Gestation and Placentation in Two New World Opossums: *Didelphis virginiana* and *Monodelphis domestica*

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ABSTRACT   Estrus, gestation, and placentation were studied in the Virginia opossum *(Didelphis virginiana)* and the gray short-tailed opossum *(Monodelphis domestica)*, representatives of the prototypic, New World marsupial family Didelphidae. *Didelphis* is a large (3,000 g), pouched opossum that exhibits a 28-day estrous cycle, spontaneous estrus and ovulation, and a high ovulation rate (60 ova per cycle). *Monodelphis* is a small (100 g), pouchless opossum with male-induced estrus and ovulation and a moderate ovulation rate (12 ova per cycle). Implantation occurs on day 10 of the 13-day gestation period in *Didelphis* and on day 12 of the 15-day gestation in *Monodelphis*. Gestation and placentation, including the ultrastructural features of trophoblastic apposition to the uterine mucosa, are very similar in *Didelphis* and *Monodelphis*. Differences, such as a larger increase in uterine volume and a more highly folded endometrium following implantation in *Monodelphis* are minor. They appear to be related to the smaller maternal body size and the relatively larger uterine load of embryos in *Monodelphis*. Because body size is one of the major variables among New World marsupials, the descriptions of gestation and placentation presented here for *Didelphis* and *Monodelphis*, provide a basis for comparison in future studies of didelphid marsupials.   © 1993 Wiley-Liss, Inc.

Marsupials and eutherians represent the two major evolutionary lines of extant mammals that diverged from common pantotherian ancestors during the Cretaceous, nearly 100 million years ago (Clemens '77). The distinctions between the mammalian Infraclasses Metatheria (marsupials) and Eutheria (all other viviparous mammals) are primarily reproductive. However, metatherians do not, as the name implies, represent an intermediate mammalian grade or a primitive state relative to a more advanced eutherian mode of reproduction (Renfree, '81; Hayssen et al., '85). Rather, they represent a distinct, alternative expression of the essential mammalian coupling of viviparity and lactation. As such, marsupials hold special significance in the study of mammalian reproduction that is further enhanced by their place in mammalian evolution and biogeography.

With the exception of the Virginia opossum *(Didelphis virginiana)*, which occurs only in the Nearctic Region, marsupials are found only in the Australian and Neotropical Regions. Only three of the 15 marsupial families are found in the New World, but together they (Didelphidae, Microbiotheriidae, and Caenolestidae) represent about 30% of the world's total of 250 species. Both North and South America have been regarded as the place of origin for marsupials. The oldest undisputed fossil marsupials (extinct members of Didelphidae) first appear in the mid-Cretaceous deposits of North America and late Cretaceous deposits of South America (80 million years b.p.) (Marshall, '82), long before the oldest marsupials (Oligocene) of Australia. The discovery of a marsupial fossil in Antarctica (Woodburne and Zinmeister, '82) strongly supports the notion of a southern dispersal route of South American marsupials into Antarctica and then into Australia prior to the Eocene separation of these Continents (Marshall et al., '87).

Didelphidae is an important family of marsupials. It contains more species ('70) than any other (Kirsch, '77); Macropodidae (kangaroos) with 60 species is second. Didelphidae is also the prototypic marsupial family. Ancestral marsupials of the late Cretaceous were didelphids very similar in dental morphology to the neotropical opossums of today. Therefore, the reproductive adaptations and patterns revealed in this group are viewed as primi-

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tive relative to other marsupials and, as such, they are of considerable interest to comparative reproductive biology.

THE MARSUPIAL MODE OF REPRODUCTION

Among the many differences in reproductive characteristics that exist between marsupials and eutherians, perhaps none is more absolute than that seen in the arrangement of the urogenital ducts in marsupials (Fig. 1). Ureters pass medial to the two lateral vaginae, which open posteriorly into a urogenital sinus. Following copulation, spermatozoa pass through the two separate lateral vaginae, cervices, and uteri on their way to the upper oviducts where fertilization takes place. Nothing similar to this morphology is seen in the eutherian reproductive tract, which is characterized by a single vagina and laterally located ureters. This difference represents a true metatherian-eutherian dichotomy (Tyndale-Biscoe and Renfree, '87).

During parturition marsupial fetuses take a surprising route to the urogenital sinus. Rather than follow the large lateral vaginal canals, as do spermatozoa following copulation, they pass through a centrally located pseudo-vaginal canal or birth canal (Fig. 1). This canal forms during parturition in the loosened connective tissue that lies between the vaginal culs de sac and the anterior end of the urogenital sinus. The canal closes after birth and epithelial layers of the culs de sac and urogenital sinus grow to cover the openings (Tyndale-Biscoe, '66; Tyndale-Biscoe and Renfree, '87).

Fundamental differences in reproductive tracts notwithstanding, the most striking and biologically important expression of the metatherian-eutherian dichotomy is seen in the relative length of gestation and the size and developmental stage of the neonate. For eutherians, time devoted to gestation approximates or exceeds, that required of the mother between birth and weaning of her litter (Glazier, '90). Marsupials, on the other hand, give birth to very small, embryonic neonates following a short gestation of negligible energy investment (Thompson and Nicoll, '86), which is followed by an extended period of lactation. For example, gestation in the Virginia opossum (Didelphis virginiana) lasts only 13 days but the young are not weaned until they are 100 days old. Many of the reproductive adaptations discussed in this paper, including placentation, are related to the fundamental brevity of marsupial gestation.

The birth weight of the marsupial litter is, on average, less than 0.09% of maternal body mass, compared to 15% or more for litters of eutherians. Compensatory time for development of marsupial young
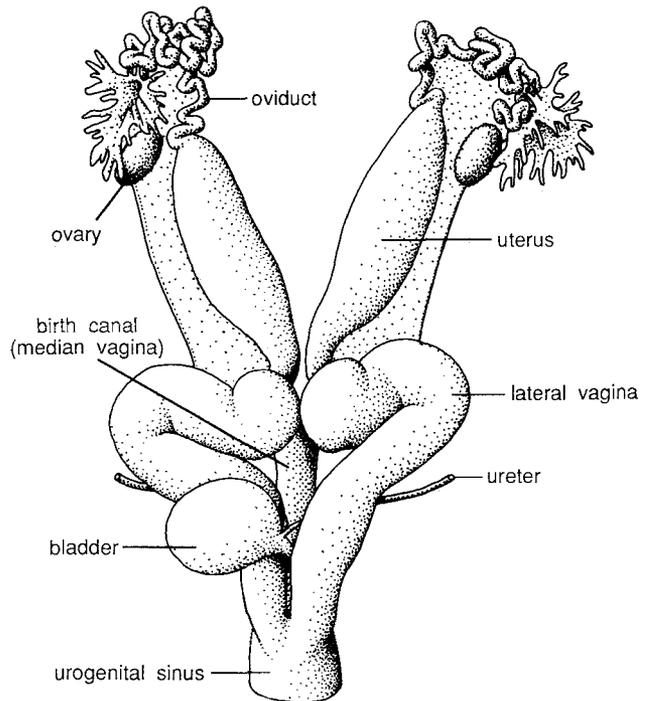

Fig. 1. The female reproductive tract of *Monodelphis domestica*. Drawing by Dave Dennis.

is provided during lactation, which averages about 40% longer than in eutherians of similar size (Hayssen et al., '85). Thus, for marsupials lactation is the dominant mode of energy transfer to the developing young and it is in marsupials that we see extensive development of this essential mammalian feature.

Discussion of gestation and placentation in New World didelphids is difficult because knowledge of most species is limited to geographic distribution and basic natural history. Until recently most reproductive studies of this largest of all marsupial families were focused on a single species, the Virginia opossum, *Didelphis virginiana* (hereinafter, *Didelphis*). Unfortunately, this large (1–3 kg) opossum is not a typical New World didelphid. Most (57/70) are small (50–300 g), pouchless species of the Neotropics, such as the gray short-tailed opossum, *Monodelphis domestica* (hereinafter, *Monodelphis*). Following the initial studies of Fadem et al., ('82), this small (60–150 g), pouchless species has become a popular and widely used marsupial for laboratory studies. Consequently, our understanding of reproduction in *Monodelphis* is rapidly approaching that in *Didelphis*, so that we now have the opportunity to consider variation and diversity in reproductive processes within the family Didelphidae.

Very little is known of the natural history or ecology of *Monodelphis (M. domestica)*. It is distributed

throughout the Caatinga region of southeastern Brazil, a semi-arid area vegetated with scattered small trees and shrubs, where it is most often trapped around mesic rock outcroppings (Streilein, '82). *Monodelphis* has omnivorous feeding habits (fruit, invertebrates, insects, and small vertebrates), similar to those of *Didelphis*. *Didelphis* is the largest of the didelphids and is found at higher latitudes (44 N) than any other New World marsupial. This terrestrial omnivore forages in brushy lowland areas during hours of darkness and retreats to a den (often an abandoned woodchuck burrow) during the day.

Didelphis and *Monodelphis* comprise a very small sample of Didelphidae, but comparisons between these species are instructive because they represent two natural groups of contrasting body size and pouch development. Body size varies by two orders of magnitude among New World opossums, a factor that was probably important in the evolution of the pouch and in the adaptive radiation in Didelphidae. Nowhere is this more evident than in the patterns of lactation and postnatal care.

Lactation in marsupials proceeds in two phases: the teat attachment phase and the nest phase (Fig. 2). Upon locating a teat, the neonate takes the teat into its well-developed oral cavity where it becomes firmly affixed. The teat is not voluntarily released during the first 60 days of life in *Didelphis* (teat attachment phase). Thereafter until weaning (90–100 days of age), young are left in the nest at night while the mother forages for food (nest phase). Small marsupials, such as *Monodelphis*, often lack a pouch and the teat attachment phase occupies less than one-third of the total lactational period, about 14 of 56 days in *Monodelphis* (Kraus and Fadem, '87) (Fig. 2). At the age didelphid young are left in the nest, they are thinly furred, ectothermic, and blind (Hunsaker and Shupe, '77). Under these conditions, a secure nest and maternal care is essential. The fact that female *Monodelphis* build larger and more complete nests than males (Fadem et al., '86) is of interest in this regard.

The pouchless condition is apparently primitive (Tyndale-Biscoe and Renfree, '87) and its persistence in small marsupials might reflect strong selection for leaving the young in a nest at an early age (Harder, '92). Young of small marsupials can equal up to 50% maternal weight by the time that they are left in the nest. By comparison, litters of larger, pouched species represent only about 20% of maternal weight by the time they are no longer carried by the mother (Russell, '82).
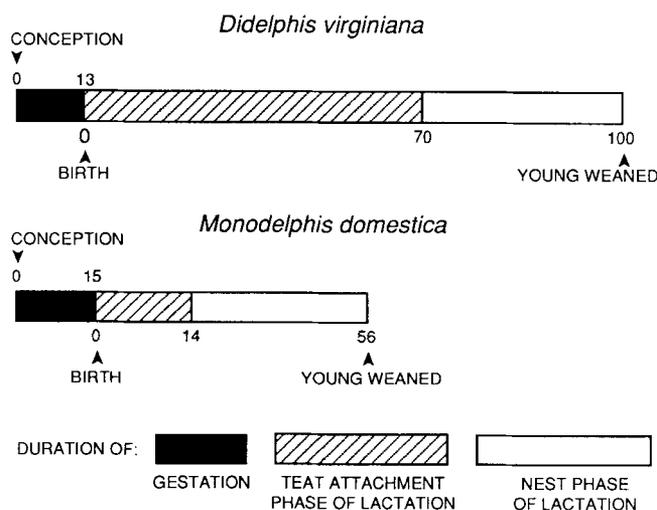

Fig. 2. Comparison of the temporal features (in days) of the reproductive cycles of *Didelphis virginiana* and *Monodelphis domestica*.

COMPARATIVE REPRODUCTIVE BIOLOGY OF DIDELPHIS AND MONODELPHIS

Estrous cycles, estrus, and breeding

Didelphis is seasonally polyestrous and polyovular with an estrous cycle length of approximately 28 days (Hartman, '23; Jurgelski and Porter, '74). If conception does not occur, females can cycle and ovulate spontaneously 6 or 7 times during the year, but restricted to January through July in northern regions. Females will also show post-lactational estrus within 5–14 days after removal of pouch young (Harder and Fleming, '82). Males make a smacking sound while cautiously investigating and nuzzling a female in estrus (36 hours in duration, Reynolds, '52). The male mounts the estrous female holding onto her hind legs with his feet; the two usually fall on their right side during the course of copulation (Hunsaker and Shupe, '77).

Following ovulation, corpora lutea (CL) develop from the ruptured follicles and secrete progesterone, which stimulates uterine secretory activity conducive to gestation. The luteal phase of the cycle (defined by elevated progesterone levels) follows ovulation and lasts about 11 days in *Didelphis* (Harder and Fleming, '81) and, in unmated females, it is followed by the follicular phase, leading to estrus and ovulation about 17 days later. The reproductive cycle of *Didelphis* is similar to that seen in a majority of marsupials in which the luteal phase occupies less than 60% of the cycle length (Tyndale-Biscoe and Renfree, '87).

Female *Monodelphis* do not exhibit a true estrous cycle. Barbara Fadem ('85) was first to show that

female reproductive activity was stimulated by male factors. Subsequent studies revealed that isolated females remain anestrous but will come into urogenital estrus and mate within 4–11 days after exposure to only male pheromones (Fadem, '87). Recent studies have shown that females will not reach puberty unless they are exposed to male pheromone (Stonerook and Harder, '92).

Estrus is routinely induced in female *Monodelphis* through a cage switching procedure in which the female is placed in a cage previously occupied by a mature male (Fadem, '87). Urogenital estrus is recognized as the first day that cornified epithelial cells predominate in smears collected daily from the urogenital sinus (Fadem and Rayve, '85). In our colony, urogenital estrus occurs an average of 5.3 ± 0.5 ($\pm$ SEM, n = 35) days after the first day of cage switching. Our time-lapse video recordings have revealed that first copulation occurs an average of 2.0 $\pm$ 0.2 days after pairing at urogenital estrus and lasts for 3 to 9 minutes (4.3 ± 0.2). Leukocytes are normally abundant in urogenital smears taken the morning after copulation, confirming the observations of Baggott et al., ('87). Approximately 86% (30/35) of copulations occur during the dark phase and 68% of these (20/30) occur within the first 4 hours after lights are off. Estrus in *Monodelphis* lasts 18 hours (Baggott et al., '87) to 36 hours (Trupin and Fadem, '82).

True (obligatory) male-induced estrus is unknown in marsupials other than *M. domestica*. There is certainly no evidence of it in *Didelphis*. However, a recent report of the stimulatory effect of male urine on the occurrence of estrus in woolly opossums *(Philander opossum)* (Perret and M'Barek, '91), suggests that such pheromonal effects should be examined in other neotropical marsupials.

Ovulation and conception

The ovulation rate in *Didelphis* is among the highest known for mammals. An average of 30 CL (range = 15–58) per ovary (Fleming and Harder, '83) indicates a release of 60 ova per cycle. Many of the ova are not viable or fertilized, as the total number of embryos counted in utero is about 22 (Hartman, '23; Reynolds '53). *Didelphis* ovulates spontaneously within 12–15 hours after the onset of behavioral estrus (Rodger and Bedford, '82a) and fertilization occurs very soon thereafter in the upper oviduct.

The early observations on *Monodelphis* (Baggott et al., '87) indicated that eggs were released about 18 hours after mating, but in a subsequent study, preovulatory oocytes were collected 18–24 hours af-

ter copulation and fertilized eggs from the ampulla of the oviduct 24–30 hours after mating (Baggott and Moore, '90). Ovulation in *Monodelphis* appears to be neither spontaneous nor induced, in the usual sense. Females brought to urogenital estrus by cage switching in our colony (pheromonal stimulus only) will not ovulate unless paired with a mature male (Stonerook and Harder, '92). However, Baggott et al. ('87) reported that proestrous females caged with males will ovulate even when copulation is prevented by removal of the male. Thus, it appears that some form of physical contact, short of intromission, is a necessary and sufficient stimulus for ovulation.

Although an average of about 22 embryos are observed in utero in *Didelphis,* fewer young are born, crawl to the pouch, and secure an unoccupied, functional teat. Litters of 12 to 14 are observed, but the average mid-lactational litter size in *Didelphis,* which ranges from 8.7 (Hamilton, '58) in the north to about 6.3 (Hartman, '28; Burns and Burns, '57) in the south, is considerably less than the normal number of teats (13). Apparently this is because the most anterior teats are not always functional (Reynolds, '52; Cutts et al., '78).

Unlike the pattern seen in *Didelphis,* the number of ova released at ovulation in *Monodelphis* does not greatly exceed the number of mid-gestational embryos or litter size (Table 1). The ovulation rate, as indicated by CL per female killed during gestation (n = 12), was 11.9 ± 0.8, which was not much higher ($P > 0.05$) than the number of embryos (9.8 ± 1.1) in the same females or even the average mid-lactational litter size in our colony, 7.1 ± 0.4 (n = 68). The maximum number of CL observed in our opossums was 16; Baggott et al., ('87) observed up to 14 ovulations per cycle in *Monodelphis*.

Baggott et al. ('87) estimated that fertilization occurs within 2 hours of ovulation in *Monodelphis,* because the fine mucin coat, which is deposited on the zona pellucida soon after ovulation by epithelial cells of the oviduct, appears to block fertilization. Rodger and Bedford ('82a) suggested that the early deposition of mucin on the zona in *Didelphis* might constitute a block to polyspermy and this might also be the case in *Monodelphis* (Phillips and Fadem, '87; Baggott and Moore, '90). Although spermatozoa are released from the epididymis in pairs (Phillips, '70; Krause and Cutts, '79; Temple-Smith and Bedford, '80), they separate in the oviduct just prior to fertilization (Rodger and Bedford, '82a,b), so that each oocyte is fertilized by a single spermatozoon.

TABLE 1. *Statistics on Reproduction and Development in* Didelphis virginiana *and* Monodelphis domestica

Characteristic	Didelphis	Monodelphis	References[1]
Body weights			
Adults (g)	1,000–3,000	60–150	Reynolds, '52; Cothran et al., '85
Neonates (mg)	130	106.8 ± 7.2[2]	Hartman, '28; Fadem et al., '82
Duration (days)			
Estrous cycle	29	0[3]	Fleming and Harder, '81a; Fadem, '87
Estrus to—			
Unilaminar blastocyst	4	5	McCrady, '38; Baggot and Moore, '90
Rupture of the shell membrane	10	11–12	Krause and Cutts, '85
Parturition	13	15.2 ± 0.3	McCrady, '38;
Lactation	100	56	Gardner, '82; Kraus and Fadem, '87
No. of—			
CL per cycle	60	11.9 ± 0.8	Fleming and Harder, '83
Embryos/female	22	9.8 ± 1.1	Hartman, '23
Litter size	8	7.1 ± 0.4	Holmes and Sanderson, '65
Teats	13–15	11–13	Robinson et al., '91

[1]The first reference given for any given characteristic refers to *Didelphis* and the second is for *Monodelphis*.
[2]All mean values ± SEM were obtained from observations of *Monodelphis* in this study.
[3]No true estrous cycle; estrus and ovulation are induced by male stimuli.

GESTATION AND PLACENTATION

Gestation period and early embryonic development

The length of gestation in *Didelphis* is remarkably short for a 2-kg animal. Two estimates, both based on the interval from observed copulation to parturition, are in close agreement: 12 days 19 hours (McCrady, '38) and 13 days ± 6 hours (Reynolds, '52). This is among the shortest gestation periods known for any marsupial. By comparison, gestation in the 300-g wooly opossum *(Caluromys philander)* lasts 24 days (Perret and M'Barek, '91); the longest gestation periods are seen among the diverse kangaroo group (Macropodidae), ranging from 21 to 26 days (Tyndale-Biscoe and Renfree, '87).

Based on time-lapse video recordings in our laboratory, the interval from copulation to parturition in *Monodelphis* is 15.2 ± 0.3 days (n = 16). This estimate of a 15-day gestation period in *Monodelphis* is equivalent to the 13.5 to 14 days between fertilization and parturition reported by Moore ('92), assuming an interval of 24–36 hours between copulation and fertilization.

One of the most unusual features of marsupial reproduction is the apparent lack of maternal recognition of pregnancy (Sharman, '70), at least among didelphids and other families in which the luteal phase occupies less than 60% of the cycle length (Type I cycle of Tyndale-Biscoe and Renfree, '87). All temporal, morphometric, and physiological aspects of the estrous cycle studied thus far in *Didelphis* remain unchanged during gestation (Hartman, '23; Renfree, '75; Fleming and Harder,

'81a,b). For example, gestation follows the course of the luteal phase such that parturition coincides with functional regression of the CL. A comparison of progesterone and estradiol profiles on equivalent days of the estrous cycle and gestation revealed no significant differences between the two reproductive states, except for a change in the estradiol to progesterone ratio at the time of parturition (Harder and Fleming, '81). If suckling is prevented by removal of young after birth, the estrous cycle progresses through the follicular phase leading to estrus at the expected time (Fleming and Harder, '81). This pattern stands in marked contrast to that in eutherian mammals in which pregnancy extends the life of the CL, interrupts the estrous cycle, and imposes alterations in the physiology of the mother.

Gestation, placentation, and embryonic development in *Didelphis* have been thoroughly examined, beginning with the work of Hartman ('16) and continuing to the electron microscopy of Krause and Cutts ('85). By contrast, *Monodelphis* has been under laboratory investigation for little more than a decade and gestation has received relatively little attention. The purpose of this section is to present our observations of placentation and uterine development during gestation in *Monodelphis* and to compare those with related descriptions of *Didelphis*.

Methods employed in our study were routine and will be described only briefly. Pheromonal induction of estrus was achieved by cage switching (Stonerook and Harder, '92). In accord with standard procedures (McCrady, '38; Fleming and Harder, '81a; Krause and Cutts, '85; Baggott and Moore,

'90) gestation was dated from the time of copulation (day 0) as documented in time lapse video recordings. Sexually mature females (n = 25) were obtained from a breeding colony in the Department of Zoology and scheduled 3 per day (except 4 on day 9 and 6 on day 13) for necropsy on days 3, 6, 9, 11, 13, or 14 of gestation. Volumes of freshly excised, pregnant uteri were obtained by displacement of saline. Uteri were then flushed with saline, to remove embryos, and fixed by immersion in buffered formalin and processed for standard hematoxylin-eosin histology. Uteri selected for electron microscopy were fixed by immersion or perfusion with glutaraldehyde and processed, with minor modifications, according to Enders and Enders ('69). Uterine gland density and the uterine epithelium index were obtained by line intersect sampling with a Merz grid (Merz, '67) of three uterine sections per female. Uterine gland density was recorded as the total gland tissue per line per field of view. The epithelium index, which reflects the surface area of the uterine lumen, was obtained as a ratio of the number of line intersects of epithelium over the number of line intersects of myometrium on the same section of uterus.

Early embryonic development in marsupials differs from the eutherian pattern in that the zygote does not cleave in the oviduct but moves rapidly to the uterus following deposition of the mucoid coat. Also, a unilaminar blastocyst develops directly without a morula stage and a bilaminar blastocyst forms with the differentiation of the endoderm (Tyndale-Biscoe and Renfree, '87; Selwood, '89).

The marsupial embryo is enclosed in a shell membrane, which Hartman ('16) concluded was secreted by the lower oviduct. However, subsequent studies have shown that shell glands are restricted to the endometrium (Hughes and Hall, '84) and that the shell membrane develops around the embryo during the first several days of uterine life (Baggott and Moore, '90). The shell membrane is a tough, transparent, proteinaceous, enzyme-resistant material (Hughes, '77) consisting of a mat of interwoven fibers (Krause and Cutts, '83). It is permeable to large molecules in the uterine fluid (Hughes and Shorey, '71). The shell membrane persists for at least the first two-thirds of gestation and isolates embryos from direct contact with maternal tissues. During this time the embryonic vesicles float free in the uterine lumen and are presumably nourished by uterine secretions which are rich in proteins, especially albumins and pre-albumins (Renfree, '75).

Embryos collected from Monodelphis on day 3 (after copulation) were primarily in the 8-cell stage

with an average diameter of 0.27 ± 0.05 mm (n = 15); unilaminar blastocysts (0.31 ± 0.03 mm diameter) were collected from females on day 6 of gestation. Our observations confirm the stages documented by Baggott and Moore ('90) in their detailed study (>200 embryos) of cleavage patterns and development during the first 6 days of gestation. The cleavage pattern seen in Monodelphis embryos is similar to that described for Didelphis (Hartman, '19; McCrady, '38), although McCrady ('38) observed the blastocyst stage somewhat earlier, following the sixth cleavage on day 4 of gestation.

Monodelphis embryos collected on day 9 of gestation were in the primitive groove stage. The embryonic vesicle, still enclosed in the shell membrane, had increased to 0.62 ± 0.04 mm, reflecting expansion of the blastocyst after day 6. Development of the mesoderm and the embryonic disc occurs between days 6 and 10 of gestation (Moore, '92). The stage of development seen in Monodelphis embryos on day 9 is equivalent to that expressed on days 7–8 of gestation in Didelphis (stages 17–18 of McCrady, '38).

The shell membrane was present and intact in nearly all Monodelphis embryos collected from 3 females on day 11 of gestation but it was absent from embryos collected on day 12 of gestation. Moore ('92) reported that breakdown of the shell membrane and superficial implantation in Monodelphis occurs 10.5 days after fertilization (11–12 days after copulation). Rupture of the shell membrane occurs on day 10 of gestation in Didelphis (McCrady, '38; Krause and Cutts, '85), an event in marsupials that marks the beginning of organogenesis and close contact between fetal and maternal tissues. At this time the well-vascularized yolk sac elaborates and, with the chorion, makes intimate contact with the uterine epithelium to form the yolk sac (choriovitelline) placenta (Krause and Cutts, '84a).

Our primary goal in the study of late gestation in Monodelphis was to examine placentation and uterine responses to direct contact with embryonic tissue following breakdown of the shell membrane on days 11–12. We did not record detailed observations of embryonic development in Monodelphis between days 12 (implantation) and 15 (birth). However, development followed the general pattern described for days 10 and 13 in Didelphis, stages 29–34 of McCrady ('38). For example the naso-oral groove and anterior limb buds were seen on day 12 of Monodelphis gestation and the tongue and distinct digits on forelimbs were evident on day 14.

Placentation

The so-called "marsupial-placental dichotomy" is false and misleading terminology when, in fact, marsupials and eutherians are united in possession of the placenta, which is defined as: "any intimate apposition or fusion of the foetal membranes to the uterine mucosa for physiological exchange" (Mossman, '37). Although the invasive, chorioallantoic placenta of eutherians differs markedly from the yolk sac placenta of many marsupials (Hughes, '74), the distinctions are not absolute and considerable variation in placental morphology may be seen within the two infraclasses.

The noninvasive yolk sac (or choriovitelline) placenta of didelphid marsupials is characterized by a small allantois that does not contact the chorion (Krause and Cutts, '84b). This type of placenta has two primary divisions: 1) the vascular yolk sac, consisting of trophoblastic cells (trophectoderm), vascularized mesoderm, and endoderm; and 2) the non-vascularized yolk sac, which is formed of trophectoderm and endoderm only (Krause and Cutts, '85). The margin of the discoidal vascular yolk sac is limited by the sinus terminalis (Fig. 3).

The yolk sac placenta of *Monodelphis* appears to establish a close apposition with the uterine mu-
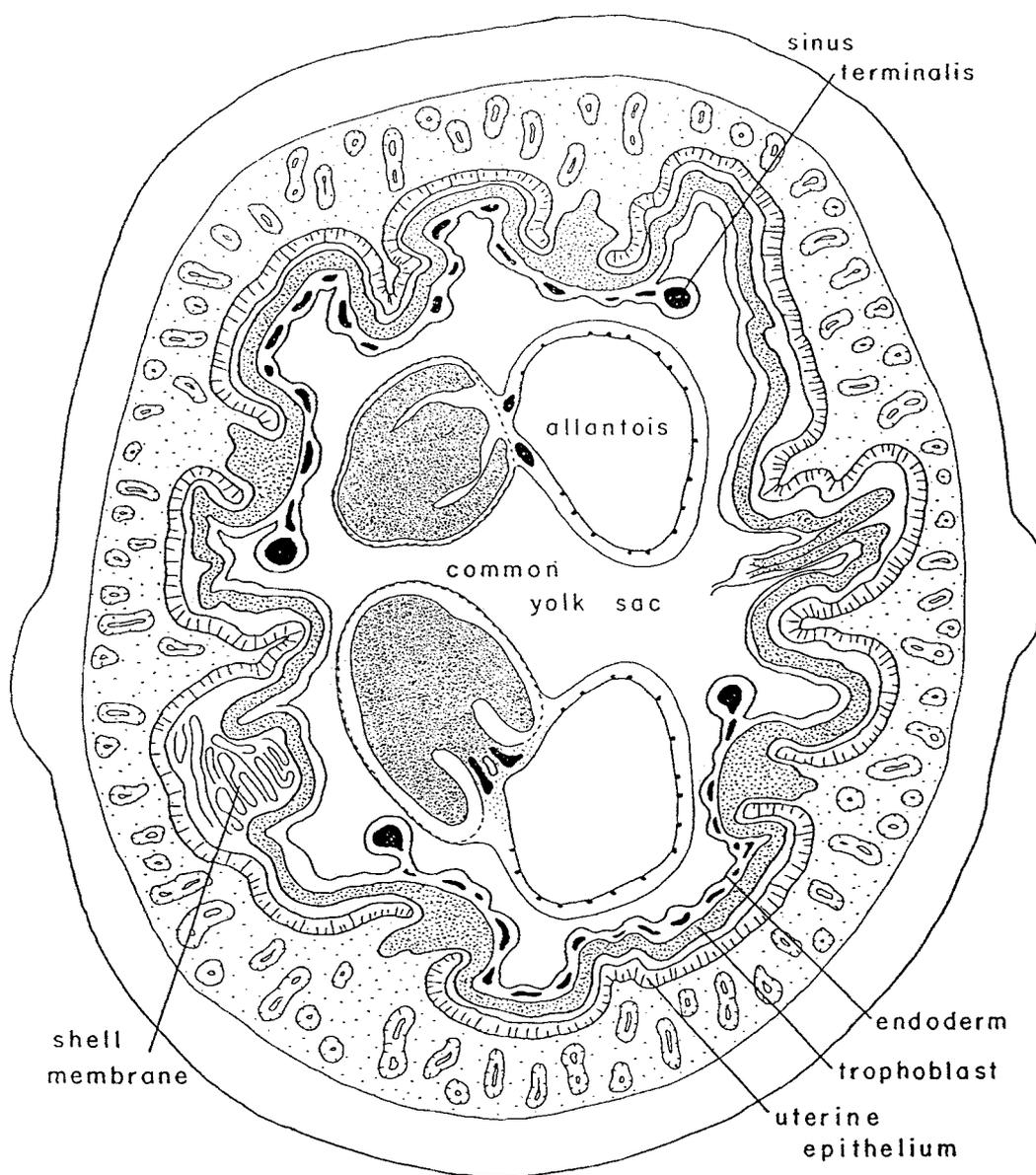

Fig. 3. Drawing of the placenta of *Philander opossum* (Reproduced from Enders and Enders, '69, with permission).

cosa immediately following break down of the shell membrane. The placentae of all embryos examined in day 12 of gestation (first day without the shell membrane) had already spread into the crypts of the highly folded uterine mucosa. Adhesion of the vascularized yolk sac membrane to the underlying uterine epithelium was apparent in fresh material collected on days 13 and 14 of gestation, and although it was possible to pull these tissues apart without apparent damage to them, fragments of trophoblastic cells were occasionally seen attached to uterine epithelium in histological preparations. Similar trophoblastic cell fragments were seen in preparations of day 11 *Didelphis* placentae, suggesting that adhesion of these tissues occurred during late gestation (Fleming and Harder, '81a).

Placentation on days 13 or 14 of gestation in *Monodelphis* follows the general pattern seen in the other two didelphids studied to date: *Didelphis* and the four-eyed opossum *(Philander opossum)*, a medium-sized (800 g) didelphid common throughout much of the northern neotropics. The vascular yolk sac is seen as a thin membrane that is usually applied to the convoluted folds of uterine mucosa. Fixation results in varying degrees of separation of these tissues, but juxtaposition is adequate in most sections from *Monodelphis* to reveal

conformation of trophoblastic cells to the rounded apical surfaces of epithelial cells (Fig. 4). A similar pattern is seen in *Didelphis* (Fig. 5) wherein epithelial cells make honeycomb-shaped impressions on the surface of the trophectoderm (Krause and Cutts, '85).

In electron micrographs of *Monodelphis*, microvilli may be seen on the opposed surfaces of the uterine epithelium and trophoblastic cells; however, they do not interdigitate and are occasionally separated by extracellular material (Fig. 6). A similar relationship has been observed in *P. opossum* (Enders and Enders, '69) and *Didelphis*, although microvilli are apparently absent from some of the trophoblastic cells in the latter (Krause and Cutts, '85).

Except for bandicoots (Peramelidae), marsupials are generally viewed as having a non-invasive placenta and exceptions are, therefore, worthy of note. Enders and Enders ('69) presented evidence that in the didelphid, *P. opossum*, trophoblastic giant cells along the margin of the vascular yolk sac penetrate the uterine epithelium and extend into endometrial stroma, sometimes reaching the basement membrane surrounding maternal blood vessels. These investigators also suggested that enlarged fibroblasts in regions of epithelial penetration might constitute a primitve decidual reaction. An even
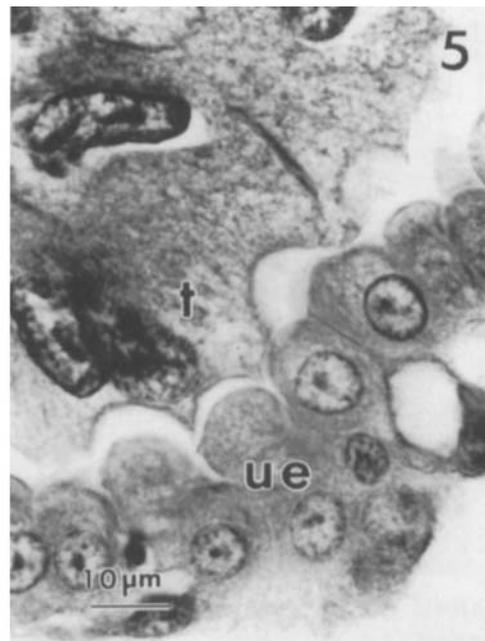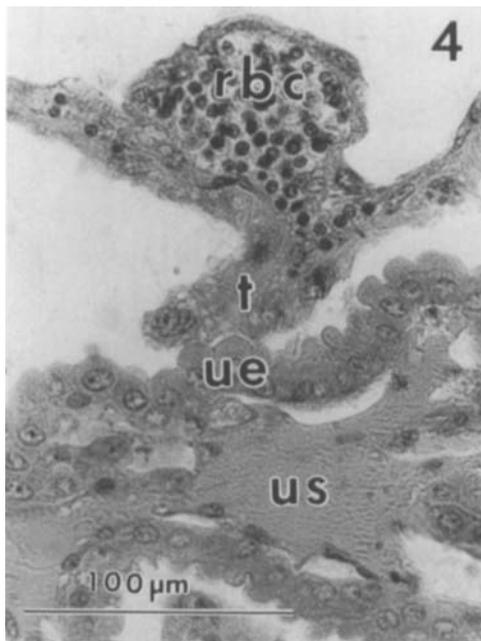

Fig. 4. Apposition of the trophoblast (t) to the rounded apical surfaces of uterine epithelial cells (ue) in *Monodelphis* on day 14 of gestation. The vascularized yolk sac placenta with red blood cells (rbc) in a vessel is shown. us, uterine stroma.

Fig. 5. Apposition of trophoblast (t) to the rounded apical surfaces of uterine epithelial cells (ue) in *Didelphis* on day 11 of gestation (photograph by M. Fleming).
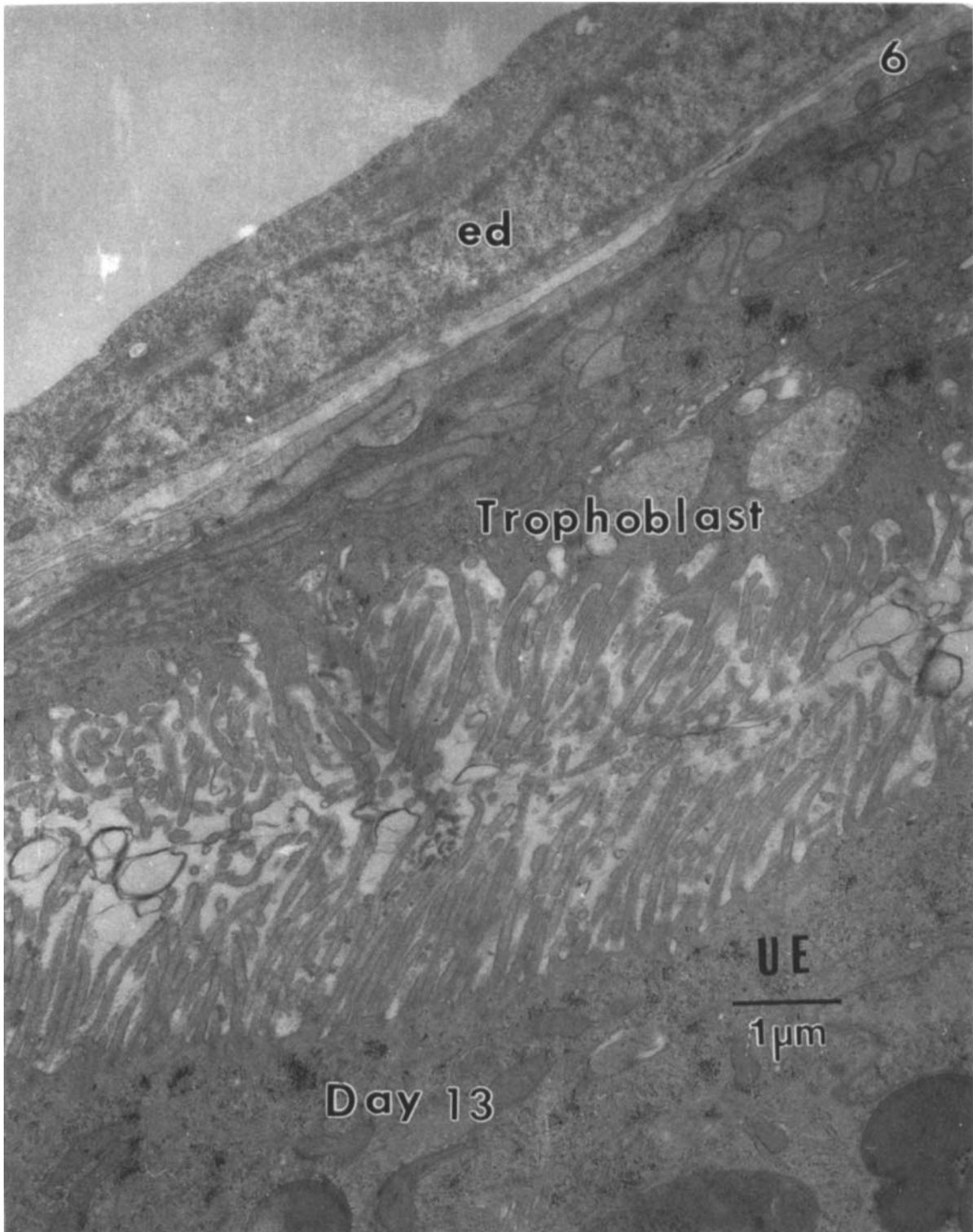
Fig. 6. Microvilli on the apical surfaces of trophoblast and uterine epithelium (ue) come into close contact but do not interdigitate in *Monodelphis* on day 13 of gestation; ed, endodermal cell.

more striking departure from the usual noninvasive nature of the yolk sac placenta (outside of Peramelidae) was observed in *Dasyurus viverrinus* by Hill (1900); trophoblast cells of the bilaminar yolk sac penetrate the uterine epithelium and form a syncytium which surrounds maternal blood vessels. We did not observe trophoblastic penetration of the uterine epithelium in *Monodelphis,* nor has it been observed in *Didelphis* (Fleming and Harder, '81a; Krause and Cutts, '85).

Embryonic growth proceeds rapidly during the period of organogenesis (shell membrane rupture to birth), an interval that is remarkably similar for two opossums of widely divergent adult body sizes, i.e., 4 days (maximum) in *Monodelphis* (100 g) and 3 days (maximum) in *Didelphis* (3,000 g). The time from shell rupture to birth is also shorter in these opossums than in most other marsupials, e.g., 8 days in the 5,000-g tammar wallaby *(Macropus eugenii)* (Renfree, '80). Neonatal weights for these species are 107, 130, and 370 mg, respectively, not much lower than the neonatal weight (740 mg) of the largest living marsupial, the gray kangaroo *(Macropus giganteus)* with an adult female body weight of 30 kg. These comparisons serve to emphasize the embryonic nature of the marsupial neonate and the heavy reliance placed on postnatal growth and development in this group of mammals.

Uterine changes during gestation

Uterine changes seen during gestation in *Didelphis* parallel those associated with the normal course of the estrous cycle (Hartman, '23; Renfree, '75; Fleming and Harder, '81a,b). However, *Didelphis* is the largest member of Didelphidae and conclusions based on size-related parameters, e.g., uterine volume, might not hold for some of the smaller members of the family. Therefore, a morphometric analysis of uterine factors seemed appropriate for this initial study of gestation in *Monodelphis.*

Uterine volume increased markedly during the course of gestation in *Monodelphis* with the largest increase evident following implantation on day 12 of gestation (Fig. 7A). Wet uterine weight (minus embryos and placentae) increased by an equivalent amount: from 0.38 ± 0.04 g on day 3 of gestation to 2.01 ± 0.32 g on day 14. Neonatal size in marsupials does not scale with maternal body size and, therefore, the relative load of embryos in utero varies with maternal body size. For example, 10 near-term *Didelphis* fetuses (130 mg each) are only about 0.04% of the maternal body weight (3 kg) or about 6.5% of uterine weight; while in *Mono-*
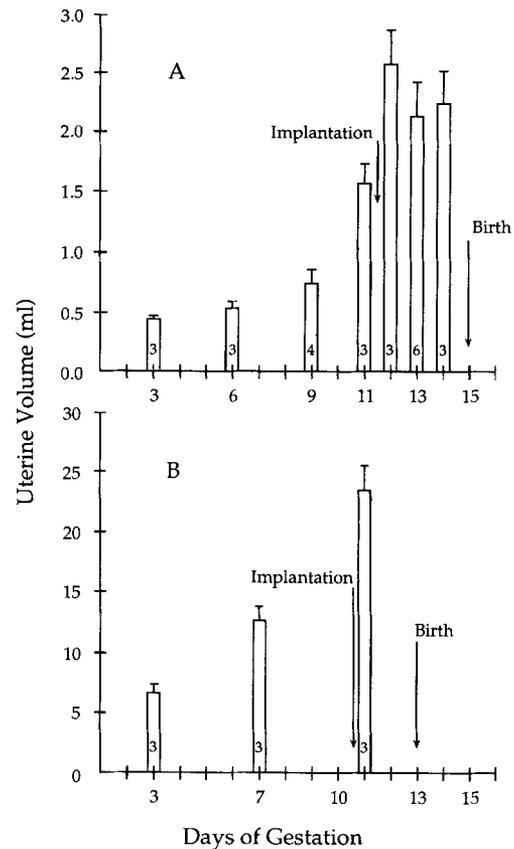

Fig. 7. Changes in uterine volume of *Monodelphis* (A) and *Didelphis* (B) during gestation.

delphis, the same number of 100-mg fetuses would represent a combined mass equal to 1.4% of maternal body weight (70 g) and 50% of uterine weight. The impact of the larger relative mass of embryos in small opossums is seen in uterine growth during gestation; uterine volume increased 5-fold in *Monodelphis* compared to only a 3.5-fold increase in *Didelphis* (Fig. 7B).

The relative abundance of uterine (endometrial) glands was markedly higher on day 3 of gestation in *Didelphis* (Fig. 8) compared to that seen in preovulatory females (Fig. 9). The index of uterine gland density remained high (70.1 ± 2.4) from day 3 through 9 of gestation in *Monodelphis* and then gradually declined ($P < 0.05$) to 53.6 ± 1.9 on day 14. Uterine gland density in *Didelphis* (Fleming and Harder, '81a) also decreased ($P < 0.05$) following implantation. The drop in uterine gland density in both species suggests that the nutritive role of the glandular epithelium declines during late gestation and is supplanted by secretions of the uterine epithelium (Fleming and Harder, '81a).

Pseudostratified columnar epitheilum covers the

Fig. 8. Section of a *Didelphis* uterus collected on day 3 of gestation showing distribution of glands in the endometrium (en). m, myometrium; ue, uterine epithelium.
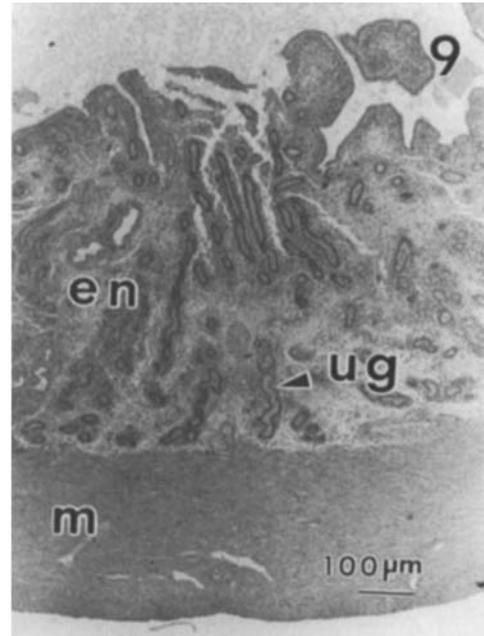

Fig. 9. Section of a nonpregnant *Didelphis* uterus collected during the early follicular phase (day 18) of the estrous cycle showing myometrium (m), endometrium (en), and scattered uterine glands (ug).

luminal surface of pregnant *Monodelphis* uteri prior to implantation. Ciliated cells are evenly distributed throughout this epithelium and appear to alternate with nonciliated cells (Fig. 10). A similar situation seems to exist in *Didelphis* (Krause and Cutts '85). Following implantation, the surface epithelium becomes cuboidal and lacks ciliated cells. Abundant rough endoplasmic reticulum and cytoplasmic inclusions indicate secretory activity in epithelial cells after implantation on day 12 (Fig. 6). An apical endocytotic complex on trophoblastic cells appears to sequester electron-dense material from the uterine lumen in *Didelphis* (Krause and Cutts, '85). The lateral and basal membranes of trophoblastic cells in *Monodelphis* show elaborate infoldings similar to those observed in *Didelphis* (Krause and Cutts, '85) and *P. philander* (Enders and Enders, '69). Such infoldings can be associated with absorption and transport of macromolecules through the trophoblast (Krause and Cutts, '85).

The sharp increase in endometrial-epithelial folds following breakdown of the shell membrane and implantation on day 12 of gestation in *Monodelphis* was the most striking histomorphological change observed in this study. The luminal surface of uteri collected on days 3 through 9 of gestation was variously folded in fixed cross sections (Figs. 11, 12),

but in all cases the folds were relatively thick and included uterine glands. By contrast, the lumina of uteri collected on days 13 and 14 of gestation were completely filled with deep, convoluted folds of thin endometrium. Embryos were found among these folds, each with an extensive elaboration of the yolk sac covering a circular area of folded endometrium.

The endometrial folds contained very thin, nonvascularized extensions of stroma overlaid with epithelium (Figs. 13, 14), but lacked uterine glands, which were concentrated instead in the basal region of the endometrium. An index of epithelial surface area increased from 1.66 on day 9 to 6.90 on day 13 of gestation, an increase of 316% (Fig. 15A). This marked increase in surface area following implantation might have been stimulated by contact with placentae following breakdown of the shell membranes or by other factors such as hormones of embryonic or maternal origin.

The increased folding of the endometrium following implantation presumably facilitates maternal-embryonic exchange. The folds that have been seen in late gestation in *Monodelphis* appear to be far more elaborate and extensive than what has been observed in *Didelphis* or *P. opossum* (Krause and Cutts, '85; Enders and Enders, '69). In fact, the epithelium index in *Didelphis* increased by only 126%
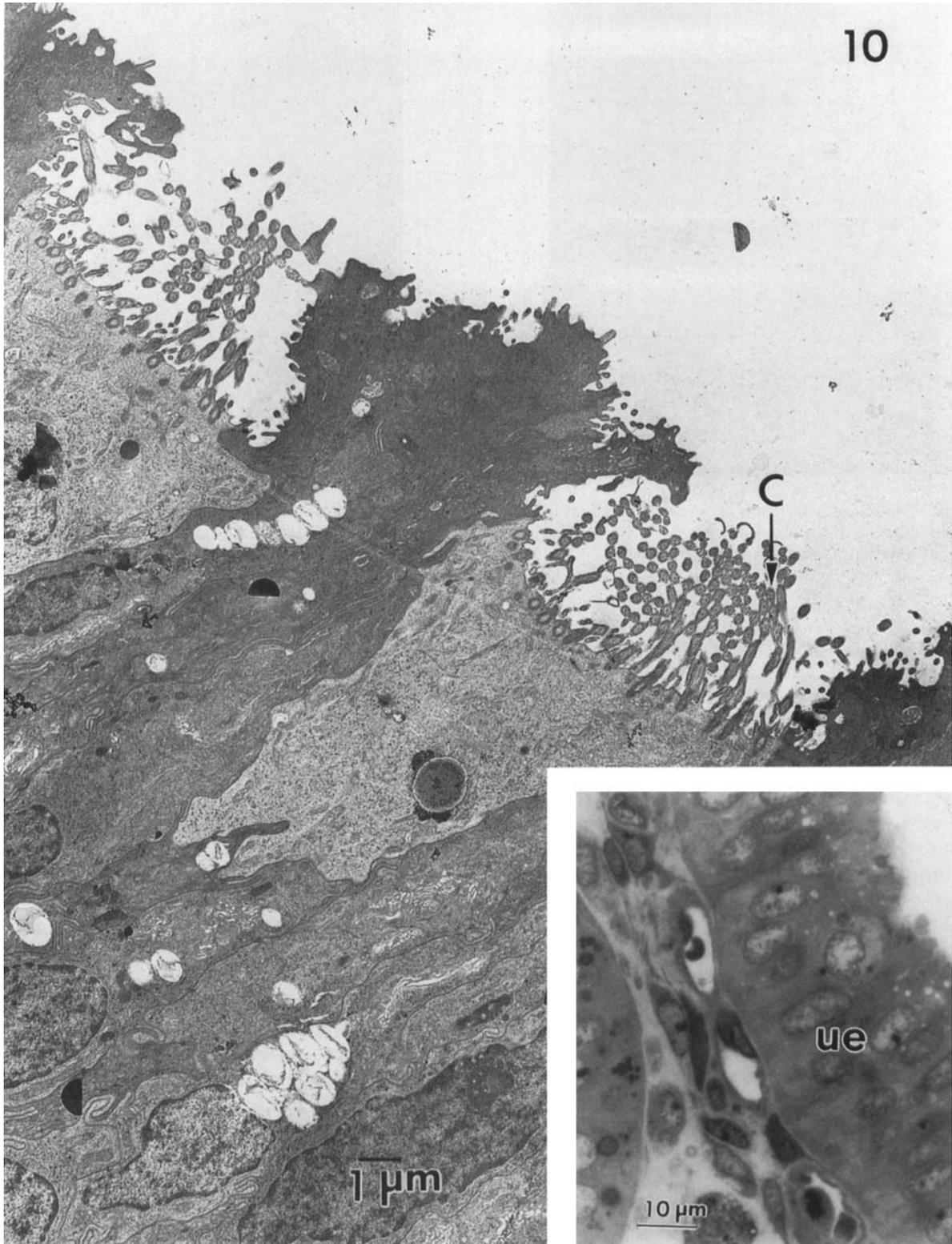
Fig. 10. On day 6 of gestation in *Monodelphis,* the uterine epithelium (ue, **inset**) is pseudo-stratified columnar. Ciliated cells (c) are abundant and appear to be spaced at regular intervals.
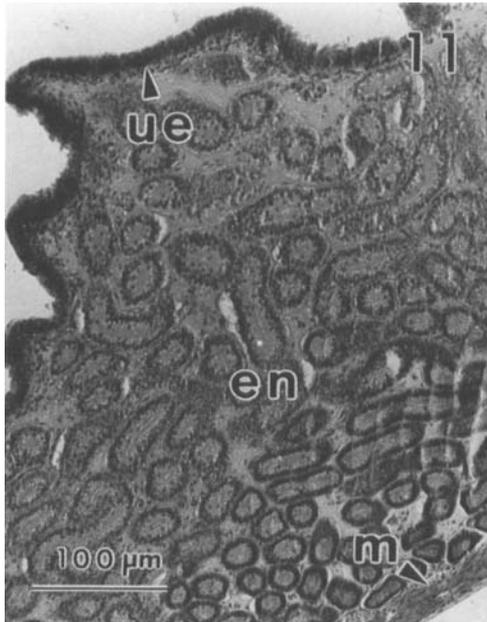
Fig. 11. Section of a *Monodelphis* uterus collected on day 6 of gestation showing myometrium (m), glandular endometrium (en), and relatively unfolded epithelium (ue).

Fig. 12. Section of a *Monodelphis* uterus collected on day 9 of gestation showing the maximum extent of endometrial folding observed prior to implantation on day 12. Uterine glands extend into these relatively thick folds.

Fig. 13. Section of a *Monodelphis* uterus collected on day 13 showing elaborate folds of uterine epithelium (ue) and closely apposed trophoblast (arrows).

following implantation (i.e., from 1.80 on day 7 to 4.07 on day 11, Fig. 15B) compared to the 316% increase in this index over the equivalent time period in *Monodelphis*. This greater increase in late term endometrial folding might reflect the relatively larger litter mass (relative to uterine mass) in *Monodelphis* and a need for greater surface area for physiological exchange.

CONCLUSIONS

Comparative reproductive biology of didelphid marsupials is compromised by the limited taxonomic breadth of studies conducted to date. Fortunately, the most thoroughly studied opossums, *Monodelphis* and *Didelphis,* represent two major groups within Didelphidae that differ markedly in body size, pouch development, and geographic distribution and, therefore, their reproductive patterns offer some insights into the extent of didelphid adaptive radiation. Understanding this is particularly important in light of the ancestral position of Didelphidae in marsupial evolution.

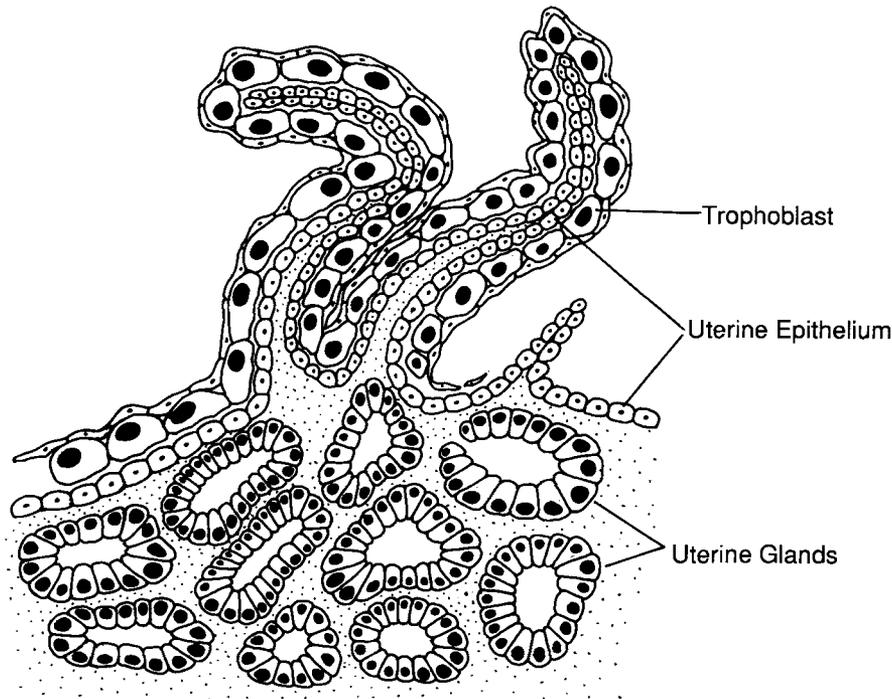*Didelphis* and *Monodelphis* differ most clearly in

Fig. 14. Drawing showing the relationship between the highly folded uterine epithelium and adjacent yolk sac placenta. Drawing by Dave Dennis.

the regulation of estrus and ovulation. *Didelphis* is seasonally polyestrous with spontaneous estrus and ovulation, while *Monodelphis* females are dependent upon male pheromone for estrus and upon some form of male stimulus for ovulation. The reasons and existence of these differences remain unclear, but they parallel those seen among eutherian mammals wherein pheromonal regulation of estrus and induced ovulation are most often interpreted relative to the socio-ecological characteristics of individual species. There are also significant differences between *Didelphis* and *Monodelphis* in ovulation rate and post-ovulatory loss of eggs that might reflect fundamental differences within Didelphidae in the regulation of oogenesis, follicular atresia, and ovulation.

The similarities between *Didelphis* and *Monodelphis* relative to gestation and placentation are far more apparent than are differences, and the differences are, for the most part, related to length of gestation and maternal body mass. The 2-day difference between the gestation period of *Monodelphis* (15 days) and that of *Didelphis* (13 days) is apparently established during the first few days after ovulation. The interval between copulation and fertilization is perhaps only about 12 hours longer in *Monodelphis* (Baggott and Moore, '90; Rodger and Bedford, '82a). However, the unilaminar blastocyst

stage is reached on day 4 in *Didelphis* (McCrady, '38) but not until day 6 in *Monodelphis* (Baggott and Moore, '90).

Placentation, seen as the close apposition of the choriovitelline membrane to the uterine mucosa, is very similar in *Monodelphis* and *Didelphis*. Trophoblastic cells conform and even adhere to the rounded apical surfaces of epithelial cells overlying the endometrium. In both species, the uterine epithelium appears to gain prominence in nutritive support of the embryo following implantation. The major difference between the two species is that the folding of the uterine mucosa is more extensive and elaborate in *Monodelphis* than in *Didelphis*. This difference and the larger increase in uterine volume during gestation are probably related to the larger relative mass and, presumably, physiological demands of near term *Monodelphis* embryos.

Didelphid marsupials are morphologically conservative. The general body plan, appendages, and dental morphology are remarkably similar across a wide array of species and body sizes. The comparisons set forth in this study suggest that conservatism among didelphids extends to the major features of gestation and placentation and that our understanding of these events in *Didelphis* and *Mononodelphis* might well apply to other members
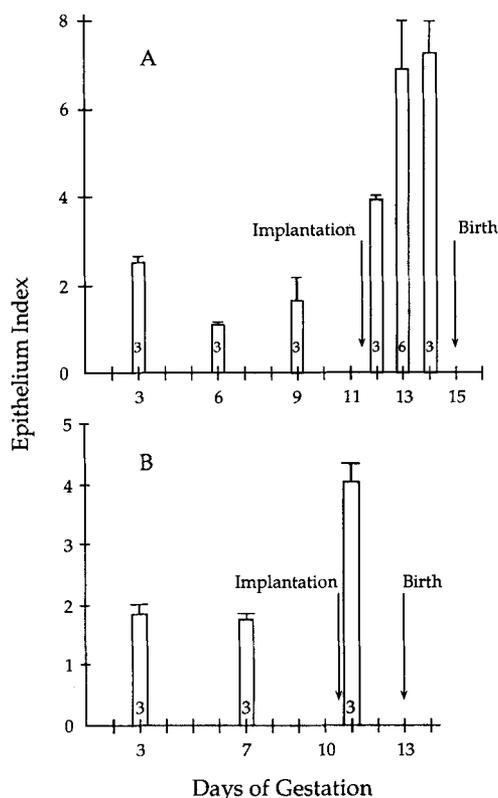
Fig. 15. Changes in the index of uterine epithelium surface area during gestation in *Monodelphis* (A) and *Didelphis* (B).

of Didelphidae. By contrast, the reproductive events that precede and follow gestation (e.g., estrus, ovulation, pouch development, and patterns of neonatal care) show considerable variation among didelphids examined thus far. Understanding of these and other reproductive processes in Didelphidae will require the study of many species representing the full array of environments encountered by marsupials in the New World.

ACKNOWLEDGMENTS

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