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A timetable of embryonic development, and ovarian and uterine changes during pregnancy, in the stripe-faced dunnart, *Sminthopsis macroura* (Marsupialia: Dasyuridae)

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Summary. Aged stages (63) were available for establishment of a timetable of embryonic development of the stripe-faced dunnart. On Day 0 oocytes reaching maturity were found in the ovary. Within ±24 h of time 0 (time of minimum morning weight) polymorphonuclear leucocytes appeared and spermatozoa were last detected in the urine of 70% of females. Embryos were collected at intervals during pregnancy by hemihysterectomy and the embryos in the contralateral uterus either were examined at a later stage of pregnancy or allowed to develop to term.

Cleavage to the unilaminar blastocyst stage with around 32 cells took 3 days with a cleavage arrest of 24 h at the 4-cell stage. Expansion of the unilaminar blastocyst occurred over the next 3 days. Primitive endoderm cells appeared on Day 6, fully bilaminar blastocysts by the end of Day 7 and trilaminar blastocysts on Day 8. Shell loss and implantation of 13–15-somite stage embryos occurred on Day 8 and organogenesis over the next 2–3 days. The gestation period was 9·5–12·0 days with most births occurring between 10·5 and 11·0 days.

Major steps in embryonic development were correlated with stages in the development of the corpora lutea, which were maximal in size, and possibly in secretory activity, when the embryos were at the bilaminar blastocyst stage. Regression commenced when the embryos were at the primitive streak stage. At the time the corpora lutea were maximal the uterine epithelium reached its greatest height and the endometrium was thick and folded. Later in pregnancy villous-like projections of the epithelium formed, and the luminal epithelial cells became rounded.

Two cell populations, a tier of 8 smaller cells above the yolk mass and a tier of 8 larger cells around the sides of the yolk mass appeared at the 16-cell stage. From the 16-cell stage to the blastocyst stage, with 150–200 cells, two cell populations distinguished by size, cell cycle time, cytoplasmic appearance and position relative to the yolk mass were present. The two populations were indistinguishable in blastocysts with >200 and <2000 cells. They reappeared in blastocysts with >2000 cells, as the darker cells of the embryoblast, and as the paler cells of the trophoblast. The darker cells lay in the yolky hemisphere and the paler cells in the non-yolky hemisphere.

Keywords: embryo; development; uterus; corpus luteum; marsupial

Introduction

Embryonic development in marsupials has characteristic features that distinguish it from development in eutherian mammals. Cleavage in marsupials begins in the uterus, and follows a precise and stylized pattern that varies between species, but which is always associated with elimination of yolk in the early divisions (Hill, 1910; Hartman, 1916, 1919; Selwood & Young, 1983; Selwood, 1986a). Because blastomere–blastomere adhesion is not a feature of early cleavage, no morula is formed.

Instead of the compaction and cavitation processes that occur in eutherians, blastocyst formation is characterized by blastomere–zona adhesion followed by blastomere–blastomere adhesion (Selwood, 1989b) with the development of cell junctions between blastomeres (Lyne & Hollis, 1977). The result is a unilaminar blastocyst with no inner cell mass and all cells forming a protoderm (McCrady, 1938) of apparently identical cells. The embryo is enclosed within a shell until just before implantation.

The rate of development of the various stages also has some unusual features. Comprehensive timetables of development of marsupial embryos have been prepared for only two species, the Virginia opossum, *Didelphis virginiana* (McCrady, 1938) and the brown antechinus, *Antechinus stuartii* (Selwood, 1980). For both species, studies of uterine and ovarian histology during pregnancy have also been made (Martinez-Esteve, 1942; Woolley, 1966a; Fleming & Harder, 1981). Less detailed timetables consisting of estimates of the duration of some stages of development have been prepared for the brush-tail possum, *Trichosurus vulpecula* (Hughes & Hall, 1984) and the tammar wallaby, *Macropus eugenii* (Tyndale-Biscoe & Renfree, 1987). A comparison of these timetables (Selwood, 1989a) shows that the pre-implantation period occupies 60–80% of gestation with implantation occurring at early somite stages in most marsupials examined. Cleavage to the 32-cell stage is twice as long (6–7 days) in some species as in others (3–$3\frac{1}{2}$ days). The relatively long cleavage period of 6 days in the brown antechinus is partly due to a temporary arrest of development for 3 days at the 4-cell stage (Selwood, 1980; Selwood & Young, 1983). It is not known for other species such as the tammar wallaby whether the long cleavage period of 7 days is due to a long cell cycle time or to a similar cleavage arrest. The length of the unilaminar blastocyst stage is the most variable, ranging from 4 to 30% of the gestation period. Sources of variation in the length of the unilaminar blastocyst stage are diapause and the time of appearance of the primary endoderm cells, which may occur soon after cleavage is complete or many generations of cells later (Selwood, 1989a).

In the present study a timetable of development has been prepared for the stripe-faced dunnart. Accurately aged embryos, from fertilization to birth, are described and the stages of embryonic development correlated with development of the corpus luteum and uterus. To prepare the timetable it was necessary to establish when ovulation occurred and to determine whether arrests were also a feature of development in this species.

Materials and Methods

The 36 stripe-faced dunnarts (*Sminthopsis macroura*) used in this study were obtained during the months of October, November and early December in 1980 and 1981 from a colony established and maintained by Woolley (1990). Reproductive condition was monitored as described by Woolley (1990). Females (body weight 17–29 g) were weighed (to the nearest 0·1 g) and a urine sample was collected twice each day (morning and late afternoon) throughout the oestrous and gestation periods and examined for the presence of cornified epithelial cells, polymorphonuclear leucocytes and spermatozoa (in females that had been mated). At the height of oestrus, when epithelial cells were present in the urine in large numbers and body weight had increased (see Fig. 3 in Woolley, 1990) a male was introduced into the female's cage. Once copulation was observed and/or spermatozoa detected in the urine of the female, the male was removed, usually after 1 or 2 days.

Embryos were collected on each day throughout gestation. By utilizing the divided condition of the marsupial reproductive tract it is possible to remove one side (ovary, oviduct and uterus) and either remove the other side later in the same pregnancy or allow the animal to give birth. This technique was used successfully to develop timetables of development for the Virginia opossum (Hartman, 1928) and brown antechinus (Selwood, 1980). Surgical procedures for the stripe-faced dunnart are described by Woolley (1990). Routinely, the right side of the tract was removed first. Generally, surgical removal of part of the tract was performed at intervals of 24 h after the time of morning assessment of reproductive condition. In a few cases when more precise timing was desirable, for instance at the start of development, surgery was performed between 5 and 12 h after the morning assessment. Of the 36 animals used in this study, 23 provided embryos from one side of the tract and those in the other side were allowed to develop to term; 12 provided embryos at two stages during the one pregnancy. Of the 23 hemi-hysterectomized animals, 10 provided embryos in a subsequent pregnancy.

Embryos, dependent upon the stage of pregnancy, were either flushed or dissected out of the uterus and placed in warm Dulbecco's phosphate-buffered saline. They were counted, examined and measured using a Wild inverted

microscope for embryos up to 0·5 mm in diameter, and a Zeiss stereo microscope for larger embryos. After fixation for 1–2 h in Karnovsky's solution counts were made of the number of cells in whole embryos up to the 32-cell stage, and in flat mounts (Clark, 1966) up to the bilaminar blastocyst stage, beyond which accurate counting became impossible. Further counts of cell numbers were made from sections of 16 embryos in early stages of bilaminar blastocyst formation. These embryos were embedded in plastic resin, sectioned at 1–1·5 μm and stained with toluidine blue. Whole mounts stained with haematoxylin and eosin were prepared of embryos at each stage of development.

The reproductive tracts (ovary, oviduct, uterus) were fixed and sectioned, and Graafian follicles, corpora lutea and tubal eggs were counted and measured, as described by Woolley (1990). The greatest width of the uterus (as shown in Fig. 7 in Woolley, 1990) was measured *in situ*. Transverse sections of the uterus were prepared from segments removed midway along the long axis of each uterus. The height of the epithelium lining the uterus and the endometrial glands was measured with a Leitz micrometer eyepiece calibrated against a stage micrometer. The uterine epithelium was measured in the thicker part of the endometrium (either mid-dorsal or mid-ventral surface of the uterus) and the gland epithelium in glands close to the uterine epithelium in the thick portion.

Results

Preparation of the timetable

At oestrus, a transient increase of 4·5–17·5% (mean 9·5, $n = 36$) in body weight was observed. Mating usually occurred when body weight was declining after the oestrous peak, and females would no longer accept a male once the body weight had reached a minimum after the oestrous peak (Woolley, 1990). Maturing oocytes were found on the day before the minimum weight was recorded after oestrus, and tubal eggs on the day of minimum weight. Ovulation therefore probably occurs on the latter day, which has been designated Day 1 of development, the day before being Day 0.

A timetable of development (Fig. 1) was prepared by matching the time of minimum morning weight (time 0) for all animals. The days when cornified epithelial cells and polymorphononuclear leucocytes appeared in the urine, when cornified epithelial cells declined in numbers and when the last spermatozoa were found in the urine were recorded on the appropriate day (Fig. 2) before or after time 0. These features have all been used as indicators of ovulation or start of development in this and other dasyurids (Selwood, 1980, 1987). The day of minimum morning weight was found to be the most accurate indicator of the day of ovulation; embryos from only 7 cases (see below) did not match the timing of 56 other cases so timed. The appearance of polymorphonuclear leucocytes and the last appearance of spermatozoa were valuable but less reliable indicators of the day of ovulation with about 70% of the animals showing these signs within 24 h of time 0 (Fig. 2). In 70% of animals cornified epithelial cells appeared 1–2 days before time 0 and declined in numbers on the 2nd or 3rd day after ovulation. The days when various embryonic stages were obtained, young born or other evidence of the day of parturition, such as weight drop, or red blood cells and uterine secretion in the urine (Woolley, 1990) were found, were also recorded (Fig. 2). The surgical procedures provided a possible 80 cases of embryos timed relative to day of minimum morning weight, another embryonic stage or parturition. Of these 63 were used for preparation of the timetable. Records were incomplete in 8 cases, 7 yielded unfertilized eggs and two cases where dead fetuses were found when animals were examined on a second occasion were not used.

Gestation period

Development was very rapid and gestation was completed during the 11th day in most animals (Fig. 2). When estimation of the length of gestation was based on time of observation of pouch young, the gestation period was 10·7 days ± 0·67 (s.d.), $n = 9$ (range 9·5–12 days). When no young were found, the time of parturition was based on the observation of other signs such as weight drop and the presence of uterine secretion or red blood cells in the urine (Woolley, 1990). Here the gestation period was 10·6 days ± 0·77 (s.d.), $n = 9$ (range 9·5–12·0 days).

The incidence of live young decreased as surgery was performed later in pregnancy. Of 10 pregnancies in which surgery was performed on Day 3 or before, 6 gave rise to young, whereas in 10

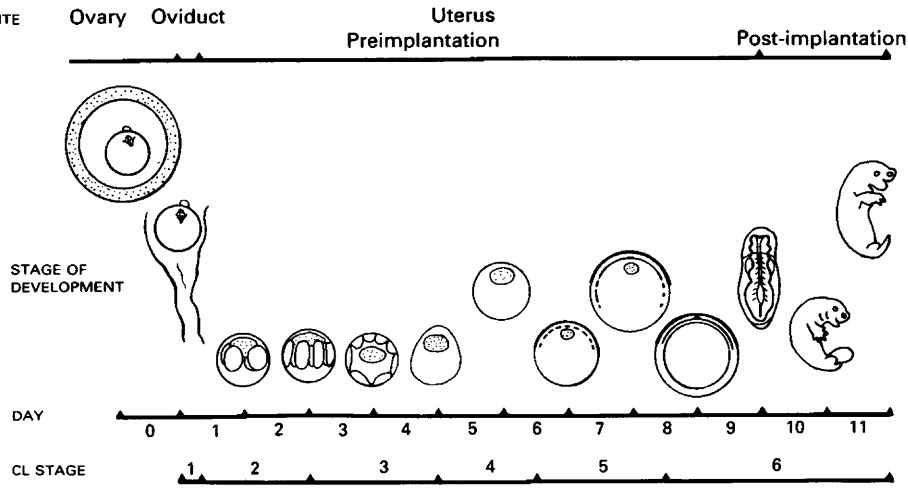

Fig. 1. Timetable of development of the stripe-faced dunnart matched with developmental stages of the corpus luteum.

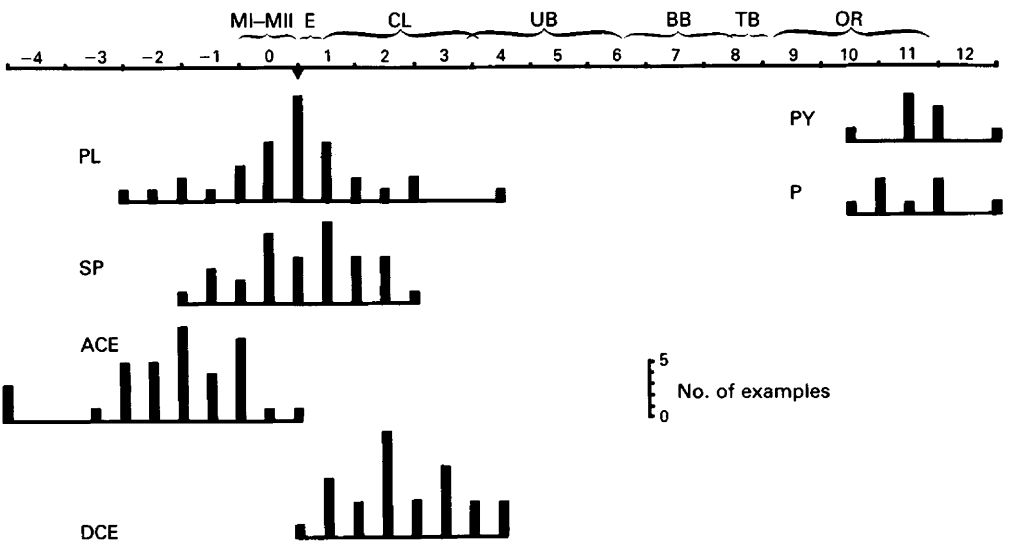

Fig. 2. Results of monitoring the reproductive condition of stripe-faced dunnarts during oestrus and pregnancy correlated with developmental stage. Time 0 (▼) is the time of minimum morning weight for the 63 cases that were used to derive the timetable in Fig. 1. The timetable is summarized here as stages of development (MI–MII = meiosis, E = eggs in oviduct, CL = cleavage stages, UB = unilaminar blastocyst, BB = bilaminar blastocyst, TB = trilaminar blastocyst, OR = organogenesis). The bars show the number of individuals on each day, at the a.m. or p.m. assessment of reproductive condition, in which polymorphonuclear leucocytes (PL) and cornified cells (ACE) first appeared, spermatozoa (SP) last appeared, cornified epithelial cells (DCE) started to decline in numbers, in the urine, and young (PY) were born or evidence was obtained that parturition (P) had occurred.

in which surgery was performed on Day 4 or later, only 4 gave rise to young. In 8 of 10 pregnancies in which two surgical interventions were performed, live, normal embryos were found on the second occasion. In the other two cases, in which the embryos were dead, they were collected on the estimated day of birth. Ovariectomy and hemihysterectomy did not appear to affect the rate of development as rates were similar whether or not there had been an earlier surgical intervention during pregnancy, but did appear to affect survival on the day of birth. The mean number of embryos (8·4 ± 2·8 (s.d.), $n = 10$) in the right uterus (which was removed first) was higher than but not significantly different from the mean number (6·3 ± 4·5 (s.d.), $n = 10$) in the left uterus. In 5 of these latter 10 cases, the uterus was removed on Day 11 and very low numbers of embryos were found, thus lowering the mean number of embryos.

Timetable of development

The number of animals examined on each day of development followed by the total number of oocytes or embryos obtained are indicated below in parentheses after the day number. For each day of development, the most advanced stage found is illustrated in Fig. 1. Day 0 represents the period from −24 h to time 0 and Day 1 represents the period from time 0 to +24 h and so on. Mean values for measurements are followed by the standard deviation.

Day 0 (2/33). On Day 0, oocytes reaching maturity were found in the ovary. In one animal, the oocytes were in various stages of prophase I of meiosis. Oocytes had an irregular, relatively yolk-free cortex containing the germinal vesicle and an internal mass of very yolky cytoplasm. They were covered on the antral side by at least 2–3 layers of cumulus cells. In the other animal, oocytes were at more advanced stages of meiosis from late prophase 1, to formation of the first polar body and metaphase II. Most prophase I oocytes had 1 or 2 layers of cumulus cells on the antral side of the oocyte and fine eosinophilic granules lying under the cumulus cells. Spaces were appearing between the zona pellucida and cumulus cells. As meiosis I progressed, eosinophilic granules increased in number and the oocytes became increasingly free of cells. Completely naked oocytes were found from telophase I to metaphase II.

Day 1 (12/134). Tubal eggs undergoing fertilization (3/30) were obtained from animals examined at 5 h, 7 h and 12 h after the morning reproductive assessment. At 5 h, eggs were found throughout the ampulla; at 7 h they were found from the ampulla to just on entry into the uterus; and at 12 h in the lower end of the oviduct. Fertilization occurred in the ampulla and appeared similar to that described for the brown antechinus by Selwood (1982) except that no incidence of polyspermy was found.

By the time the zygotes entered the uterus they were at the pronuclear stage; the yolk was polarised; and they were covered by a mucoid layer and a sticky shell (2/29). In one specimen examined at 12 h on Day 1 all the zygotes had entered the uterus. The zygote, which measured 198·9 ± 31·9 by 224·5 ± 13·6 μm ($n = 10$), was surrounded by a distinct perivitelline space. Further dimensions of the zygote and subsequent cleavage stages are recorded in Table 1. The zona width was irregular in early cleavage stages and so an average width for each egg was used to determine the mean. The membrane-bound yolk mass was oval in shape and the measurements given in the table are the average of 2 diameters.

Animals examined at the end of Day 1 had zygotes in which the yolk mass was separating from the remainder of the cytoplasm (2/19), 2-cell embryos (2/20) and 2–4-cell embryos (3/36), indicating that the first two cleavage divisions were completed during Day 1. Cleavage up to formation of the unilaminar blastocyst with about 32-cells has been described in previous in-vitro studies (Selwood, 1986b, 1989b) and will, therefore, be summarized here and not described in detail, except for features not previously documented.

Day 2 (4/35). Embryos were found in the rounded separate 4-cell stage (2/16), the late 4-cell stage with cells flattened in preparation for the next division (1/13) and undergoing the next meridional division (1/6) to the 8-cell stage. The total diameter of the embryo had increased by the

Table 1. Dimensions (mean ± s.d.) of live embryos of the stripe-faced dunnart during cleavage

Stage	No.	Diameter (μm)		Width of investments (μm)			Diameter of yolk mass (μm)
		External	Internal	Shell	Mucoid layer	Zona pellucida	
1-cell	10	289·1 ± 5·8	267·7	1·4 ± 0·3	7·9 ± 1·9	1·4 ± 0·4	—
2-cell	6	291·1 ± 3·4	258·7	2·0 ± 0·27	11·1 ± 0·2	3·1 ± 0·27	153·0 ± 7·6
4-cell	6	328·6 ± 8·1	302·2	2·5 ± 0·9	8·0 ± 3·8	2·7 ± 1·3	154·3 ± 16·8
8-cell	11	335·3 ± 8·4	309·1	3·1 ± 1·3	7·7 ± 3·8	2·3 ± 0·7	134·4 ± 12·1
16-cell	8	335·7 ± 6·6	309·3	2·4 ± 0·9	9·0 ± 1·0	1·8 ± 0·2	147·5 ± 5·4
32-cell	6	337·9 ± 10·0	313·3	1·9 ± 0·4	7·5 ± 1·9	2·9 ± 1·1	144·3 ± 10·4
64-cell	5	356·8 ± 15·0	342·2	1·9 ± 0·4	3·7 ± 1·7	1·7 ± 0·0	140·0 (av.)

beginning of Day 2, due partly to increase in width of the shell and zona layers but also to expansion within the zona pellucida (Table 1).

Day 3 (5/56). Embryos with 4–8 cells were found early on this day (2/20). One of these animals had embryos at the rounded 4-cell stage 24 h previously, suggesting that the 4-cell stage lasted about 24 h. The fourth (2/24) and fifth (1/12) divisions were also completed during this day. During cleavage the yolk mass showed a slight decrease in size (Table 1). The fourth division was unequal and latitudinal and produced two tiers, each of 8 cells.

Towards the end of the latitudinal 5th division the wall of the blastocyst was completed, so that all the zona pellucida was lined by flattened cells. Usually, the zona pellucida in the immediate vicinity of the yolk mass was the first to be completely lined, by the smaller cells of the upper tier, whereas the zona pellucida opposite the yolk mass was the last part to be lined by the larger cells of the lower tier. When cell numbers were counted in sectioned embryos 10 incomplete blastocysts had 22–32 cells and 6 complete blastocysts had 28–38 cells. Completion of the blastocyst wall and expansion of the blastocyst was associated with expansion inside the zona and compression of investments, particularly the mucoid layer (Table 1).

Day 4 (4/27). During Day 4 expanding, spherical, unilaminar blastocysts with two populations of cells, based on counts of cells in whole and flat mounts (Table 2) were found. Small dark-staining cells were associated with the yolk and larger paler-staining cells were found in the non-yolky hemisphere. Occasionally dark, obviously degenerate, rounded cells were found lying in the blastocoele in addition to a yolk mass measuring 132–168 μm. In larger 'egg-shaped' specimens no obvious differences were found between the cells of the blastocyst (Table 2). A small number of cells in mitosis was found in both types of cells in blastocysts with >60 cells. The number of cells in mitosis, together with the relative numbers of dark and pale cells, suggested that, from the 32-cell stage, the dark cells of the upper tier were dividing more slowly (24 h/cycle) than the pale cells of the lower tier (8 h/cycle).

Day 5 (3/30). Blastocysts retained the 'egg-shape' during most of the 5th day but began to round up at the end of this day. Cell numbers increased to about 1000 (Table 2) and the yolk mass measured 94·9 ± 17·9 μm ($n = 5$). All the cells of each blastocyst were uniform in appearance throughout this day.

Day 6 (3/16). The primitive endoderm cells first appeared on this day after blastocysts expanded further and became spherical in form (Table 2). All 3 uteri examined on this day contained a mixture of 'egg-shaped' blastocysts, similar to those on Day 5, and spherical blastocysts of two types. In smaller spherical blastocysts no differences were found between cells in different parts of the blastocyst, either in whole specimens, living or fixed, or in whole mounts. In larger spherical blastocysts two equally abundant cell types, dark and pale, separated by a sutural line were seen in stained whole mounts, and in some whole specimens. The major difference between the two cell types was in the intensity of staining and amount of granular material in the cytoplasm. The changes in blastocyst shape and relative numbers of dark and pale cells on Days 4, 5 and 6 suggest

Table 2. Blastocyst characteristics and cell numbers during expansion of the unilaminar blastocyst to the time of appearance of the primitive endoderm cells

	Blastocyst characteristics					
				Cell numbers		
Day	Shape	No.	Size (mm)	Total	Embryoblast (Dark cells)	Trophoblast (Pale cells)
4	Round	5	0·37 ± 0·1	36–66	16–19	20–47
	Round	12	0·41 ± 0·04	135–141	30–34	105–107
	'Egg-shaped'	6	0·43 ± 0·10 0·53 ± 0·11	224	Not distinct	Not distinct
5	'Egg-shaped'	8	0·68 ± 0·04 0·58 ± 0·04	224–356	Not distinct	Not distinct
		5	0·77 ± 0·09 0·85 ± 0·07	351–986	Not distinct	Not distinct
	Round	5	0·82 ± 0·03	579–895	Not distinct	Not distinct
6	Round	9	0·98 ± 0·16	1015–1697	Not distinct	Not distinct
		8	1·25 ± 0·06	2207–2481	739–1274, primitive endoderm cells appear	1408–999

that, on Day 4, when the pale cells were dividing faster, the part of the wall occupied by them expanded to form the wider part of the 'egg-shaped' blastocyst. The blastocyst rounded up late on Day 5 because the increase in number of the dark cells, until they equalled the number of pale cells, caused the pointed end of the 'egg-shaped' blastocyst to expand. The dark cells were the cells of the embryonic area or embryoblast and the pale cells were the extraembryonic cells or trophectoderm. In the larger blastocysts primitive endoderm cells were being proliferated. They were found in the hemisphere of dark cells in one blastocyst with 2481 cells at three sites where 9, 30 and 60 endoderm cells were present. One cell was also rounding up at each of 5 other sites. Primitive endoderm cells became more intensely stained, rounded up and bulged into the blastocoele before separating from the overlying cells. The cells above and in the immediate vicinity of the endoderm cells became rectangular in shape, more intensely basophilic and had no visible nucleoli. They form the primitive ectoderm or epiblast. In another blastocyst with 2865 cells, primitive endoderm cells were developing at one site but no differentiation of the embryoblast cells could be detected in this specimen and it was not possible to distinguish pale and dark cells.

Day 7 (2/35). The primitive endoderm cells continued to proliferate during the 7th day to form an incomplete lining of the blastocyst. Of the 2 animals examined on this day, one had mostly oval blastocysts, 0·9 × 1·4 mm diameter, with 1500–2200 cells. These blastocysts showed early stages of endoderm cell proliferation and transformation of embryoblast cells into primitive ectoderm cells. The other animal had mostly rounded blastocysts, 1·75–2·0 mm (1·93 ± 0·09 mm, $n = 10$), with a distinct rounded or oval embryoblast. When a yolk mass was visible, it was associated with the embryoblast or with the hemisphere containing the embryoblast. At this stage all the remaining external embryoblast cells had transformed into primitive ectoderm cells. Under the primitive ectoderm, endoderm cells formed a continuous layer of flattened cells, and outside the embryoblast area, endoderm cells extended as a discontinuous layer or reticulum under the trophectoderm to just past the equator of the blastocyst. Several sites of primitive endoderm cell proliferation, with 3–20 rounded cells at each site were found near the free margin of the cell reticulum. Isolated primitive endoderm cells were found under the remainder of the trophectoderm. It was impossible to count accurately cell numbers in these specimens, but estimated cell numbers in two were about 4500 and 7500 respectively.

Day 8 (3/23). The bilaminar blastocyst was completed during the first half of this day. Complete bilaminar blastocysts ranged in size from 2·2 to 3·0 mm in diameter. These blastocysts

were fully lined by primitive endoderm cells, although the lining of the hemisphere opposite the epiblast was in the form of a reticulum rather than a continuous epithelium. A yolk mass with an average diameter of 7 μm was visible in the smaller blastocysts but not in the larger ones. As the blastocysts increased in size, the embryoblast changed from a circular structure with a diameter of 1·5 mm to an oval structure up to 1·6 × 1·8 mm. In the epiblast of these most advanced blastocysts, greatly increased cell densities were found in the central area as a precursor to primitive streak formation.

The trilaminar blastocyst developed during the second half of the 8th day. The primitive streak was well defined in a pear-shaped epiblast between 1·9 and 3·0 mm in length. These blastocysts ranged in size from 3·2 × 3·6 mm to 3·5 × 3·8 mm and the mesoderm had reached the edge of the epiblast. The primitive endoderm had formed a continuous epithelium lining all parts of the blastocyst.

Day 9 (1/9). The earliest stage seen of somite development was found in 9 blastocysts from one female. The somites were difficult to count in some specimens and the estimate varied between 13 and 15. Each embryo was flat, with a greater length varying between 3·9 mm and 4·8 mm. The heart, in the form of a single external tube, was beating. The head, which was covered by the head fold of the amnion in the more advanced specimens, was recessed into the blastocyst. The neural canal was closed anteriorly except for the neuropore, and open posteriorly behind the level of the heart. A primitive streak was present at the posterior end of the embryo and the tail fold of the amnion was beginning to develop. The embryo had three pharyngeal arches. The lens and otic placodes were visible in one specimen, and fore-limb folds were present in all. These blastocysts were without a shell and were in the early stages of implantation, although all could be detached easily from the uterine endometrium. The blastocysts tended to collapse when removed from the uterus and the diameter varied between 3·5 and 4·5 mm. The sinus terminalis was developed at about the level of the equator of the blastocyst. It formed the boundary between the vascular trilaminar yolk sac, within which lay the embryo, and the bilaminar yolk sac, which was adhering to the uterine endometrium.

Day 10 (1/4). The next stage was seen in 4 embryos with crown–rump (CR) lengths of 2·0, 2·8, 3·0 and 3·0 mm. These embryos were completely lifted off the yolk sac and lying on their sides. Each was fully enclosed within the amnion and had a small allantoic bud, 0·8–1·0 mm in diameter. In the smallest embryo, the fore-limb bud was paddle-shaped and the hind-limb bud was a ridge. In the 3 largest specimens, the fore-limb bud had 5 digits, and the hind-limb was paddle-shaped. Two pharyngeal clefts could be seen and the heart was beating. The mouth was open and the eye and ear primordia were visible.

Day 11 (4/13). Embryos with CR lengths between 3·4 and 4·9 mm and head lengths (HL) between 1·3 and 1·9 mm were found early on this day. The tongue was protruding and the nostrils prominent. No pharyngeal arches were visible externally. A pronounced cervical swelling was present. The forelimbs had 5 distinct clawed digits and the hind limb buds were paddle-shaped. The tail was becoming elongated. The allantois was about one-third the length of the embryo and had a long stalk.

The most advanced stage in embryonic development was found later on this day in 4 embryos with CR lengths of 5·2, 5·2, 5·3 and 5·4 mm and a HL of 2·0 mm for all specimens. These embryos were very active and the constant flexing of the body made the CR length measurement difficult to obtain in live specimens; those given represent the embryo in its fully flexed stage. The allantois was about two-thirds or more the length of the body. The eyes, which had a faint ring of retinal pigmentation, nostrils and sides of the mouth were covered by the epitrichium.

Young were born during this day. Except for the absence of the extra-embryonic membranes and the loss of the epitrichium the form of the young was similar to that of the most advanced embryonic stages. CR lengths were 4·02 ± 0·5 mm (n = 5) (range = 3·4–4·7) in fixed specimens.

Corpus luteum

Formation of the corpus luteum was slow, with maximal size being reached about half way through the gestation period when the embryos were at the bilaminar blastocyst stage. Regression began soon after, when the embryos were at the primitive streak stage. Six stages in formation and regression could be recognized, each stage being correlated with a new phase of differentiation of the embryos. The length of each stage in days is shown in Fig. 1.

Stage 1. Mean diameter of corpora lutea: 234–323 μm
Embryonic stage: tubal and undivided uterine eggs

Following release of the ovum the Graafian follicle decreased in size (Woolley, 1990). The site of rupture of the follicle, but no extravasated blood, could be seen (Fig. 3a). Both the thecal and granulosa cell layers were thickened and the antral cavity reduced. Cellular debris was sometimes present in the cavity and among the granulosa cells, the boundaries of which were indistinct and the nuclei crowded (Fig. 3b). Mitotic figures were not visible in the thecal or transformed granulosa cells and, contrary to the observation by Godfrey (1969), no polymorphonuclear leucocytes could be seen in the cavity. Vascularization appeared to be limited to peripheral vessels lying in the theca.

Stage 2. Mean diameter of corpora lutea: 235–389 μm
Embryonic stage: early cleavage.

During early cleavage, luteal (transformed granulosa) cells, the boundaries of which remained indistinct, filled the central cavity (Fig. 3c, d). This process was usually completed by the 4-cell, and always by the 8-cell, embryonic stage. Mitotic figures, probably in cells of thecal origin, were seen only rarely.

Stage 3. Mean diameter of corpora lutea: 304–403 μm
Embryonic stage: late cleavage to early blastocyst

The boundaries of the luteal cells became distinct and clusters of cells were formed as thecal elements irrupted and moved progressively towards the centre of the corpus luteum (Fig. 3e, f). Hypertrophy of luteal cells occurred and again mitotic figures, probably in cells of thecal origin, were seen only rarely. Blood vessels penetrated between the clusters of luteal cells. The irruption of thecal elements may begin in individuals with embryos at the 2-cell stage but in the majority it did not occur before the embryos were at the 4-cell stage. Penetration of connective tissue to the centre of the corpus luteum was complete in individuals with 64-cell embryos.

Stage 4. Mean diameter of corpora lutea: 286–483 μm
Embryonic stage: unilaminar blastocyst

Further enlargement of the luteal cells occurred and vacuoles appeared in the cytoplasm (Fig. 4a, b). A new cavity containing loose connective tissue formed in the centre of the corpus luteum.

Stage 5. Mean diameter of corpora lutea: 406–580 μm
Embryonic stage: bilaminar blastocyst

The luteal cells reached their greatest size and vacuolation was maximal (Fig. 4c, d). The corpus luteum was well vascularized and spaces (? lymphatic vessels) could be seen between the clusters of luteal cells and in the central core of connective tissue. Plasma progesterone concentrations were highest (up to 9·25 ng ml^{-1}) during this stage (pilot study, L. A. Hinds, personal communication).

Stage 6. Mean diameter of corpora lutea: 346–488 μm
Embryonic stage: trilaminar blastocyst, embryos in organogenesis

Loss of vacuolation of the luteal cells and closure of the spaces in the core and between the clusters of luteal cells began when the embryos reached the primitive streak stage and was virtually complete by the time implantation occurred. Late in pregnancy the luteal cells were shrunken and the tissue dense (Fig. 4e, f).

Uterus

Progressive enlargement of the uteri occurred during pregnancy and histological changes in the endometrium could be correlated with the stage of development of the corpora lutea (Table 3). In

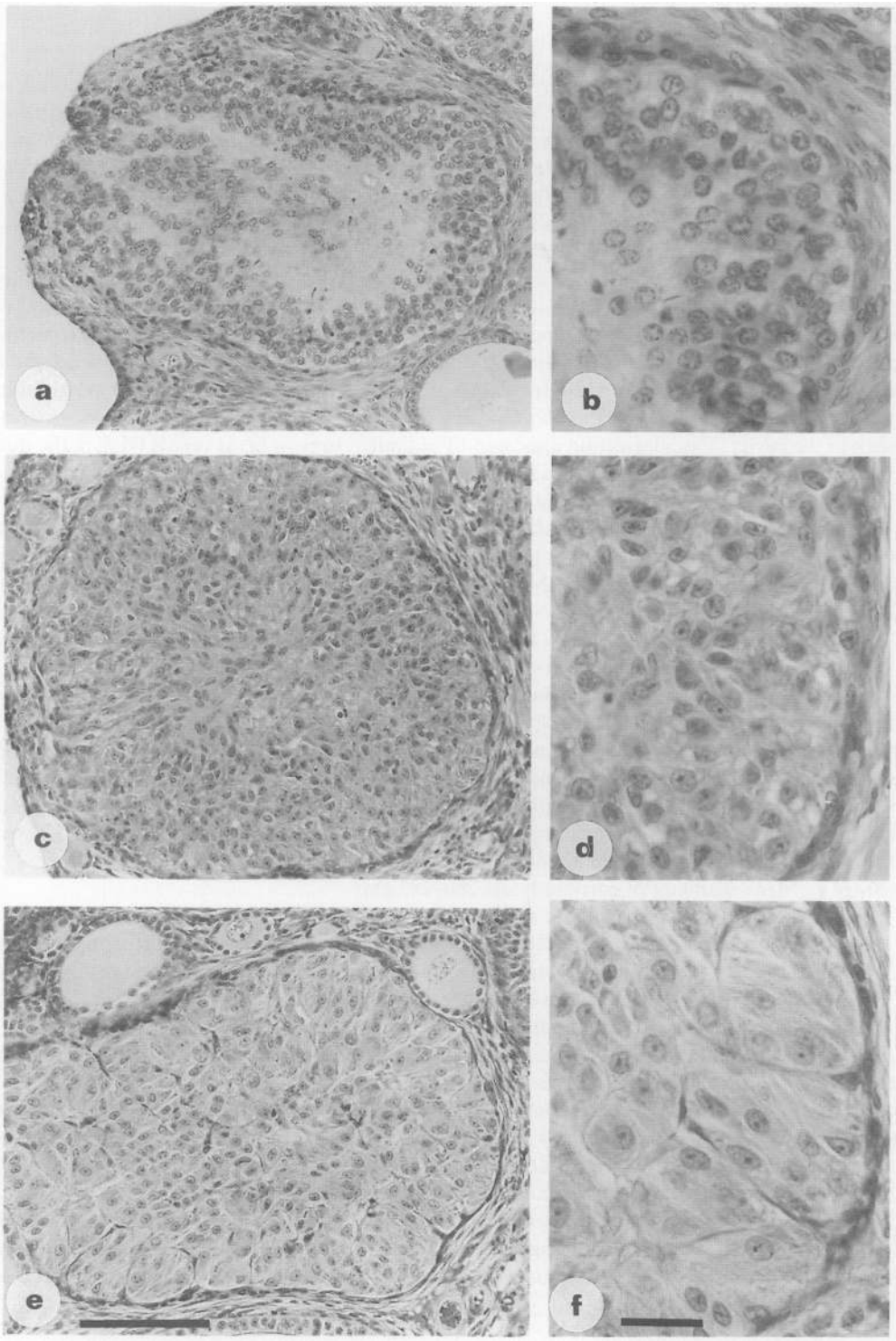

Fig. 3. Development of the corpus luteum in stripe-faced dunnarts. **(a,b)** Stage 1; **(c,d)** Stage 2; **(e,f)** Stage 3. (a,c,e) Sections through central region of entire corpus luteum. Photomicrographs at same magnification. Scale line = 100 μm. (b,d,f) Sections through periphery of corpus luteum. Photomicrographs at same magnification. Scale line = 25 μm.

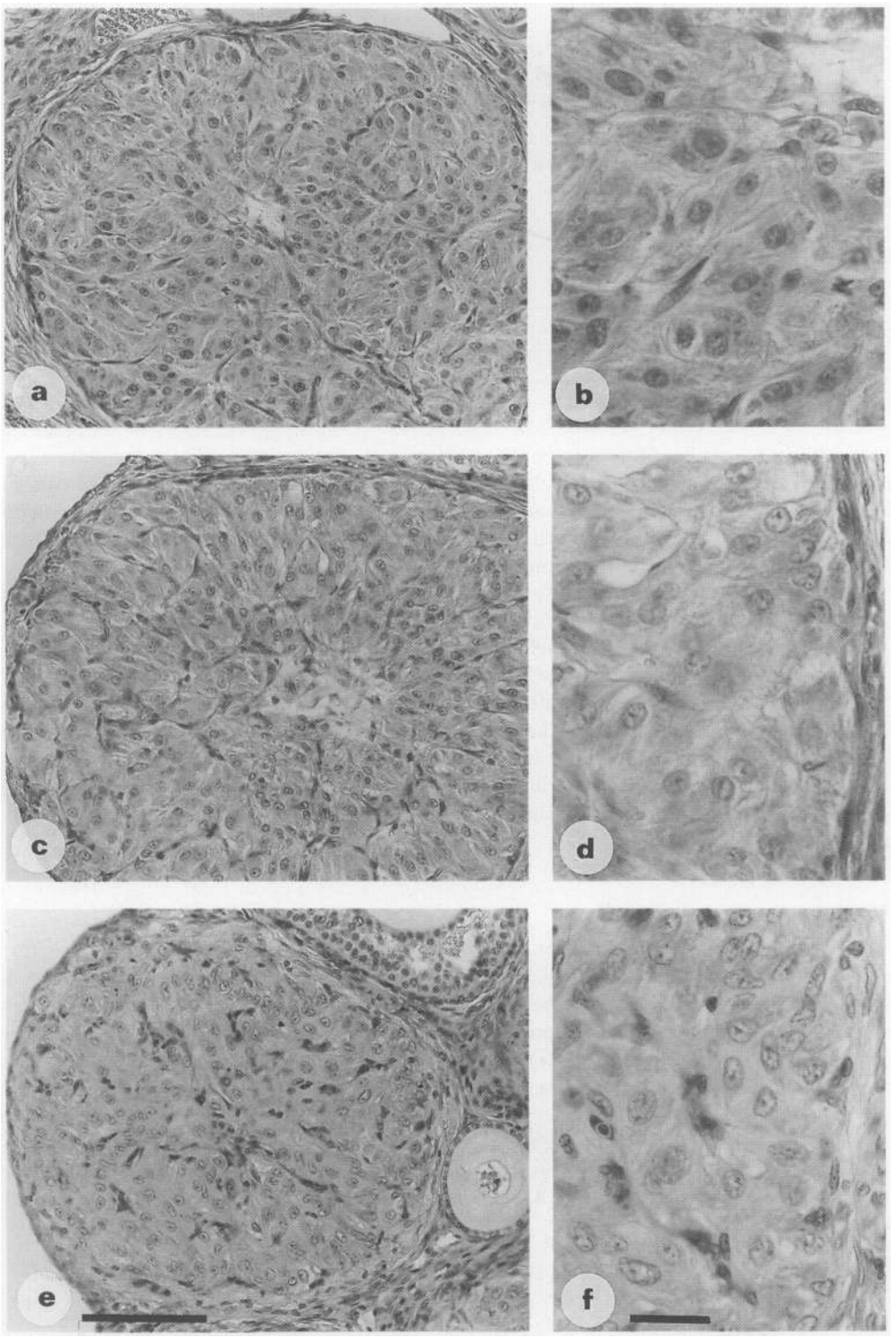

Fig. 4. Development of the corpus luteum in stripe-faced dunnarts. **(a,b)** Stage 4; **(c,d)** Stage 5; **(e,f)** Stage 6. (a,c,e) Sections through central region of entire corpus luteum. Photomicrographs at same magnification. Scale line = 100 μm. (b,d,f) Sections through periphery of corpus luteum. Photomicrographs at same magnification. Scale line = 25 μm.

Table 3. Changes in the width of the uterus and height of the epithelium lining the uterus and endometrial glands of pregnant stripe-faced dunnarts

Corpus luteum stage	No. of animals	Width of uterus (mm)		Height of epithelium (μm)			
				Lumen		Glands	
		Mean	Range	Mean	Range	Mean	Range
1	7	6·8	5·0–9·0	17·6	17·0–21·5	19·6	17·0–21·5
2	9	8·0	6·0–9·0	23·8	21·5–25·7	21·0	17·0–21·5
3	4	9·4	8·5–10·0	37·5	25·7–42·85	21·4	17·0–25·7
4	10	11·0	9·0–11·0	45·0	42·85–47·1	24·9	21·5–30·0
5	7	11·7	10·0–13·0	41·6	38·6–42·85	28·75	25·7–34·5
6	6	14·4	11·5–18·5	(see text)		(see text)	

the early stages (1 and 2) of formation of the corpora lutea the epithelium lining the uterine lumen and the endometrial glands was low columnar, pseudostratified in the lumen lining and ciliated in the glands. Numerous mitotic figures could be seen in both regions. The density of the endometrial stroma was very variable, both between individuals and between adjacent parts of the one uterus. Macrophages were common in the stroma.

The endometrium became thicker and folded, the stroma very loose, and the epithelial cells taller as development of the corpora lutea proceeded. The lumen epithelium reached its greatest height at Stage 4, and the gland epithelium at Stage 5, of the development of the corpora lutea. Mitotic figures in epithelial cells became less common after Stage 3 of corpus luteum formation and by Stage 5 had ceased. At this stage mitotic figures could be seen in connective tissue cells immediately below the lumen epithelium, in which villus-like projections were forming. The epithelial cells on these projections became rounded and the height of the epithelium decreased before implantation of the embryos which occurred during corpus luteum Stage 6. The epithelium became highly modified where it was in contact with the fetal membranes. Desquamation of the gland epithelium commenced before parturition.

Anomalies

Information from 7 cases did not fit the timetable as outlined above. In each case the embryos developed faster than shown in the timetable, and development of both the corpus luteum and uterus were accelerated. Embryos reached the 16–32-cell stage by Day 2 (1/12) instead of Day 3 and the uterine and corpus luteum development were as for Day 3 (Stage 3); the 128-cell, unilaminar blastocyst stage in early expansion (2/21) by Day 3 instead of Day 4, with uterus and corpus luteum appropriate to Day 4 (Stage 3) or Day 6 (Stage 4); the 0·7 × 0·9 mm 'egg-shaped' unilaminar blastocyst with about 1000 cells by Day 4·5 (1/11) instead of late on Day 5, with uterus and corpus luteum appropriate to Day 5 (Stage 4); the advanced bilaminar blastocyst 2·2–3·0 mm in diameter (2/19) by Day 6 instead of Day 8 with uterus and corpus luteum appropriate to Day 8 (Stage 5). One animal, which had 2–4-cell embryos as normal on Day 1, gave birth to young on Day 9 instead of Day 10 or 11.

Discussion

The rate of development in the stripe-faced dunnart is very rapid and the gestation period of 9·5–12 days is the shortest known for any mammal. Previously the shortest gestation reliably known was 12·5 days for each of two species of bandicoot, *Perameles nasuta* (Stodart, 1966) and *Isoodon macrourus* (Lyne, 1974), and for the Virginia opossum *Didelphis virginiana* (Hartman, 1928). While

only 7 out of the 63 cases used to prepare the timetable did not fit, the results for these 7 were very consistent; they indicated that in some animals gestation was completed in 9·5 days. Most births occurred between 10·5 and 11·0 days.

For other marsupials in which the rate of embryonic development has been studied (Selwood, 1989b), the embryos were free for 60–80% of the gestation period; in the stripe-faced dunnart, they were free for 73% of the gestation period. Most of the free period was occupied by cleavage and the unilaminar blastocyst stage, a pattern similar to that found in other species. The greater length of cleavage in the brown antechinus, compared with other previously studied marsupials, is due partly to a temporary cleavage arrest of about 3 days at the 4-cell stage and partly to a long cell cycle time of 12–18 h for other stages (Selwood, 1980, 1989b; Selwood & Young, 1983). Similarly, in the stripe-faced dunnart the 4-cell stage is the longest and lasts for about 24 h, compared to 8–12 h for other stages. Cleavage *in vitro* is slightly slower, taking 70 h from the early uterine zygote to the 32-cell stage (Selwood, 1987) compared to 60–65 h in this study. It seems likely that in marsupials, as in eutherian mammals, one of the cleavage divisions will take longer than any other, and in species that arrest *in vitro* during cleavage, arrest occurs at this division. Both the brown antechinus and the fat-tailed dunnart (*S. crassicaudata*) (Selwood, 1987) show arrest *in vitro* at the 4-cell stage but there is no arrest in *S. macroura*. In all three species, however, the 4-cell stage is the longest cleavage stage. Wide variation in the gestation period is found among dasyurid marsupials, from around 11 days in the stripe-faced dunnart to 25–31 days in the brown antechinus (Selwood, 1980) and up to 6 or more weeks in other small dasyurids (see Woolley, 1988). Investigation of embryonic development in species with long gestation periods may also show periods of arrest in cleavage and unilaminar blastocyst stages as has been shown previously in the brown antechinus (Selwood, 1980).

Because a variable period of storage of spermatozoa after mating of from 1 to 14 days is found in dasyurid marsupials (Woolley, 1966a; Selwood, 1987; Selwood & McCallum, 1987), the day of copulation is not always a satisfactory indicator of the onset of embryonic development. Weight and pouch features (Woolley, 1966b) and cornified epithelial cells and polymorphonuclear leucocytes (Godfrey, 1969; Woolley, 1990) change in a characteristic manner during the oestrous cycle and have been used with varied success to detect ovulation. It appears from these and other studies that, in each species, different characteristics assume more importance in specifying the day of ovulation. This study showed that weight change was most useful in the stripe-faced dunnart, but the disappearance of spermatozoa from, and the appearance of polymorphonuclear leucocytes in the urine provided additional supportive evidence. In contrast, in the brown antechinus, decline in the numbers of cornified epithelial cells is most useful, with the disappearance of spermatozoa, and the appearance of polymorphonuclear leucocytes providing supportive evidence (Selwood, 1980, 1982). These methods for assessing the time of ovulation, while laborious, are accurate to within 12–24 h and are particularly valuable when animal numbers are limited.

It is generally accepted, and this study provides additional evidence, that unilateral hysterectomy and ovariectomy do not affect the rate of development. The death of full-term embryos and the failure to find live pouch young after surgery, however, suggests that this procedure may affect parturition, possibly because of the reduction in number of embryos or level of ovarian hormones (see discussion by Woolley, 1990).

Major phases in embryonic development were correlated with stages in the development and regression of the corpus luteum and associated changes in uterine size and glandular development were seen. Parallels have been found in other marsupials (Woolley, 1966a; Lyne & Hollis, 1979; Fleming & Harder, 1981; Tyndale-Biscoe & Renfree, 1987) although information on embryonic development is generally not as complete as in this study. The 7 anomalous cases suggest that acceleration of development of the corpus luteum caused acceleration of uterine and embryonic development, with the embryos sometimes lagging a day behind the corpus luteum. Studies on hormone profiles during pregnancy (Tyndale-Biscoe & Hinds, 1989) show a correlation between development of the corpus luteum and uterus and progesterone concentrations, all of which

increase during blastocyst expansion and peak at the time of primary germ layer formation. The pilot study on progesterone concentrations suggests that a similar relationship will be found in the stripe-faced dunnart.

It has been suggested (Hill, 1910; Hartman, 1919) that the two populations of cells that arise during cleavage in marsupials give rise to embryonic cells (embryoblast) and extra-embryonic cells (trophoblast). These populations are distinguished by differences in size and cleavage rate in the Virginia opossum (Hartman, 1916, 1919; McCrady, 1938) or by size and histological differences in various dasyurid marsupials (Hill, 1910; Selwood, 1980, 1987, 1989b; Selwood & Young, 1983). Before the primitive endoderm cells develop from the cells of the embryoblast, however, all the cells of the blastocyst became identical in appearance. This caused McCrady (1938) to describe the cells of the blastocyst at this stage, as protoderm cells. When the primitive endoderm cells appear, the cells in that part of the blastocyst wall differentiate as primitive ectoderm cells and the cells in the remainder of the blastocyst form the trophectoderm (Hill, 1910; Hartman, 1916, 1919; McCrady, 1938; Lyne & Hollis, 1977; Selwood, 1980, 1986a). Because of the intervention of a blastocyst stage with identical cells, and the absence (Hartman, 1919; McCrady, 1938) or disappearance of the yolk mass (Selwood, 1986a) in the blastocysts, it has been impossible to say which cells give rise to embryoblast and which to trophoblast. In the stripe-faced dunnart, however, certain features suggest strongly that the tier of smaller cells above the yolk mass gives rise to the embryoblast. These features include retention of distinguishing characteristics in the two cell populations well into blastocyst development, the persistence of the yolk mass until the time of primitive endoderm cell generation and the proximity of the yolk mass to the embryoblast, or the hemisphere containing the embryoblast. The tier of cells to the side of the yolk mass forms the trophoblast. Hill (1910), because of the occasional association of the yolk mass with the embryoblast in the native cat, suggested that the fourth division was determinate and gave rise to embryonic versus extra-embryonic cells. This may be so, as destruction of some cells during cleavage gives rise to fractional embryos (Selwood, 1986b). Alternatively, a positional effect may operate in marsupial embryos as in eutherian mammals (Tarkowski & Wroblewska, 1967). The small cells over the yolk mass are the first cells to have maximum contact with their neighbours and thus may form a two-dimensional equivalent to the inner cell mass of eutherian mammals.

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