index, based on the sum of the average 2-dimensional maximum diameters of corpora lutea (CL) per ovarian pair, was obtained for each animal. However, because diameters from paraffin-processed and frozen-sectioned ovaries are not comparable, direct measurements were restricted to paraffin-processed ovaries. When ovaries from an individual were processed separately for paraffin histology and cryostat histology, the mean diameter of all CL from the paraffin- infiltrated ovary was used as an estimate of the mean diameter for the contralateral, frozen-sectioned ovary and multiplied times the number of CL in this ovary to obtain the estimated sum of maximum diameters, the CL mass index.

Statistical Analyses

Values were expressed as means (X) ± standard error of the mean (S.E.M.), and differences between pregnant and nonpregnant animals from equivalent days or differences among days were considered significant at P < 0.05 unless otherwise stated. Except for steroid levels, differences between reproductive states were compared with the Wilcoxon Rank Sum Test and Dunn's Multiple Comparisons (Hollander and Wolfe, 1973). Relationships of treatments with time were tested with Jonckheere Ordered Alternatives Test (Hollander and Wolfe, 1973),
linear regression (Sokal and Rohlf, 1969), or Wilcoxon Rank Test for
Umbrella Alternatives (Mack and Wolfe, 1976). Daily steroid concentra-
tions between reproductive states were compared with Student's t-test
(Sokal and Rohlf, 1969).
RESULTS

Reproductive Cycles and Ovarian Morphology

The mean length of a cycle in which gestation occurred (29.0 ± 1.8 days, N = 9) was statistically equivalent to the mean length of nonbreeding cycles (29.1 ± 1.2 days, N = 15) (Wilcoxon Rank Sum); cycle duration ranged from 22 to 42 days regardless of reproductive state, i.e., estrous cycle or gestation.

Ovarian weights on Day 3, 7, or 11 were not significantly different relative to reproductive condition (Table 3) (Wilcoxon Rank Sum), although a significant difference (P<0.005) occurred among these 3 days (Kruskal-Wallis Rank Sum); Day 7 ovaries (613 ± 30 mg) were significantly heavier (P<0.01) than Day 3 ovaries (394 ± 20 mg) (Dunn’s Multiple Comparisons).

Each ovary contained 30.6 ± 1.1 CL, ranging from 15 to 58 CL per ovary (N = 55) or 43 to 85 CL per animal (N = 24) (Fig. 6). No difference relative to reproductive state occurred in the number of CL per ovary (Wilcoxon Rank Sum). Luteinized follicles might have been included in the CL counts because anovulatory ova apparently degenerated after Day 3, and luteinized follicles could no longer be distinguished from CL. Five, Day 3 ovaries retained evidence of ova within at least one luteinized follicle (Fig. 7) (range: 1 to 4). Between reproductive states no differences in mean diameter of CL per ovary occurred (Table 3) (Wilcoxon Rank Sum). However, among Days 3, 7, and 11, the mean maximum diameters were significantly different...
Fig. 6. Cross-section (8 μm) of an opossum ovary 7 days postovulation. Thirteen corpora lutea (CL) occurred in this section with several antral follicles (F) (hematoxylin and eosin; 12 X).
Fig. 7. Cross-section (8 μm) of an anovulatory follicle during luteinization 3 days postestru. Granulosa cells (G) were undergoing hypertrophy, reducing the size of the antrum (A) that still contained the ovum (arrow) (hematoxylin and eosin; 54 X).
Table 3. Opossum ovarian weight (mg) and maximum diameter (mm) of corpora lutea during the luteal phase of the estrous cycle and during gestation.a

<table>
<thead>
<tr>
<th>Reproductive State</th>
<th>Days Postestrus</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 3</td>
<td>Day 7</td>
</tr>
<tr>
<td>Ovarian Weight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrous Cycle</td>
<td>382 ± 32</td>
<td>(4)</td>
<td>592 ± 28</td>
</tr>
<tr>
<td>Gestation</td>
<td>403 ± 14</td>
<td>(6)</td>
<td>634 ± 224</td>
</tr>
<tr>
<td>Maximum Diameter of Corpora Lutea</td>
<td>1.42 ± 0.13</td>
<td>(130)</td>
<td>1.85 ± 0.03</td>
</tr>
<tr>
<td>Estrous Cycle</td>
<td>1.44 ± 0.02</td>
<td>(105)</td>
<td>1.87 ± 0.17</td>
</tr>
<tr>
<td>Gestation</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Mean ± S.E.M. Sample size is in parentheses.
Day 3 CL were significantly smaller than either Day 7 CL \((P<0.01)\) and Day 11 CL \((P<0.05)\), irrespective of reproductive state (Dunn's Multiple Comparisons).

All CL from frozen-sectioned ovaries stained uniformly with oil red O on Days 3, 7, and 11. Luteal cells were faint pink andpronouncedly counter-stained with hematoxylin. In contrast, all granulosa cells of antral follicles were stained intensely with oil red O indicating concentrated lipid deposition in these cells during folliculogenesis that might terminate at ovulation.

Peripheral Steroid Levels

The profiles of circulating estradiol during opossum reproductive cycles (Fig. 8) were characterized by a pre-estrous maximum value of \(23.8 \pm 1.9 \) pg/ml of serum on Days -2 and -1 and wide fluctuations from estrus \((9.0 \pm 1.3 \) pg/ml, \(N = 36)\) through Day 14 \((10.6 \pm 2.1 \) pg/ml, \(N = 16)\); mean values ranged between 5.4 and 16.8 pg/ml in gestation and the luteal phase of the estrous cycle. No differences occurred between the two reproductive states on individual days except on Day 2 when estradiol concentrations from pregnant animals \((N = 6)\) were significantly higher than from nonpregnant animals \((N = 7)\), 15.5 \pm 3.3 and 5.4 \pm 1.5 pg/ml, respectively (Student's t-test).

Mean pre-ovulatory (Days -6 through -1) serum concentration of progesterone in 16 animals \((1.0 \pm 0.2 \) ng/ml, \(N = 26)\) was less than the 3.0 \pm 0.7 ng/ml measured in estrous samples from the same animals. Progesterone concentration for all pre-estrous samples was 2.1 \pm 0.3 ng/ml \((N = 41)\). After estrus, circulating progesterone had a distinct unimodal profile (Fig. 9) with peak values of 13.6 \pm 1.3 (Day 8) and 17.8 \pm 3.4 ng/ml (Day 9) in nonpregnant and pregnant.
Fig. 8. Peripheral concentrations of estradiol (pg/ml) in serum of captive opossums during the pre-estrous phase (▲-▲), the luteal phase of the estrous cycle (●-●), and gestation (○-○-○-○). Sample sizes for pre-estrous (L), luteal phase of the estrous cycle (C), and gestation (G) means ± S.E.M. are below the abscissa.
Fig. 8.
Fig. 9. Peripheral concentrations of progesterone (ng/ml) in serum of captive opossums during the pre-estrous phase (△—△), the luteal phase of the estrous cycle (●—●), and gestation (○—○—○—○—○). Sample sizes for pre-estrous (L), luteal phase of the estrous cycle (C), and gestation (G) means ± S.E.M. are below the abscissa.
Fig. 9.
animals, respectively. On Day 18 progesterone returned to low levels, averaging $2.4 \pm 0.4$ ng/ml ($N = 17$). No significant differences between reproductive states in serum progesterone concentration occurred on individual days (Days 0 through 14 and Day 18) (Student's $t$-test).

Circulating progesterone concentrations were significantly correlated with the index of luteal mass, the sum of the maximum mean diameter of each CL in both ovaries (Fig. 10). This correlation was highly significant ($P < 0.001$) for individuals ($N = 8$) with exact measurements from CL in both ovaries that were processed for paraffin histology ($t = 6.188; 6$ df). Similarly, progesterone levels were highly correlated with index of luteal mass based on measurements of CL from a paraffin-sectioned ovary and diameter estimates of CL from the contralateral, frozen-sectioned ovary ($t = 3.881; 21$ df; $P < 0.001$).

The average estrogen:progesterone ratio (E:P ratio) was highest 3 and 4 days before estrus (33 pg/ng) and declined to average ratios less than 3 pg/ng by Day 4 and remained near this Day 4 level until Day 10 when the ratio began to increase (Fig. 11). No significant differences in this ratio occurred on any day between the two reproductive states (Wilcoxon Rank Sum). However, the Day 12 gestation ratio represented a significant peak compared to the periparturient samples on Days 10, 11 and Days 13, 14 (Wilcoxon Rank Test for Umbrella Alternatives). The luteal phase of the estrous cycle did not exhibit a similar E:P ratio peak.

**Biochemical Composition of the Uterus**

No significant differences occurred between pregnant and nonpregnant uteri in tissue composition of water, glucose, or protein
Fig. 10. Linear regression of peripheral progesterone concentration (P₄ ng/ml) and CL mass index in the opossum. Line A (O--O) was calculated from the sum of maximum average 2-dimensional diameters from both paraffin-processed ovaries. Mean maximum diameters of CL from paraffin-processed ovaries were used as standards when ovaries from an individual were separated for paraffin and frozen-section histology (Line B: •—•).
Fig. 10.

\[ y = 0.267x - 15.90 \]
\[ r = 0.930 \]
\[ N = 8 \]

\[ y = 0.20x - 10.869 \]
\[ r = 0.698 \]
\[ N = 23 \]
Fig. 11. Mean ratios (pg/ng) of peripheral concentrations of estradiol and progesterone in the serum of captive opossums during the pre-estrous phase (▲—▲), luteal phase of the estrous cycle (●—●), and gestation (○—○—○—○). Sample sizes for pre-estrous (L), luteal phase of the estrous cycle (C), and gestation (G) mean ratios are below the abscissa.
Fig. 11.
on Day 3, 7, and 11 (Wilcoxon Rank Sum). However, glycogen levels were significantly lower (P<0.05) on Day 7 of pregnancy (Table 4). On Day 3 and 11, glycogen was statistically equivalent in the two reproductive states. Both percent water and protein composition demonstrated positive (P<0.01) relationships with time over the sampling period (Jonckheere Ordered Alternatives Test). Among days, glycogen levels were significantly different (P<0.01; Kruskal-Wallis Rank Sum); Day 7 was significantly higher than Day 3 or Day 11 (Dunn's Multiple Comparisons). Glucose concentrations were not significantly different among Days 3, 7, and 11 (Kruskal-Wallis Rank Sum).

**Uterine Morphology and Histology**

Volumes of pregnant and nonpregnant uteri (Table 5) were equivalent on each sampling day (Wilcoxon Rank Sum). From Day 3 to Day 11, volumes increased significantly (P<0.05) with time (Jonckheere's Ordered Alternatives Test). Endometrial widths compared between reproductive states were statistically equivalent on Days 3 and also on Day 7 but were significantly smaller in pregnant animals on Day 11 (Wilcoxon Rank Sum). Neither pregnant nor nonpregnant uteri demonstrated an increasing relationship between endometrial width and time (Jonckheere's Ordered Alternatives Test). Among days, endometrial widths on Day 7 were significantly larger than widths on Day 3 (P<0.05; Kruskal-Wallis Rank Sum; Dunn's multiple Comparisons).

No differences in the uterine gland Index occurred between pregnant and nonpregnant animals on each day (Table 5) (Wilcoxon Rank Sum). This Index was correlated significantly with endometrial width (P<0.001; \( y = 0.04 x + 4.08; r = 0.728; t = 4.251; 16 \text{ df}) and with progesterone.
Table 4. Median and range of percent water and uterine constituents (mg/g dry weight) throughout the luteal phase of the estrous cycle and gestation in the opossum. Sample size is in parentheses.

<table>
<thead>
<tr>
<th>Uterine Component</th>
<th>Day 3</th>
<th></th>
<th></th>
<th>Day 7</th>
<th></th>
<th></th>
<th>Day 11</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nonpregnant</td>
<td>Pregnant</td>
<td></td>
<td>Nonpregnant</td>
<td>Pregnant</td>
<td></td>
<td>Nonpregnant</td>
</tr>
<tr>
<td>Percent Water</td>
<td>83.8</td>
<td>81.8</td>
<td></td>
<td>84.7</td>
<td>83.5</td>
<td></td>
<td>87.4</td>
<td>87.6</td>
</tr>
<tr>
<td></td>
<td>82.6 - 85.1</td>
<td>81.5 - 82.8</td>
<td></td>
<td>84.5 - 86.8</td>
<td>76.7 - 88.3</td>
<td></td>
<td>86.4 - 90.5</td>
<td>80.8 - 88.3</td>
</tr>
<tr>
<td></td>
<td>82.6 - 85.1</td>
<td>81.5 - 82.8</td>
<td></td>
<td>84.5 - 86.8</td>
<td>76.7 - 88.3</td>
<td></td>
<td>86.4 - 90.5</td>
<td>80.8 - 88.3</td>
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<tr>
<td>Glucose</td>
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<td>13.6</td>
<td></td>
<td>5.3</td>
<td>4.2</td>
<td></td>
<td>7.6</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>6.3 - 8.1</td>
<td>5.7 - 17.3</td>
<td></td>
<td>5.0 - 6.1</td>
<td>3.6 - 8.8</td>
<td></td>
<td>4.9 - 11.6</td>
<td>4.2 - 6.9</td>
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<tr>
<td></td>
<td>6.3 - 8.1</td>
<td>5.7 - 17.3</td>
<td></td>
<td>5.0 - 6.1</td>
<td>3.6 - 8.8</td>
<td></td>
<td>4.9 - 11.6</td>
<td>4.2 - 6.9</td>
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<tr>
<td>Glycogen</td>
<td>2.4</td>
<td>4.0</td>
<td></td>
<td>10.2*</td>
<td>6.1*</td>
<td></td>
<td>4.7</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>2.1 - 2.7</td>
<td>3.1 - 5.9</td>
<td></td>
<td>8.5 - 15.8</td>
<td>4.4 - 7.8</td>
<td></td>
<td>3.7 - 5.8</td>
<td>3.2 - 4.9</td>
</tr>
<tr>
<td></td>
<td>2.1 - 2.7</td>
<td>3.1 - 5.9</td>
<td></td>
<td>8.5 - 15.8</td>
<td>4.4 - 7.8</td>
<td></td>
<td>3.7 - 5.8</td>
<td>3.2 - 4.9</td>
</tr>
<tr>
<td>Protein</td>
<td>133</td>
<td>109</td>
<td></td>
<td>141</td>
<td>151</td>
<td></td>
<td>185</td>
<td>191</td>
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<tr>
<td></td>
<td>108 - 159</td>
<td>93 - 149</td>
<td></td>
<td>79 - 160</td>
<td>85 - 190</td>
<td></td>
<td>112 - 208</td>
<td>158 - 245</td>
</tr>
</tbody>
</table>

*Significantly different at P<0.05.
Table 5. Volume (ml), endometrial width (mm), and gland index (mm) of opossum uteri on equivalent days of gestation and the estrous cycle.\(^a\)

<table>
<thead>
<tr>
<th>Reproductive Condition</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 11</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Volume</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrous Cycle</td>
<td>6.0</td>
<td>12.7 ± 0.9</td>
<td>20.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.7 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Gestation</td>
<td>6.7 ± 0.7</td>
<td>12.7 ± 1.2</td>
<td>23.5 ± 2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Endometrial Width</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrous Cycle</td>
<td>4.41 ± 0.80</td>
<td>7.13 ± 0.82</td>
<td>6.50 ± 0.47*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.42 ± 0.25</td>
<td></td>
</tr>
<tr>
<td>Gestation</td>
<td>5.74 ± 0.21</td>
<td>5.18 ± 0.48*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Gland Index</strong>(^b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrous Cycle</td>
<td>1.00 ± 0.16</td>
<td>2.05 ± 0.08</td>
<td>0.83 ± 0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.76 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>Gestation</td>
<td>0.77 ± 0.04</td>
<td>0.96 ± 0.07</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Mean ± S.E.M. Sample size is in parentheses. Values with superscripts are significantly different (P < 0.05).

\(^b\)Nine transects were selected randomly from each animal.
concentrations in the serum (P < 0.05; y = 0.12x + 3.67; r = 0.51; 
t = 2.373; 16 df).

The endometria appeared identical in pregnant and nonpregnant 
uteri on Day 3 (Fig. 12). Densely packed, convoluted uterine glands 
and relatively little stromal material characterized uteri on this 
day. The significant increase in endometrial width on Day 7 was the 
apparent result of stromal deposition and glandular proliferation, 
irrespective of reproductive status (Fig. 13). Day 11 pregnant uteri 
had compacted, dense endometria compared to the endometria from 
nonpregnant animals (Fig. 14). In particular, the luminal third 
of nonpregnant uteri had flocculent and diffuse stromal material. 
Although no significant differences were demonstrated, blood vessels 
and uterine glands appeared larger and more numerous in pregnant animals, particularly along the subluminal margin.

Maternal-Embryonic Associations

The presence of the chorio-vitelline membrane on Day 11 resulted 
in no detectable anatomical change in the size, conformation, or 
structure of the uterine surface epithelium layer, the sole surface 
of maternal cellular contact with embryonic tissues. The uterine 
epithelial cells were consistently rounded apically regardless of the 
juxtaposition of trophoblasts on lateral and apical surfaces (Fig. 15).

Separation of these two layers apparently occurred during 
fixation and processing. Despite this separation, trophoblasts 
occasionally either maintained contact with the maternal epithelium 
(Fig. 16), or trophoblasts ruptured, thereby leaving cell fragments
Fig. 12. Cross-section (8 μm) of the uterine wall of an opossum on Day 3 of the estrous cycle (A) and Day 3 of gestation (B). M = myometrium; E = endometrium; L = lumen (hematoxylin and eosin; 45 X).
Fig. 13. Cross-section (8 μm) of the uterine wall of an opossum on Day 7 of the estrous cycle (A) and Day 7 of gestation (B). M = myometrium; E = endometrium; L = lumen (hematoxylin and eosin; 45 X).
Fig. 14. Cross-section (8 µm) of the uterine wall of an opossum on Day 11 of the estrous cycle (A) and Day 11 of gestation (B). M = myometrium; E = endometrium; L = lumen (hematoxylin and eosin; 45 X).
Fig. 15. Apposition of uterine epithelium (E) with trophoblasts (T) of the choriovitelline membrane. Individual trophoblasts conformed to the shape of the epithelial cells. S = stroma (hematoxylin and eosin; 8 μm; 360 X).
Fig. 16. Apposition of uterine epithelium (E) with trophoblasts (T) of the chorio-vitelline membrane. One trophoblast (arrows) maintained contact with the epithelium through fixation. S = stroma (hematoxylin and eosin; 8 µm; 360 X).
attached to the luminal surface of the uterine epithelium (Figs. 17 and 18). These cell fragments stained deeply with alcian blue, indicating a concentrated layer of mucopolysaccharides associated with this interface (Fig. 19). Trophoblasts stained lightly, and uterine epithelium failed to stain with alcian blue.
Fig. 17. Apposition of uterine epithelium (E) with trophoblasts (T) of the chorio-vitelline membrane. Trophoblastic cell fragments still adhered to the apical surface of the epithelial cells (arrows) after fixational separation. S = stroma (hematoxylin and eosin; 8 μm; 180 X).
Fig. 18. Apposition of uterine epithelium (E) with trophoblasts (T) of the chorlo-vitelline membrane. Trophoblastic cell fragments still adhered to the apical surface of the epithelial cells (arrows) after fixational separation.

S = stroma (hematoxylin and eosin; 8μm; 360 X).
Fig. 19. Apposition of uterine epithelium (E) with trophoblasts (T) of the chorio-vitelline membrane. Trophoblastic cell fragments stained darkly for mucopolysaccharides (arrows); trophoblastic cytoplasm was faintly stained for mucopolysaccharides (alcian blue and hematoxylin; 8 μm; 360 X).
DISCUSSION

Recognition of pregnancy by the maternal environment entails alteration of physiological and histological events that occur during the estrous cycle in the absence of breeding. Recognition, as such, requires both production of novel physical, physiological, and/or hormonal stimuli by the conceptus and maternal receptor mechanisms to perceive these stimuli and initiate physiological changes. Lack of differences between gestation and the luteal phase of the estrous cycle indicates the adequacy of the estrous cycle to accommodate the conceptus. The intent and design of this study was to examine systematically maternal components, i.e., circulating estradiol and progesterone levels, uterine protein, glucose, glycogen, and water concentrations, and uterine and ovarian histomorphology, periodically during gestation and equivalent days of the estrous cycle. A further consequence of this sampling scheme was the characterization of the postovulatory profile for each component at trimester intervals.

Reproductive Endocrinology

The pattern of circulating ovarian steroids in the opossum during gestation and the luteal phase of the estrous cycle corresponded roughly to the estrous cycle profiles in sheep (Stabenfeldt et al., 1969), in guinea pigs (Challis et al., 1971b), and in monkeys (Neill et al., 1967) (also, see review by Short, 1972). Comparable features
Included a clearly defined pre-estrous surge of estradiol and a unimodal, short-term progesterone peak, highly correlated with maximum luteal area. Although a few scattergrams of peripheral progesterone concentrations are available, no metatherian reproductive cycle has been characterized previously by serial sampling to establish the mean pattern and variation of this hormone. Also, no values on circulating estradiol concentration in a marsupial have been published. This study documented the opossum's daily hormonal levels during both reproductive states as statistically equivalent, except Day 2 estradiol, thus supporting overall the equivalence hypothesis. Similarly, the daily ratios of estradiol to progesterone were equivalent through gestation and the luteal phase.

The estradiol profile was accented by a peak 1 to 2 days before estrus and a rapid decline on the day of estrus, a sequence perhaps necessary for behavioral receptivity and ovulation. Large populations of anovulatory antral follicles apparently maintained the continually fluctuating production of estradiol throughout the reproductive cycle.

The difference in estradiol levels between the 2 reproductive states on Day 2 might have resulted from asynchronous fluctuations of mean levels and small sample sizes. Each day containing less than 10 samples per reproductive state, i.e., Days 1, 2, 4, 8, and 9, were characterized by large differences between mean values. Day 2 contained the smallest composite sample (N = 13) and thus, perhaps, was artifically subject to statistical significance. Alternatively, breeding might have induced increased estradiol release. Evidence for this explanation included the similar mean values on estrus and
and Day 3, but higher mean values on Both Days 1 and 2 of gestation as compared to equivalent days of the estrous cycle. Therefore, breeding activity could have produced a temporary rise (48 hr) in peripheral estradiol after estrus, perhaps to facilitate tubal transport of zygotes.

In contrast, the relatively large sample (N = 24) on Day 12, the day of parturition, showed a large (120%) rise in estradiol during gestation, but a statistically significant difference between the two reproductive states did not occur. The profiles on this day also departed from the relatively parallel pattern established during the previous 7 days. This trend during gestation in estradiol concentration or, more importantly, the rise in E:P ratio might be biologically important for parturition at the uterine level.

Mean unconjugated total estrogens fluctuated between 3.8 and 15.9 pg/ml of plasma in the pregnant tammar wallaby sampled on either Day 11, 15, 20, or 25 after removal of the pouch young (Renfree and Heap, 1977). Administration of estradiol benzoate (10μg/day i.m.) to the tammar wallaby during seasonal quiescence caused an increase in the amplitude of uterine contractile activity in addition to imposing a cyclic patterns in vivo (Young, 1977). However, in vitro preparations of uterine strips obtained from estrogen-dominated, late follicular phase, and immediately postestrous quokkas exhibited a tendency for low, irregular, infrequent but often pronounced uterine contractions. Thus, definition of contractile patterns induced by estrogen was relative to prior hormonal conditioning.
Levels of progesterone, the steroid responsible for maintenance of the secretory endometrium, were statistically identical during pregnancy and the postovulatory estrous cycle. This result, in conjunction with the high correlation with the luteal mass index, suggested that the CL were the sole gestational source of progesterone. Continuously increasing levels of progesterone occurred during the first and second trimester of pregnancy while the embryo was a free-floating blastocyst, dependent on uterine secretions for nutrients. The precipitous (200%) decline in progesterone during the 4 days prepartum coincided with the initiation of chorio-vitelline membrane elaboration, suggesting a reduced dependence on uterine gland secretion as a nutrient source might occur during late gestation. Furthermore, the index of glandular abundance was highest on Day 7 rather than during late gestation, the trimester of greatest nutrient demand.

Cook et al. (1977b) reported maximum circulating levels of progesterone up to 12 ng/ml plasma during the luteal phase of the untreated cycle and over 80 ng/ml plasma in gonadotrophin-primed female opossums. In samples from the present study, a maximum level of 34 ng/ml of serum was recorded and 13 samples were greater than 20 ng/ml of serum during normal reproductive cycles. These values contrasted sharply with peripheral levels reported from two monovulatory Australian species, the only other metatherians examined for peripheral steroid concentrations. The brush-tailed possum had maximum levels of 4.5 ng/ml of plasma on Day 12 of the cycle (parturition Day 17.5), ranging from 0.35 ng/ml on estrus to 0.9 ng/ml on Day 20 with a unimodal pattern, as measured by radioimmunoassay (Shorey and Hughes, 1973a).
Thorburn et al. (1971) found similar levels with competitive protein-binding techniques; they recorded a maximum level of 5.0 ng/ml between Days 11 and 15. Circulating progesterone in the tammar wallaby appeared to follow a biphasic profile, reaching mean peak levels ($N = 3$ or 4) of 614 pg/ml of plasma on Day 11 and of 902 pg/ml on Day 25, 1 to 2 days before parturition (Renfree and Heap, 1977). Based on progesterone concentration within CL sampled on approximately alternate days from Day 10 to Day 25 after pulling pouch young ($N = 2$ to 13), Renfree et al. (1979) further characterized this pattern as having a minor peak on Day 17 (24.4 ng/mg), a major peak on Day 22 (32.0 ng/mg), and declining values on Day 25 (22.2 ng/mg). From these latter two studies, the complementary Day 25 relationship between increased peripheral concentration and decreased luteal concentration of progesterone appears contradictory to conventional models of periparturient reductions in progesterone unless a concurrent prepartum rise in estrogens also occurred. These reciprocal changes in steroid levels could effect a shift in the E:P ratio.

The antagonistic, or synergistic, action of estrogen and progesterone on the sheep and rodent uterus might be dictated by the ratio of these two hormones rather than their absolute concentrations (Challis, 1971; Challis et al., 1971 a, b). In accord with these eutherian models of gestation, the opossum demonstrated a large E:P ratio peak 3 and 4 days before estrus, a decline to mean levels below 2 pg/ng by Day 4, and maintenance of low ratios until near term. Notable, no significant differences between E:P ratios on individual days occurred despite the intervention of parturition. However,
ratio profiles during the final trimester were patterned differently in the two reproductive states. Pregnant animals showed a significant peak in E:P ratio on Day 12; a similar peak did not occur in the estrous cycle. The initiation of parturition might not be a response simply to critical E:P ratios on a particular day but the culmination of a continuous and dynamic pattern of these steroid ratios from Day 10 through Day 14. The profile during this period rather than daily point estimates is the determinate feature, according to this hypothesis.

Historically, the role of the metatherian conceptus in determination of the maternal physiological condition has been assumed to be insignificant (Sharman, 1970), a necessary tenet of the equivalence hypothesis. However, two independent lines of evidence have suggested more recently that, at least locally, the maternal system might be altered by the presence of the embryo. First, steroidogenesis has been identified in embryonic membranes and in the embryo. Bradshaw et al. (1975) and Renfree and Heap (1977) synthesized progesterone from pregnenolone in chorio-vitelline tissue from the quokka and tammar wallaby, respectively. In two plasma samples from the embryonic tammar wallaby, corticosteroid (0.9 and 1.5 ng/ml) and androgen (0.8 and 0.0 ng/ml) concentrations were measured one to two days before birth (Catling and Vinson, 1976). Second, the uterine myometrium had unique contractile patterns during late gestation in the quokka (Heller, 1972, 1973) and in the tammar wallaby (Young, 1977). The sensitization of the myometrium to initiatory stimuli for periparturient contractions, possibly oxytocin or vasopressin, was mediated apparently by an
embryonic factor in these species. In the opossum, the unique E:P ratio profile from Days 10 through 14 also might be indicative of local uterine alterations related to parturition. Steroid production by the conceptus or myometrial motility in the adult have not been examined in this species, but supportive data from other marsupials suggest that the embryo potentially influences the termination of gestation. Parturition remains an enigma within the contest of the equivalence hypothesis because no estrous cycle correlate of this event exists and, therefore, must require a conceptus-induced mechanism.

Biochemical Composition of the Uterus

Renfree (1975) distinguished no differences in opossum endometrial weight or uterine exudate protein composition and concentration during the first 8 days of pregnancy and the estrous cycle. With alternative criteria, this study equated early, mid-, and late gestational uteri with their estrous cycle correlates. Similar levels of water, glucose, or protein on equivalent days of the estrous cycle and pregnancy emphasized the similarity of both reproductive states in the opossum. Apparently, the levels of these uterine components present during the luteal phase of the estrous cycle were adequate for embryonic development and probably reflected similarities in hormonal profiles that regulated these constituents. Uterine water content gradually increased before implantation in the rat (Surani and Heald, 1971) in response to estrogen stimulation (Boettiger, 1946; Bitman et al., 1965). Local glucose concentration generally was maintained by exogenous circulating levels. However, embryonic events, such as implantation in the rat, increased glycolysis significantly (Surani and Heald, 1971). Although the dynamics
of carbohydrate metabolism were not examined in this study, the complementary relationship of glucose and glycogen suggested a shift towards glycogenic mechanisms during midpregnancy and midluteal phase of the estrous cycle. Renfree (1975) identified no unique protein electrophoretic bands in these two reproductive states. In eutherians, a significant rise in proteins occurred only after implantation (Drasher, 1953).

The significantly different levels of glycogen in the Day 7 pregnant and nonpregnant uteri indicated that the reproductive tract responded to the presence of the blastocyst. Walaas (1952) found glycogen to be restricted largely to the myometrium, functioning as an energy reserve for uterine contractions. Therefore, a lower level of glycogen in pregnant uteri, compared to equivalent-day nonpregnant uteri, probably indicated an increase in contractions and motility during midpregnancy. Fertilized and unfertilized eggs arrived in the uterus before Day 3 encased in a cortical layer; blastocysts expanded to 33 times their original volume by Day 7, whereas unfertilized eggs tended to shrink (McCrady, 1938). Thus, maternal response might be based solely on physical recognition of the expanded blastocyst, but a unique hormonal regime or biochemical recognition of embryonic metabolism also might have elicited a glycolytic response.

In this study, no differences occurred in the size of corpora lutea, progesterone concentrations, estradiol concentrations (except Day 2), and the E:P ratio on equivalent days during the estrous cycle and gestation. Therefore, although some have shown that myometrial activity (Schofield, 1955, 1957) and glycogen dynamics (Walaas, 1952;
Stelnetz et al., 1957; Bitman et al., 1965) might be moderated largely by endocrine mechanisms, embryonic production of hormones or modification of maternal steroid production in the opossum has not been demonstrated. Only in the quokka and the tammar wallaby has an endocrine function been demonstrated in the chorlo-vitelline membranes of the marsupial embryo (Bradshaw et al., 1975; Renfree and Heap, 1977, respectively). Increased uterine contractibility and, hence, lower glycogen concentrations might be induced by locally-produced steroids modifying the uterine motility (Heller, 1972, 1973) but not detectable peripherally.

The reliance of the preimplantation eutherian upon uterine nutrients has been documented through embryo culture techniques. Absorption by rodent and rabbit embryos of specific nutrients, such as glucose (Renard et al., 1980; Flynn and Hillman, 1978), pyruvate and lactate (Brinster, 1965; Quinn and Wales, 1973), choline (Pratt, 1980), amino acids (Epstein and Smith, 1973), electrolytes (Biggers et al., 1977), and water (Tuft and Bovling, 1970) have been reported. In each case, transport of nutrients was accomplished without disruption of the golgi lemma, the acellular adhesive layer surrounding the eggs, which is composed of secretions from the follicle, oviduct, and uterus (Bovling, 1954). Hughes and Shorey (1973) determined that molecules between 306 and 460,000 molecular weight diffused across the cortical layer, mucopolysaccharide coat, and zona pellucida in the brush-tailed possum embryonic vesicle. In addition, the 200-fold increase in volume of the opossum blastocyst before elaboration of embryonic membranes on Day 8 (McCrady, 1938) indicated that dynamic interchange between maternal and embryonic systems occurred, but resultant production of
a unique gestational compound has been demonstrated only in the tammar wallaby. Therefore, although exchange of compounds undoubtedly occurs between maternal and embryonic compartments, evidence does not support these as stimuli for the apparent glycogen mobilization observed in this study.

If the opossum uterus detects the expansion of blastocysts and embryo-spacing contractile movements are initiated, myometrial glycogen reserves might diminish during midpregnancy. Differential uterine response to objects with diameters similar to preimplantation blastocysts was demonstrated by Boving (1954; 1971; 1972) in the rabbit. Through waves of circumferential contractions radiating from sites of distention, each blastocyst was distributed equidistant from neighbors. Rats also experienced a decline in uterine glycogen during this preimplantation period (Day 4 to 6) while the blastocysts were being spaced (Rajalakshmi et al., 1972).

Histology

The early and midgestational uteri of the opossum were identical histomorphologically to their estrous cycle counterparts, although the late gestational uterus was distinguished clearly from its nonpregnant equivalent on the basis of endometrial width, the appearance of stromal material, and subepithelial vascularization. However, each of these characteristics could be attributed to the mechanical effect of the embryos, chorio-vitelline membranes, and fluids. Compression of the endometrial layer during pregnancy apparently resulted in dense, compacted stromal material and confined the glands and blood vessels to a reduced volume of tissue. Alternatively, the apparent increase
in vascularity might have been an antigenic response to embryonic components, but the immunologic relationship of maternal and embryonic tissues in marsupials remains generally speculative (Walker and Tyndale-Biscoe, 1978).

Extensive elaboration of embryonic membranes over the uterine surface in the opossum probably insures maximum surface area for absorption of nutrients and an intricate network for securing the embryo in utero. McCrady (1938) noted the consistent orientation of all embryos that he excised after Day 10. In particular, the animal pole of the vesicles rested against the uterine mucosa, suggesting that the area vasculosa was sticky and inclined to adhere to the mucosa by Day 11. Adhesion might be accomplished in part through production of mucopolysaccharides. Although uterine glands are considered the primary source of histiotrophe and hemotrophe, Padykula and Taylor (1971) described the ultrastructure of the surface epithelial cells as indicative of transport function. The intimate contact of maternal and embryonic surfaces might be necessary for optimal transport of nutrients. The apparent adhesive properties of trophoblasts of the vascularized portion of the membrane support the contention that this relationship entails more than simple physical juxtaposition, perhaps transport facilitation. Furthermore, this progression of embryo-uterine Intimacy, from apposition to adhesion, corresponds to the pre-implantation stages that occur in eutherians (Enders and Schlafke, 1969; Enders, 1976).

Marsupials demonstrate a spectrum of reproductive complexity in terms of embryonic alteration of uterine histo-anatomy. Didelphids and
and peramelids represent the two extremes, from no apparent alteration of the uterine environment to extensive endometrial erosion, respectively (Hughes, 1974). Conventionally, the apposition of embryonic membranes with the uterine epithelium has been considered a simple juxtaposition of tissues (Hartman, 1919; Hughes, 1974; Padykula and Taylor, 1976) with nutrients apparently supplied in the luminal fluids. The final trimester of gestation represents the period of highest nutrient demand by the embryos. However, within this period, glandular tissue was significantly reduced, and volume of uterine fluid was never sufficient to draw into a capillary tube. This reduction in glandular tissue on Day 11 corresponded to the decline in peripheral progesterone concentrations and occurred in both pregnant and nonpregnant opossums. The apparent withdrawal of glandular support for the embryos at this time is reconcilable with the embryo's energetic demands only if the elaboration of the chorio-vitelline membrane and its association with maternal tissue enhances nutrient acquisition. Although the epithelial characteristics remained unchanged due to the presence of the conceptus, the morphology of the trophoblasts indicated mechanical interlocking or cellular adhesion with the epithelium. Potentially, the direct and intimate intercellular contact between luminal epithelium and trophoblasts facilitates transfer of material during the final days of gestation when glandular secretions apparently wane.
Embryo-Maternal Relationships

Synthesis of these results generates this proposed sequence for the reproductive process in the opossum. On estrus, each ovary ovulates an overabundance of ova, probably between 15 and 25, thus compensating for subsequent intra- and postpartum losses. If fertilization occurs, the free-floating embryos grow within the uterine lumen, absorbing nutrients from, and releasing wastes into, the uterine fluid. This interchange is mediated by diffusion across the extra-embryonic, acellular layers throughout the first 7 days postfertilization. During midgestation, the expansion of embryos generates myometrial contractions, thereby redistributing them from a cluster at the caudal end of the uterus on Day 3 to a random distribution on Day 8 when elaboration of the chorio-vitelline membrane occurs. During the final trimester, direct contact between maternal and embryonic tissues might facilitate the transport of compounds and provide positional support. Contact of these heterologous tissues appears to include intricate physical juxtaposition, a mucopolysaccharide interface, and adhesion of trophoblasts with uterine surface epithelial cells. Reduction in uterine glandular tissue and scarcity of uterine luminal fluids suggest that this interface might serve as the primary avenue of prepartum interchange between maternal and embryonic compartments. Parturition might be initiated by a shift in the E:P ratio after Day 10.
CONCLUSIONS

1. Length of an estrous cycle was 29.2 ± 1.0 days (X ± S.E.M.) irrespective of the breeding status of the adult female opossum. No significant difference occurred in the length of cycles between pregnant and nonpregnant animals thus demonstrating for the first time the statistical equivalence of cyclicity in these two states.

2. Ovaries contained 30.6 ± 1.1 corpora lutea during each reproductive cycle. Ovarian weights and an index of luteal mass (total maximum luteal diameters) were each equivalent on Days 3, 7, and 11 between pregnant and nonpregnant animals. This index was also highly correlated with peripheral progesterone concentrations; circulating progesterone appears, therefore, to be attributable solely to ovarian sources and not to embryonic or uterine steroid production. Corpora lutea within and among ovaries did not stain differentially with a lipid stain, oil red 0, thus indicating similar steroid metabolism in each gland.

3. The peripheral estradiol concentrations in pregnant and nonpregnant animals were equivalent on Days 0 through 14 and Day 18, except for Day 2. Copulation might have induced higher estradiol concentrations on Day 2. Alternatively, the low sample size (N = 13) might have resulted in artifactual statistical significance. Estradiol
profile was characterized by a pre-estrous peak (23.8 ± 1.9 pg/ml) on Days -1, -2 and subsequent postestrous mean values between 5.4 and 16.8 pg/ml. Similar to other mammals, the opossum apparently requires a pre-estrous rise in estrogens for ovulation and estrous receptivity. Postovulatory variation confounded functional interpretation.

4. Peripheral progesterone concentrations in pregnant and nonpregnant animals were equivalent on Days 0 through 14 and Day 18. Maximum concentration occurred on Day 8 of the luteal phase of the estrous cycle (13.8 ± 1.3 ng/ml) and Day 9 of gestation (17.8 ± 3.4 ng/ml). These peripheral steroid concentrations, the first profiles for a marsupial, supported the equivalence hypothesis in the opossum.

5. The estrogen:progesterone (E:P) ratios in both reproductive states were equivalent on Days 0 through 14 and Day 18. Maximum E:P ratio occurred on 3 and 4 days prior to estrus (33 pg/ng), and all postestrous averages were below 7 pg/ng. However, the periparturient profiles were significantly different. During pregnancy, a peak value occurred on Day 12 compared to Days 10, 11 and Days 13, 14; a similar peak did not occur in the luteal phase of the estrous cycle. These results suggest a possible mechanism of induction of parturition, an event that lacks an estrous cycle correlate and, therefore, requires a unique initiator.

6. Uterine concentrations of protein, glucose, and water were equivalent on each sampling date in pregnant and nonpregnant animals. Glycogen was significantly lower in pregnant animals on Day 7; on Days 3 and 11, glycogen concentrations were equivalent.
Mobilization of glycogen during midpregnancy was probably the result of increased myometrial activity during midpregnancy, effecting an equal distribution of individuals throughout the uterine lumen to optimize utilization of uterine surface area.

7. Endometrial widths in pregnant and nonpregnant animals were equivalent on Days 3 and 7. On Day 11, the endometrial width in pregnant animals was significantly smaller, apparently the result of compaction by embryonic elements. In support of the equivalence hypotheses, no differences between reproductive states in an index of uterine glandular mass occurred on each of the 3 sampling days.

8. Apposition of the chorio-vitelline membranes with the uterine surface epithelium resulted in no maternal cellular alterations. Trophoblasts of the vascularized membrane usually conformed exactly to the intricate contours on the luminal surface, indicating a tight mechanical association of these tissues. In addition, despite general separation of embryonic and maternal tissues, probably from fixation, some trophoblasts remained in contact with the epithelium or, more often, were ruptured, leaving embryonic membrane fragments attached to the epithelium surface. Mucopolysaccharides were present in trophoblasts and along the luminal surface of pregnant uteri but not evident within uterine epithelial cells. These results indicated adhesion of these tissues during late gestation, possibly related to transport and/or maintenance of embryonic orientation or location.
9. Physiological comparison of the two reproductive states, the luteal phase of the estrous cycle and gestation, generally supported the equivalence hypothesis of marsupial reproduction in the Virginia opossum. Specifically, daily peripheral estradiol and progesterone concentrations and E:P ratios were similar from estrus (Day 0) through Day 14. A significantly higher estradiol concentration on Day 2 of gestation might have been a response to breeding. Uterine constituents and ovarian and uterine histomorphology were also similar on Days 3, 7, or 11 of the estrous cycle and gestation. Differences in uterine glycogen concentrations (Day 7) and endometrial widths (Day 11) were attributed to biophysical responses to the expanding embryos. According to these parameters, marked alteration of maternal physiology due to the presence of the conceptus does not occur in the opossum.
APPENDIX A

METHODOLOGY

Radioimmunoassay

Two procedural alterations were incorporated into standard radioimmunoassay techniques during this study. First, the 0.1 M phosphate buffer required the use of Knox gelatin from freshly-opened packets. When gelatin from previously-opened packets was used in the estradiol (E₂) assay, antigen-antibody (³H-E₂) binding over total ³H-E₂ available dropped from 29 % to 14 % at an antiserum dilution of 1/16000 and 52 % to 27 % at 1/8000.

Second, binding in progesterone assays decreased significantly over the 3 to 4 minutes required to aliquot charcoal suspension into the 62 assay tubes. Initial zero tubes had 37 % binding (an approximate 6000 CPM difference). Binding was standardized when charcoal aliquot time was reduced to 1.5 minutes by two individuals dispensing the charcoal suspension (0.5 ml) from each end of the assay tube series and meeting at the middle tubes.

¹Knox Gelatin, Inc., Englewood Cliffs, NJ 07632
Blood Sampling

Serial blood samples were obtained consistently only by cardiac puncture. The anesthetized animal was restrained on a portable table with rubber tubing (Jurgelski, 1974) and the needle (21-ga, 1.5 in) was inserted between the third and fourth most posterior ribs 1 to 2 cm to the left of the sternum at an angle about 45° posterior to vertical and a depth of 2 to 4 cm. Blood pressure was not adequate to force blood into the syringe and slight negative pressure on the plunger was required to collect several milliliters. Anesthetization and blood sampling required 5 to 10 minutes per animal.

Fourteen animals (29%; N = 48) died within 24 hr of cardiac puncture, probably the result of direct trauma or secondary inflammation and septicemia. Postmortem examinations frequently identified the presence of Salmonella. However, 64% of this mortality occurred during the initial months of the study before sampling techniques had been refined.

Blood was obtained also from the mammary artery in the pouch of postlactational animals and from caudal veins. These locations were inadequate for daily serial samples because large hematomae formed in the pouch and caudal vessels in adults were difficult to locate. Superficial blood vessels in the limbs could not be located.
APPENDIX B

OVOTESTES IN THE VIRGINIA OPOSSUM

The primordial vertebrate gonad is ambisexual, containing both primary sex cords (presumptive seminiferous tubules) and germinal epithelium (presumptive ovarian cortex). In the developing mammalian ovary, the secondary sex cords proliferate, while the primary sex cords normally regress and either disappear or remain vestigial. After a 13-day gestation, sexual differentiation of the gonad of the Virginia opossum (*Didelphis virginiana*) had not occurred at birth, although by Day 5 postpartum, the gonad was distinguished histologically clearly as male or female (Morgan, 1943). The primary cords in the medulla of developing opossum ovaries remained unaltered by exogenous estrogen or androgen treatment from birth to Day 100 postpartum (Morgan, 1943). Hartman and League (1925) located primary cords in the ovotestis of a sex-integrad opossum; League and Hartman (1925) apparently misidentified primary cords as medium and mature anovular follicles in several ovaries. No investigator has indicated possible physiological effects of primary sex cords within the ovary or the reproductive consequence of their presence in the adult opossum.

Sixty ovaries from 33 adult opossums on known days of the estrous cycle or gestation were processed routinely for paraffin or frozen-section histology. Two females had primary tubules in the medullary portion of each ovary. In animal A, which was purchased from Florida, the tubules comprised less than 1% of the ovarian
volume 3 days postestrus. Both ovaries contained several corpora lutea (right = 44; left = 31) although the right ovary also had 14 luteinized follicles (Fig. 20). Circulating estradiol concentrations during the previous estrous cycle averaged $3.2 \pm 0.6$ pg/ml ($N = 4$). On the day of ovariectomy (Day 3), estradiol and progesterone were 12.9 pg/ml and 9.5 ng/ml, respectively.

Animal B was trapped in Ohio and ovarlectomized on Day 11 of the estrous cycle. She had given birth on the previous cycle, but the litter did not reach the pouch. Eight percent of the right ovary was composed of primary tubules, and less than 1% of the left ovary was primary tubules; the majority of each ovary was composed of large graafian follicles (Fig. 21), and mitotic figures were abundant in all paraffin-processed tubules (Fig. 22). Ovarian steroid levels during the previous cycle (Fig. 23) showed characteristic profiles of adults without ovotestis (Figs. 8 and 9). Mean estradiol concentration was $10.1 \pm 2.6$ pg/ml (range: 3.8 to 27.4 pg/ml) from estrus to 13 days postestrus, and the progesterone profile was unimodal with a peak value (18.3 ng/ml) on Day 9. However, during the 12 days before castration, the mean values of estradiol and progesterone were $18.7 \pm 6.9$ pg/ml and $0.4 \pm 0.1$, respectively.

The morphological and endocrinological alterations in these two opossums suggest a series of ovarian changes that might have been associated with primary tube proliferation. Initially, mitotically-active tubules composed a minor portion of the ovary and were associated with an increase in luteinized follicles. Estradiol and progesterone remained within normal levels. However, continued proliferation of
Fig. 20. Primary tubules (T) within the opossum ovary 3 days after estrus in animal A. Corpora lutea (CL), luteinized follicles (LF), and graafian follicles (G) also occurred (paraffin section; 8 μm; hematoxylin and eosin; 6.4 X).
Fig. 21. Graafian follicles (G) occurred in this ovary with primary tubules (T) from Animal B, 11 days after estrus. No corpora lutea were present (cryostat section; 15μm; hematoxylin and oil red 0; 6.4 X).
Fig. 22. Mitotic figures (arrows) in the primary tubules of the opossum ovary (paraffin section; 8 μm; hematoxylin and eosin; 560 X).
Fig. 23. Scattergram of peripheral progesterone concentrations in serum during gestation (○) and subsequent anovulatory cycle (□) and of peripheral estradiol concentration in serum during gestation (◆) and the anovulatory cycle (◇) from opossum B with an ovotestis.
Fig. 23.

Day Postestrus

Progesterone (ng/ml)

Estrogen (pg/ml)
the tubules terminated ovulations and luteinization resulting in post-
estrous graafian follicles, absence of corpora lutea, relatively high
estradiol concentrations, and low or undetectable progesterone
concentrations. Potentially, these changes might be induced by altera-
tions in the hypothalmic-pituitary-gonadal hormonal axis or by
reproductive senescence.
LITERATURE CITED


