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A Timetable of Embryonic Development of the Dasyurid Marsupial *Antechinus stuartii* (Macleay)

Lynne Selwood

Department of Zoology, La Trobe University, Bundoora, Vic. 3083.

Abstract

The rate of embryonic development was investigated in 278 embryos of *A. stuartii*. Ovulation occurred when the number of epithelial cells in the urine of the female began to fall at the end of oestrus. The gestation period was 27·2 days (SD, ± 1·83; SE, ± 0·55; range, 25–31 days). Most births occurred during the 26th, 27th or 28th days.

Data from sequentially sampled embryos was used in preparation of a timetable of development over 27 days from fertilization to birth. The eggs spent 24 h or less in the oviduct, where they were fertilized. After entering the uterus, they rapidly underwent the first and second cleavage divisions, and by the start of the 2nd day had reached the four-cell stage. They then appeared to undergo a period of developmental arrest for up to 4 days. Cleavage was resumed by the end of the 5th day at the latest, after which the blastocyst slowly expanded and cell numbers increased, to 1·0 mm diameter and approximately 100–300 cells at the end of the 11th day. (The unilaminar blastocyst was formed between the 20- and 50-cell stages.) A second period of developmental arrest followed, until the end of the 13th or 14th day, and was followed by slow growth, increase in cell number and differentiation so that by the 16th day the embryo had a cell number of 1000–3000, measured 1·3–1·5 mm and possessed a distinct embryonic area. The bilaminar blastocyst was completed by day 20; primitive streak and notochord formation took a further 2 days. By the end of the 22nd day, the embryo possessed a distinct notochord and the mesodermal mantle had passed the equator of the blastocyst. The first somites, the heart rudiment, neural tube, and early stages in amnion development appeared during the 23rd day. The shell had been lost and implantation, which began during the 23rd day, was complete by the end of the 24th day. Organogenesis, from the advanced primitive streak stage without notochord to birth, took 6 days.

Introduction

Little information is available on embryonic development in marsupials, and staged series of embryos for the entire period of embryonic development have only been described in one species, the American opossum *Didelphis virginiana* (Hartman 1916, 1919, 1928; McCrady 1938). In this species these authors, using successive sampling of tubal and uterine material to establish the time lapse between various stages, devised a timetable of embryonic development over the 13 days of development.

Much less information is available on Australian marsupials. Of these, most information on the rate of development is available for the tammar wallaby *Macropus eugenii* (Renfree and Tyndale-Biscoe 1973; Tyndale-Biscoe 1979) bandicoots *Isoodon macrourus* and *Perameles nasuta* (Lyne and Hollis 1976, 1977),

the quokka *Setonix brachyurus* (Sharman 1955*b*) and the brush possum *Trichosurus vulpecula* (Sharman 1961; Pilton and Sharman 1962; Hughes 1974). Even in these species, the information is based on a small number of aged stages. For most marsupials, information on the chronology of development *in utero* is restricted to estimations of the gestation period and descriptions of isolated, not aged, stages (Bancroft 1973; for reviews see Tyndale-Biscoe 1973; Lyne and Hollis 1977).

Hill (1910) made the most detailed investigation of development for any Australian marsupial, on development from the fertilized egg to the unilaminar blastocyst stage in the dasyurid *Dasyurus viverrinus.* Unfortunately, because he did not utilize successive sampling techniques and because in *D. viverrinus,* as in most Australian marsupials, the time of ovulation relative to copulation or oestrus was not known, he was unable to provide information on the rate of embryonic development. For other dasyurids, the length of the gestation period has been estimated for a large number of species (Woolley 1973) but little else is known of their embryonic development.

This study, in the dasyurid marsupial *Antechinus stuartii* Macleay, provides the most complete timetable of development for any Australian marsupial. Development is described from the fertilized tubal ovum to the newborn pouch young. The description of each stage is confined to the number of cells, where applicable, and to external features, which is appropriate for staging of embryonic material (New 1966). A more detailed histological and ultrastructural analysis of certain aspects of embryology of *A. stuartii* based on the aged material collected in this study and further material of unknown age is at present in progress.

Materials and Methods

Capture and Breeding

A. stuartii was trapped in three localities around Melbourne (Warburton, Kinglake and Lake Mountain) during 1975, 1976 and 1978, and breeding colonies were established in the laboratory. In 1975, 20 pregnant females were trapped during the breeding season. In 1976, the colony consisted of 25 females, of which 18 were mated in the laboratory, and 19 males. In 1978, it consisted of 28 females, of which 23 were mated in the laboratory, and 15 males. The colonies were maintained at 20°C and 45% RH, with the actual daylength conditions of Melbourne during July–September applying.

Each animal was housed in a polypropylene box cage with wire mesh lid, with a disposable plastic cup for a nest box, or in a glass-fronted stainless steel cage, with a detachable nest box and wire mesh back and top designed by Woolley (1973) for this species. The animals were fed on a combination of finely sliced fresh meat, live insects (maggots, crickets, mealworms) and dried cat food pellets. Water was provided *ab libitum.*

The onset of behavioural oestrus was detected from the presence of epithelial cells in the urine, by the method outlined by Woolley (1966*b*, 1971). Once cells were detected, daily urine samples from each female were scored for the number of cells per unit area on a three-point scale (+ cells present; + + many cells; + + + cell number very high). When the number of epithelial cells increased to + + or + + +, females were mated with males in the colony. A mating was deemed to be successful if actual penetration was observed or if sperm were detected in the urine of the female after mating (Woolley 1971). All females were mated at least twice, once or more when the pouch skin was flushed towards the end of oestrus (Woolley 1966*a*).

After completion of mating or capture of pregnant females, each female was checked daily for epithelial cells and sperm in the urine. Ovarian and uterine samples were taken during the latter part of oestrus and during pregnancy. For each female, a record was kept of the pouch changes described by Woolley (1966*a*) towards the end of oestrus, and during pregnancy. For those females which were allowed to give birth, the pouch was checked daily or more frequently to detect the granular shiny appearance, and the clear secretion from the nipples, which indicate the birth is imminent (Woolley

1966*a*), and for the presence of pouch young. Where no pouch young were found, the presence of the clear secretion was used as an estimate of the day of birth (Woolley 1966*a*).

Estimates of time of copulation (based on observation of copulation or the presence of sperm in the urine of the female), of the beginning of the period of declining cell numbers, and of birth can vary by up to 24 h, as observations were routinely made in the morning of each day. However, in most cases the variations would be 12 h or less, as observations were usually made at least twice a day in critical periods, e.g. when pouch young were due, in late oestrus or after mating.

Sampling of Embryos

A total of 278 embryos was collected. Embryos were sequentially sampled from 26 females: 10 in 1976 and 16 in 1978 (Table 1). For embryos to be collected on two successive occasions, pregnant females were anaesthetized with sodium amytal at a rate of 0·08–0·10 mg per gram of body weight (Woolley 1966*b*). Lower doses were administered to recently captured animals and higher doses to those which had been in captivity for a month or so. The left or right ovary and uterus were removed and placed in warm phosphate-buffered saline. After the wound was stitched up, the female was allowed to recover. The uterus was opened and the embryos, dependent upon their state of development, were flushed or dissected out of the uterus. Tubal ova were fixed *in situ*. After the number of embryos were counted (Table 1), the material was divided into two samples, one of which was photographed while the embryos were still living and the other fixed for electron microscopy or routine histological processing. Cell numbers were counted by microscopic examination in intact embryos up to the 21-cell stage, and in blastocysts which were fixed, stained and flat mounted (Clark 1966) from the 50-cell stage up to the first appearance of the endoderm. The remainder of the reproductive tract and enclosed embryos were removed at a later date, or the female was allowed to give birth.

In a further 10 females (Table 2), the reproductive tract was examined during late oestrus or in the subsequent weeks for signs of ovulation or pregnancy. Any embryos were removed from both sides of the tract and were processed as outlined above.

The timing of the initial and second sampling of embryos was determined by information on oestrus, mating and pouch development. The initial samples were taken towards the end of behavioural oestrus from females still showing high numbers of epithelial cells in the urine, in the period of declining cell numbers with or without sperm present in the urine, and in the 3 weeks after the cells and sperm had disappeared from the urine. By the successive sampling method, embryos could be aged relative to each other and relative to events in oestrus and in the breeding program.

In order to assess the effect of the surgical procedures used in this study, I determined the mean, standard error and standard deviation for the number of embryos found in each side of the reproductive tract when all embryos were removed during the first laparotomy (Table 2) or when embryos were sampled twice during development (Table 1). The means of the number of embryos in each side of the tract were compared by a t test in each case.

Preparation of Timetable

The information from the successive sampling of embryonic material was combined. Identical stages during development were matched and the periods back to tubal egg and forward to birth filled in. Stages from either end of pregnancy (i.e. tubal egg or birth) were matched and the intermediate stages then filled in. Fig. 1 shows how these results were combined for a gestation period of 27 days. As the length of the gestation period was determined in this study to be 26–27 days, the combined data for 1976 and 1978 were fitted into 25, 26, 27 or 28 days. The number and type of anomalies (i.e. those sampled embryos which could not be fitted into the timetable) were noted for each timetable. The information obtained from the embryos which were sampled in 1976 was used to compile a provisional timetable, which was used successfully for sampling of embryos of a desired stage from females of the 1978 colony. Attempts were also made to prepare timetables in which development was assumed to start on the day of the last successful copulation or on the first day of declining cell numbers. This information was combined with the data from the sequentially sampled embryos.

Results

Breeding Program and Sampling of Embryos

Scoring the number of cells present in the urine has a number of inherent diffi-

Table 1. Embryos of *A. stuartii* which were successively sampled during development

CRL, crown-rump length; GL, greatest length

Female No.	First sample Stage	*N*	Second sample Stage	*N*
6	Unilaminar blastocyst, 1·0 mm	2	Dead pouch young, day 1 (clear secretion)	2
8	Four cells	3	Pouch young, day 1 (clear secretion)	0
	Three cells	1		
9	Foetus, CRL 4·5 mm	6	Pouch young (clear secretion)	0
	Unfertilized eggs	3		
11	Bilaminar blastocyst, 3·2 mm, with primitive streak	3	Foetus, CRL 4·5 mm	3
12	Unilaminar blastocyst, 1·3–1·5 mm, with round embryonic area, 0·1 mm	5	Bilaminar blastocyst, 3·8 mm, with advanced primitive streak	5
13	Unilaminar blastocyst, 1·0 mm	1	Pouch young (clear secretion)	0
14	Unilaminar blastocyst, 1·0 mm	6	Trilaminar blastocyst, 3·7×4·5–4·0×4·5 mm, with early notochord	5
16	Bilaminar blastocyst, 3·8–4·0 mm, with advanced primitive streak	3	12-somite embryo, GL 4·5 mm, early implantation	5
17	Bilaminar blastocyst, 3·0–3·1 mm	5	Pouch young, day 1	3
	Unfertilized egg	1		
18	Bilaminar blastocyst, 3·2–3·5 mm, with primitive streak	5	Foetus with branchial arches, GL 3·5 mm	5
			Abnormal dead foetus	1
20	Fertilized tubal ova	7	Bilaminar blastocyst, 2·9×3·0×2·8–2·9×3·0×3·0, with pear-shaped embryonic area	4
21	Four cells	5	Pouch young, day 1	4
	Unfertilized egg	1		
22	Four cells	6	Foetus, CRL 4·5 mm	4
23	50–80-cell unilaminar blastocyst, 0·5×0·4–0·7×0·6 mm	4	Foetus with branchial arches, GL 3·0 mm	6
28	60–80-cell unilaminar blastocyst, 0·5×0·6–0·6×0·7 mm	4	Foetus, CRL, 3·5 mm	4
	Unfertilized egg	1	Abnormal foetus	1
29	Four cells	4	Unilaminar blastocyst, 2·0–2·1 mm, with round embryonic area, 0·4–0·5 mm	4
	Three cells	1		
	Unfertilized egg	2		
30	Unilaminar blastocyst, 1·6–1·7 mm, with round embryonic area 0·3 mm	5	Pouch young, day 1	4
31	10–16 cells	3	Bilaminar blastocyst, 2·8×2·9–3·0×3·0 mm, with pear-shaped embryonic area	5
32	Four cells	2	Unilaminar blastocyst, 0·8 mm	2
	Five cells	1	Unfertilized egg	3
	Eight cells	1		

Table 1 (*Continued*)

Female No.	First sample Stage	*N*	Second sample Stage	*N*
33	Four cells	6	Trilaminar blastocyst, 4·1×4·4–4·1×4·5 mm, advanced notochord	4
36	One cell	2	Four cells	6
	Two cells	4	Three cells	1
37	Unilaminar blastocyst, 1·0–1·1 mm	5	Bilaminar blastocyst, 3·0×3·3 mm, with primitive streak	5
41	Four cells	6	Pouch young (clear secretion)	0
42	Bilaminar blastocyst, 3·0×3·1 mm, with pear-shaped embryonic area	4	Trilaminar blastocyst, 3·8×4·0–4·1×4·1 mm, with early notochord	5
43	Bilaminar blastocyst, 3·7×3·9–3·8×4·0 mm, with advanced primitive streak	7	8-somite embryo, GL 5·0 mm, early implantation	7
44	Bilaminar blastocyst, 3·2×3·3–3·4×3·5 mm, with early primitive streak	6	Trilaminar blastocyst, 3·8×4·0–4·0×4·0 mm, with early notochord	5

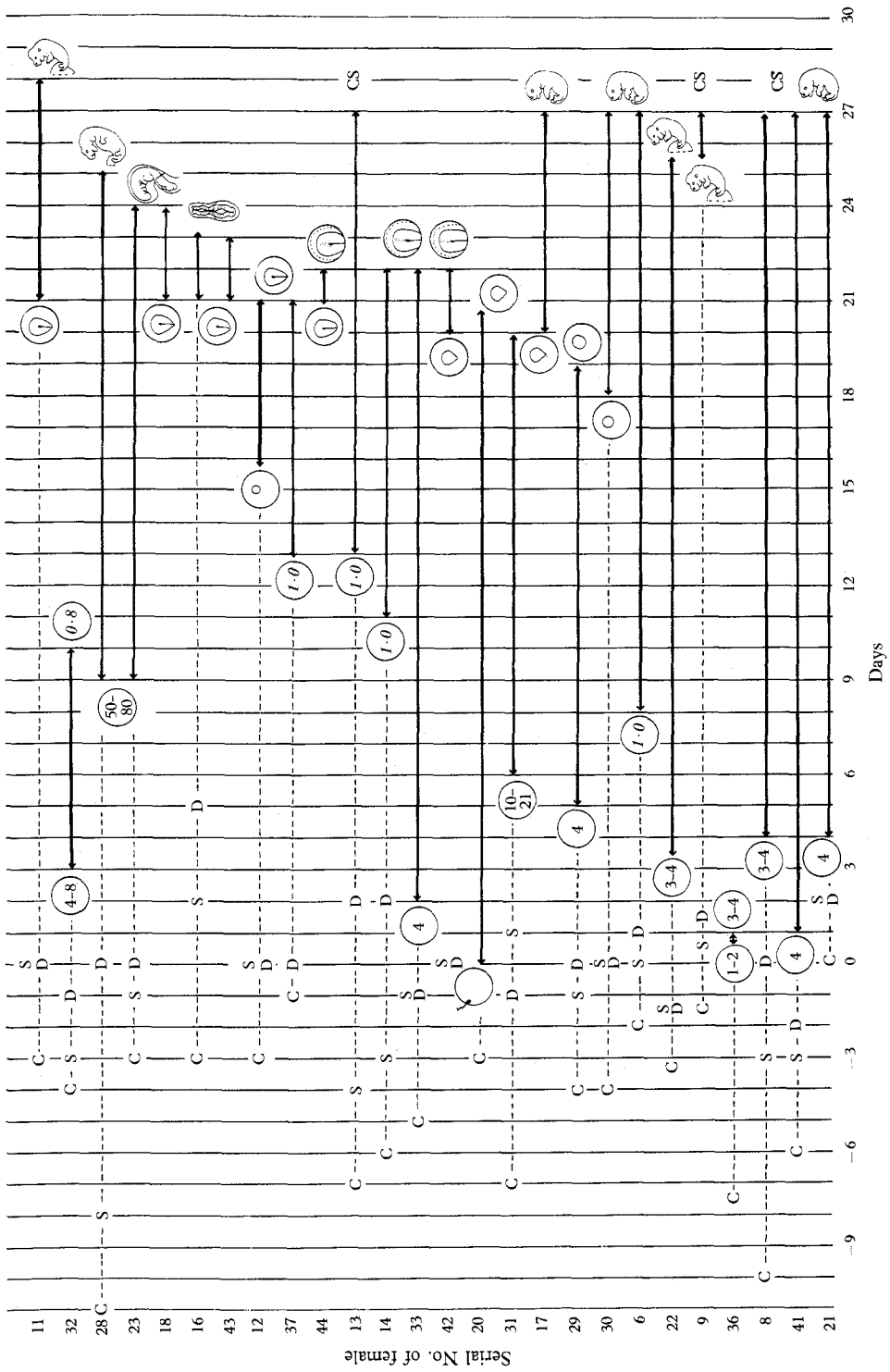
culties. The number can vary, dependent upon degree of contamination with faecal material, time when mating occurred and time of previous urination. The pattern of fluctuation in numbers varies from female to female, the onset of behavioural oestrus being marked by high numbers in some females and a gradual increase in others. Cell numbers usually declined over 3–4 days at the end of behavioural oestrus. Ovulation took place either just before or just after the start of this decline, as indicated by examination of reproductive tracts sampled about this time (Table 2).

Two females, in whose urine cell numbers were starting to fall and sperm could still be detected, had fertilized eggs in the oviduct (Fig. 1; Table 2). Nine females with few cells and no sperm in the urine had early cleavage stages in their uteri. Once the cells had disappeared from the urine, later cleavage stages were obtained.

Where both sides of the reproductive tract were removed at the same time (Table 2) the mean number of embryos in the left side was 2·7 (SD, 2·21; SE, 0·70) which was not significantly different ($P > 0·5$) from that (4·0; SD, 2·40; SE, 0·76) in the right side of the tract. The mean number of embryos of 4·7 (SD, 1·47; SE, 0·29) removed from the tract at the first laparotomy was not significantly different ($P > 0·2$) to that (3·7; SD, 1·92; SE, 0·38) removed at the second laparotomy.

Gestation Period

The length of the gestation period was estimated in three ways for the 11 females which completed pregnancy without laparotomy, and for which complete records were available. For these estimates, the onset of development was taken as the time of the last successful mating, as has been done in other dasyurids (Woolley 1973), the time when cells began to decline in the urine, and the time when sperm


Serial No. of female
11 32 28 23 18 16 43 12 37 44 13 14 33 42 20 31 17 29 30 6 22 9 36 8 41 21
Days
30 27 24 21 18 15 12 9 6 3 0 —3 —6 —9
CS
0·8
50–80
1·0
4–8
10–21
3–4
1–2
4
S
D
C

disappeared from the urine of the female. The end of pregnancy was indicated by the presence of pouch young or, where pouch young were not found, by the presence of clear secretion in the pouch, which indicates that birth is imminent (Woolley 1966*a*).

For these 11 females, the gestation period estimated as the number of days between the last successful copulation and birth ranged between 26 and 34 days, with mean 29·6 days (SD, 2·12; SE, 0·64). Slightly more than half the births took place on day 28 or 29. Estimated as the number of days from the start of the decline in number of epithelial cells in the urine to birth, the gestation period ranged from 25 to 31 days, with mean 27·2 days (SD, 1·83; SE, 0·55). Estimated as the number of days from the permanent disappearance of sperm from the urine to birth, the range was 24–30 days and the mean was 26·6 days (SD, 1·69; SE, 0·51). For the two latter methods, more than half the births took place on day 26 or 27.

Timetable of Development

Embryos were successfully sequentially sampled from 26 females (Table 1; Fig. 1). Some additional females gave abnormal embryos in either the first or the second sample; these embryos are not included in the Table nor were they used in the preparation of the timetable. The number of days between the first sampling of embryos and the last successful mating, the start of the decline in number of epithelial cells in the urine, or the permanent disappearance of sperm from the urine are also recorded in Fig. 1.

Embryos from both oviducts or uteri were taken from a further 10 females; the reproductive tracts of six females were examined in late behavioural oestrus when cell counts in the urine were high (Table 2). Relevant information on the oestrus and breeding periods for these 16 females is included in Table 2.

Sampling around the end of behavioural oestrus has shown that the period of declining cell numbers is a more reliable indicator of the onset of development than the last copulation. Fig. 1 shows that for 20 females declining cell numbers were detected within 48 h of fertilization in all except one case (No. 16). However, because this test was accurate only to within 48 h it was not suitable for estimating the age of embryos. Information from embryos aged in this way or from the last copulation could not be combined with that from embryos which were sequentially sampled during development, as it was impossible to match many of the stages. The relative age of the embryos in the timetable presented here is derived solely from the known times (in days and hours) between the two samples from each

Fig. 1. Chart showing the way in which the data from the *A. stuartii* embryos sampled twice during development were combined to make a timetable of development over 27 days. For each female, the heavy line represents the interval between the first and second sampling of embryos. The stages of development are represented diagrammatically, not drawn to scale. Day 0 is that on which development begins. C, last successful copulation. S, last day on which sperm are present in urine. D, first day of decline in number of epithelial cells in urine. CS, clear secretion from nipples (pouch young not seen). Numerals within circles are the numbers of cells; italic numerals are the length of the blastocyst in millimetres.

female as represented in Fig. 1. Embryos which were aged relative to the day of declining cell numbers can sometimes be fitted into this timetable (Table 2).

Of the four timetables of development (25, 26, 27 and 28 days) which were prepared from sequentially sampled embryos, that for a 27-day gestation fitted the data best. A summary of this timetable is presented in Fig. 2 and represents a guide to the rate of development in *A. stuartii.* Variations are outlined below and in Fig. 1.

Table 2. Results of the examination of the reproductive tract for 16 females of *A. stuartii* during late behavioural oestrus and pregnancy

n.a., not applicable, very many cells present in urine

Female No.	Development stage	No. of follicles/embryos Right side	Left side	No. of days since: Copulation	Cell Nos decline	Sperm disappear
1	Graafian follicle	5	8	–	n.a.	–
2	Graafian follicle	8	4	–	n.a.	–
3	Graafian follicle	5	7	–	n.a.	–
4	Graafian follicle	6	6	–	n.a.	–
5	Graafian follicle	5	6	–	n.a.	–
38	Graafian follicle	7	–	–	n.a.	–
7	Unilaminar blastocyst, 2·5 mm, with round embryonic area	1	0	19	15	19
	Unfertilized egg	0	2			
10	10–16 cells	3	1	11	8	8
15	10–16 cells	2	2	12	10	10
	Unfertilized egg	3	0			
19	10–21 cells	4	5	8	4	5
24	Foetus, CRL 4·0 mm	0	3	28	23	22
	Unfertilized egg	2	0			
25	Fertilized tubal ovum	5	4	11	0	–[A]
26	Four cells	6	5	8	4	4
27	10–16 cells	7	0	14	4	3
	2 cells	1	0			
	Unfertilized egg	1	2			
34	Unilaminar blastocyst, 0·9–1·0 mm	6	1	22	13	20
	Unfertilized egg	0	3			
35	Trilaminar blastocyst, 4·0×4·5–4·2×4·4 mm, early notochord	6	6	28	21	22

[A]Sperm still present in urine.

Stages of Development

(i) *Tubal eggs*

As the precise time of ovulation is difficult to detect in *A. stuartii,* the beginning of development (0 days) was taken not as ovulation but as the fertilized egg in the oviduct (Figs 1, 2).

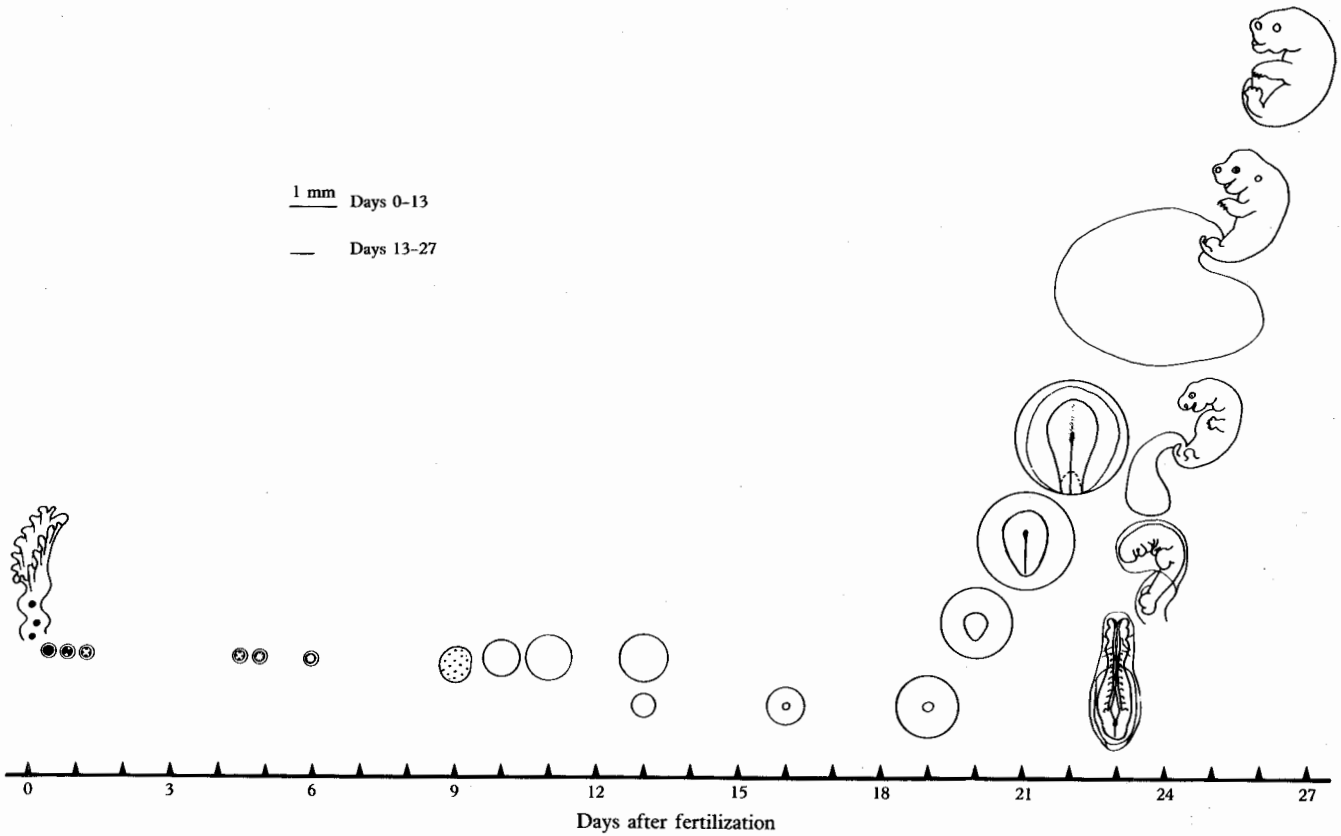


Fig. 2. Timetable of development in *A. stuartii* from fertilization in the oviduct to birth, over 27 days.

Fertilized eggs (16) were found in the oviducts of two females (Tables 1, 2). For all these tubal ova, the deposition of mucoid material was not complete and the shell had not been deposited.

The eggs were fertilized in the upper oviduct and were at progressively later stages in fertilization as they descended the oviduct. From the evidence provided by sequential sampling of females, the eggs took about 24 h after fertilization to pass along the oviduct, enter the uterus and develop to the 4-cell stage. The beginning of the decline in cell numbers was an indication of ovulation, and the disappearance of sperm from the urine was an indication that the eggs had entered the uterus. Therefore, for the 11 females concerned, if the mean gestation period estimated from the disappearance of sperm from the urine (26·6 days) is subtracted from that estimated from the decline of number of epithelial cells in the urine (27·2 days) the difference is 0·6 days, i.e. the eggs take about half a day to traverse the oviduct after fertilization. The undivided uterine egg has a mean total diameter of $242 \pm 4 \cdot 1$ μm (SE), the mean width of the zona $7 \pm 0 \cdot 74$ μm, the mean width of the mucoid layer $18 \pm 1 \cdot 02$ μm, and the mean width of the shell $4 \pm 0 \cdot 29$ μm. Sperm tails were visible in the mucoid layer of the zygote and later cleavage stages. It is not known how long after entering the uterus the egg undergoes the first division, but by the time this division was complete the 'yolk' had been eliminated.

As 3- and 4-cell stages were occasionally found together in the one uterus (Table 1) the next division, the second, presumably was not synchronized. In one female (Table 1), embryos at the 1–2-cell stage were found in one uterus, and 2·5 h later 3–4-cell stages in the other. This division occurred at the end of the first day or during the first half of the second day. Once the 4-cell stage was reached, development appeared to halt for up to 4 days (Fig. 2). In one female, No 32, the next division had started by the end of day 3 and in another, No. 29, 4-cell stages showing early signs of the next division were found on day 5 (Fig. 1).

By the end of the 5th day the third division had started (Figs 1, 2), resulting in the 8-cell stage. When division was complete, the eight cells lay flattened against the zona, forming a band around the equator enclosing the yolk. Further divisions were relatively rapid and unsynchronized, forming an incomplete blastocyst of 10–21 cells (Figs 1, 2) by the end of the 6th day; there was some variation, probably related to the time of ovulation, in the number of cells. The embryo had not yet started to grow and still had an outer diameter of about 240 μm. The blastocyst appeared to be not quite complete by the 20-cell stage but histological analysis is needed to confirm this.

(ii) *Unilaminar blastocyst*

By the 50-cell stage, the wall of the blastocyst was complete and the embryo had undergone a slow expansion in size. By the end of the 9th day (Figs 1, 2), it was a slightly oval unilaminar blastocyst ranging in size from 410 by 510 μm to 650 by 770 μm, with 50–82 cells. 'Yolk' material, 50–60 μm in diameter, and sperm in the mucoid layer were still present. The number of cells did not correlate with the size of the blastocyst, i.e. the biggest blastocysts were not necessarily those with most cells. At this stage, there seemed to be some differentiation in the wall of the blastocyst; two or three cells, which were triangular with distinct margins and quite different from the other cells, were seen in the wall of two blastocysts.

Further expansion of the blastocyst resulted in a unilaminar blastocyst about 1·0 mm in diameter (Table 1) by the end of the 11th day (Figs 1, 2). The cells now numbered between 100 and 300 and no part of the blastocyst appeared to be distinctly different from any other. 'Yolk', 50 μm in diameter, was still present inside the blastocyst. The zona and mucoid layer were no longer visible and the shell was about 3 μm wide. The blastocysts appeared to remain in this state for about 2 or 3 days. In female No. 6 (Fig. 1), 1·0-mm blastocysts were found on day 8; presumably in this animal cleavage and formation of the unilaminar blastocysts were faster and development from the unilaminar blastocyst to birth was slower than in any other.

(iii) *Formation of the bilaminar blastocyst*

From the end of day 13 or 14 onwards the blastocyst slowly expanded, so that by day 16 the blastocyst was 1·3–1·5 mm in diameter and contained between 1000 and 3000 cells. It was during this stage that the embryonic area, the site of endoderm formation (Selwood, unpublished) first became clearly visible as a clear window (about 0·1–0·2 mm in diameter) in the wall of the blastocyst. No yolk material was visible in these blastocysts. As the blastocyst had expanded the shell had become progressively thinner, to be about 2·0 μm thick in the 1·5-mm blastocyst. Sperm were still visible in some blastocysts at this stage but were not seen in any later ones.

During the next 3 days, the blastocyst and embryonic area continued to expand (Figs 1, 2), so that at the end of the 19th day the blastocyst, now about 2·1–2·5 mm in diameter, had a round embryonic area of 0·4–0·8 mm in diameter.

During day 20 (Figs 1, 2), the embryonic area, which was now 1·0 by 1·2 mm in size, became slightly pear-shaped due to an elongation of its posterior edge. The primitive streak was not visible at this stage, although histological analysis may reveal that it is present in this elongated posterior portion. In profile, at this stage the blastocyst appeared very slightly egg-shaped and ranged in size from 2·8 by 3·1 to 3·0 by 3·1 mm in diameter, its blunt end lying at the anterior or rounded side of the embryonic area. At the opposite side of the blastocyst to the embryonic area a small triangular dense patch of material 0·5 by 0·5 by 0·3 mm appeared in the wall of the blastocyst. In sectioned material the blastocyst was now seen to be bilaminar.

(iv) *Formation of the trilaminar blastocyst*

During the 21st day (Figs 1, 2) the primitive streak appeared in the posterior, elongating section of the pear-shaped embryonic area. As the blastocyst and embryonic area increased in size the latter became progressively elongated and so did the streak. By the end of the 21st day, when the streak was about 2·0 mm long, the primitive node became visible at the anterior end.

Blastocysts at this stage were still egg-shaped with an intact shell. They measured 3·6 by 3·5 mm, with an embryonic area of 2·3 by 1·8 mm, in those earlier stages where the node was not yet visible. At the end of the day, when the node was distinct, the blastocyst measured 3·9 by 3·7 mm and the embryonic area 3·0 by 2·4 mm. The triangular dense patch at the opposite pole to the embryonic area

was still visible, and in some specimens the mesoderm appeared to have reached the edge of the embryonic area.

Progressive elongation of the embryonic area and the primitive streak continued during the 22nd day (Figs 1, 2). The embryonic area was now so big that it was not fully visible in one hemisphere. Material had started to move forward from the node, and the notochord became visible ahead of the node towards the end of the day. The egg-shape persisted, and the blastocyst in the most advanced stages of notochord development measured 4·5 by 4·1 mm with an embryonic area of 5·0 by 2·0 mm. The dense patch at the opposite pole persisted, and became surrounded by a narrow clearer area 1·0 mm in diameter in these stages.

During this day a sheet of tissue, presumably mesodermal, moved out from the embryonic area towards the opposite pole. Its rate of movement was not the same for all embryos, in that in those with the most advanced notochords the mesoderm did not quite reach the equator, but in others, with less well developed notochords, the mesoderm passed the equator.

(v) *Organogenesis*

The 23rd day (Fig. 1) was a day of rapid organogenesis, and by its end a recognizable vertebrate embryo about 5·0 mm long had appeared. The neural folds had developed and met anteriorly just behind the anterior neuropore, which was still open. The presumptive prosencephalon, mesencephalon and rhombencephalon were readily distinguished. In the heart rudiment, the heart tubes had fused in the midline and the vitelline veins, each ending in a capillary plexus, were also present. From eight to twelve pairs of somites had developed, and posteriorly the primitive pit and primitive streak extended into the tail region.

The head was lifted off the blastocyst and the subcephalic pocket extended back to the level of the mesencephalon. Cephalic flexures had also developed by this stage. The head fold of the amnion had extended back to the level of the third somite and lateral folds of the amnion were present in addition to a small tail fold. Attachment of the blastocyst to the uterine wall had just started, but was not complete, as blastocysts could still be removed from the uterine lining without damage to the embryonic material or uterus. The shell had fallen away from the embryo and lay in the uterine lumen, and the vascular yolk sac now had a diameter of approximately 9·0 mm with the embryo lying slightly below centre.

By the end of the 24th day (Fig. 1), the embryo had turned on its right side and the greatest length was about 3·5 mm. Cranial, cephalic and cervical flexures were marked and the primary lumbar flexure was starting to appear posteriorly. Some broken shells were still found in the uterine lumen during this day. The anterior neuropore and otocysts had closed. Four branchial arches were visible and the maxillary process was still distinct. The beating heart was visible externally as a bent tube lying under the arches in the ventral curve of the body. The forelimb rudiment was in the form of a paddle, and the hindlimb rudiment was a lateral bud at the posterior end of the embryo. The entire embryo was enclosed by the amnion. The blastocyst was now firmly attached to the uterine lining and the sinus terminalis, vitelline veins and vitelline artery were well developed.

During the 25th day (Figs 1, 2) the secondary lumbar or caudal flexure developed, so that the embryo was now curved into a C-shape. The head was characterized by the widely gaping mouth. Two pharyngeal arches were still visible

externally and the heart had been incorporated into the body. In the forelimb, digital rudiments were present as buds, and the hindlimb rudiment was now club-shaped. A tail was present and curved ventrally under the body. A prominent allantois, almost the length of the crown-rump axis, was now present.

During the 26th day (Figs 1, 2) the head, which was slightly lifted away from the chest, became progressively differentiated and possessed an open mouth with slightly protruding tongue, open nostrils and distinctly pigmented eyes. The eyes, ears and sides of the mouth had not yet been covered with the epitrichium. The crown-rump length was now about 4·5 mm and the allantois was about one and a half times as long as the crown-rump length. Claws were present on the digits of the forelimb, but the hindlimb rudiment was in the form of a paddle without digital rudiments. In one female (11) development from the primitive streak stage to a foetus at this stage took 2 days longer than in any other.

The newborn pouch young, which were born towards the end of the 27th day (Figs 1, 2), had a crown-rump length of 4·8–5·0 mm. Although the nostrils and anterior portion of the mouth were open, the sides of the mouth, eyes and ears had been covered with the epitrichium. The head was bent further away from the chest and the hindlimb rudiment was in the form of a paddle on which ridges of four digits could be seen. Claws were present on the digits of the forelimb.

Discussion

Gestation Period

As in many marsupials where the time of ovulation relative to oestrus and copulation is unknown (Tyndale-Biscoe 1973), in dasyurids the gestation period is usually given as the number of days between the last copulation and birth. Because in this group oestrus and behavioural oestrus can be relatively extensive and because ovulation is not induced by copulation (Woolley 1973), the estimated gestation periods are usually variable (see Woolley 1973 for review table). In *A. stuartii* this situation applies, and the gestation period has been given as 30–33 days (Marlow 1961), 26–35 days (Woolley 1966*a*), 32–34 days (Wakefield and Warneke 1967) and about one month (Wood 1970). However, in this study, as sampling of the reproductive tract at intervals after mating showed that ovulation is not related to the last copulation but that it occurs just before or just after the number of epithelial cells in the female's urine starts to fall, the gestation period was estimated as the number of days between the beginning of the decline in cell numbers and birth. Compared with estimates of gestation period based on copulation in this and other studies, this gives a narrower range, of 25–31 days with a mean of 27·2 days, and is a more reliable method of estimation. The individual gestation period varied around 27 days. Variation in the length of the gestation period is common in eutherians (New 1966) and in the marsupial which has been most extensively investigated, *D. virginiana* (Hartman 1928).

As has previously been discussed, the estimation of the number of cells in the urine has a number of difficulties, but, even so, use of this test to indicate the onset of development has enabled samples to be taken at the time of fertilization, while the eggs were still in the oviduct, and later, from the uteri, at the desired stage of development. It should always be used in association with other data; for example, pouch changes during oestrus and pregnancy should be monitored.

It may be that examination of the urine at intervals of 6 h or less would indicate the time of ovulation more precisely than in this study, and would minimize false estimates of the number of cells in the urine. The disadvantage of this test is, apart from its intrinsic difficulties, that it tells when ovulation has occurred, not when it is about to occur.

Sampling of reproductive tracts at the end of oestrus, after copulation, has suggested that the final disappearance of sperm from the urine of females at the end of oestrus would be inaccurate as an indicator of the onset of development, as the earlier tubal stages would not be found. More sampling around this period is needed to clarify this point. In *A. stuartii,* as in other marsupial species which have been studied (McCrady 1938; Lyne and Hollis 1977), the egg receives a mucoid coat in which numerous sperm became embedded as the egg passes down the oviduct. It seems likely, therefore, that the incorporation of the sperm in the mucoid coat removes excess sperm from the oviduct and results in the final disappearance of sperm from the urine of the female.

Timetable of Development

In order to study the rate of development in marsupials, estimates of the age of embryonic material based on the last copulation are appropriate only in species where the females mate only once or within a short period, e.g. 12–24 h, where the females do not mate when in early pregnancy and where ovulation follows the end of behavioural oestrus within a short and known time. These conditions apply in *Trichosurus vulpecula* (Pilton and Sharman 1962; Hughes 1974), *Setonix brachyurus* (Sharman 1955*a*, 1955*b*) and *Macropus eugenii* (Renfree and Tyndale-Biscoe 1973; Tyndale-Biscoe 1979) where embryos have been aged in this way.

In polytocous marsupials, where embryos are found in both uteri, the divided condition of the uterus has allowed the collection from each female of two samples of embryos, a known time apart. This method has given information on the rate of development in *D. virginiana* (Hartman 1928), in *P. nasuta* and *I. macrourus* (Lyne and Hollis 1977) and in *A. stuartii* in this study, where it has been combined with information on the gestation period to prepare the most complete timetable for any Australian species of marsupial (Figs 1, 2).

Comparison of the numbers of embryos from each uterus has shown that the mean number obtained at the first laparotomy was not significantly different to that obtained at the second. Hartman (1919) found in *D. virginiana* that laparotomy and removal of the embryos from one side of the tract did not affect survival of the remaining embryos. Selwood (1980), by comparing the number of corpora lutea in *A. stuartii* with the number of embryos in the same side of the tract, showed that 23–27% of the eggs shed from each ovary will not be fertilized or complete cleavage to the unilaminar blastocyst stage. Most of the embryos that survive to the latter stage will survive to be pouch young. This high mortality during early development presumably accounts for the unfertilized eggs found with more advanced stages during development in *A. stuartii* (Tables 1, 2), and appears to be a feature of development in *D. viverrinus* (Hill 1910), *D. virginiana* (Hartman 1919) and *Sarcophilus harrisii* (Hughes 1979).

In addition, the absence of pouch young in some females in 1976 was probably related to the higher mortality of embryos in that year, which was due to the conditions of management of the colony (Selwood 1980).

Less variation in stage of development was found between the embryos in one uterus in *A. stuartii* than has been reported by Hill (1918), Hartman (1919) and Hughes (1979) for other polytocous marsupials. However, even in these species the variation is not usually more than two total cleavage divisions apart, and could possibly be due to the larger number of eggs shed in these species than in the *A. stuartii* used in this study, so that a longer time exists between the fertilization of the first and that of the last egg shed.

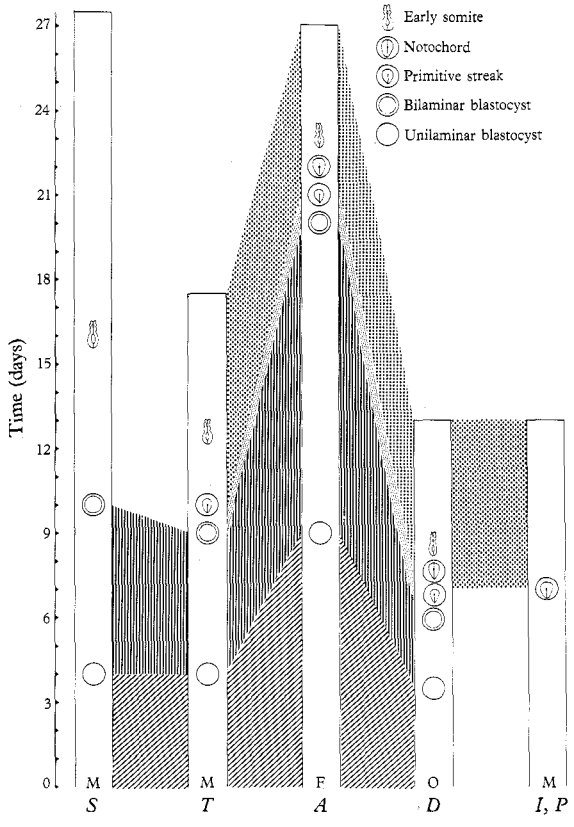


Fig. 3. Comparison of the relative rates of embryonic development in marsupials. *S, Setonix brachyurus* (Sharman 1955*b*); *T, Trichosurus vulpecula* (Hughes 1974); *A, Antechinus stuartii* (this study); *D, Didelphis virginiana* (McCrady 1938); *I,P, Isoodon macrourus* and *Perameles nasuta* (Lyne and Hollis 1977). M, mating; F, fertilization in oviduct; O, ovulation. Diagonal hatching, cleavage; vertical hatching, formation of bilaminar blastocyst; light stipple, formation of primitive streak; heavy stipple, organogenesis. The stages of development are not to scale.

The relative rates of development for the species for which most information on rate of development is available are compared in Fig. 3. In *A. stuartii,* as in other marsupials which have been studied (Hartman 1923; Sharman 1955*b*; Godfrey 1969; Tyndale-Biscoe and Rodger 1978), the egg takes about a day to traverse the oviduct. Sampling around this period suggested that in *A. stuartii* the time between fertilization and entering the uterus may be as short as 12 h.

Because of the difficulty of detecting ovulation in dasyurids, it is not possible to compare the rate of development during cleavage in *A. stuartii* with that in

other dasyurids where cleavage stages have been obtained, because in these species, *D. viverrinus* (Hill and O'Donoghue 1913) and *S. larapinta* (Godfrey 1969), embryos have been aged from last copulation to birth and the data are very confusing. Cleavage to the 16-cell stage in *A. stuartii* was about twice as slow as that reported for *D. virginiana* (Hartman 1928; McCrady 1938) and for *P. nasuta* and *I. macrourus* (Lyne and Hollis 1977) but takes approximately the same time as in *M. eugenii* (Tyndale-Biscoe 1979).

Formation of the unilaminar blastocyst takes about the same time, 3–4 days, in *S. brachyurus, T. vulpecula* and *D. virginiana* (Fig. 3) but, in contrast, in *M. eugenii* (Tyndale-Biscoe 1979) formation of the unilaminar blastocyst took 7–8 days and in *A. stuartii* it took up to 9 days. Transformation of the unilaminar blastocyst into a bilaminar blastocyst by migration of the endoderm from the embryonic area is much slower in *T. vulpecula* (Hughes 1974), *Setonix brachyurus* (Sharman 1955*b*), and *A. stuartii* than in *D. virginiana* (McCrady 1938) (Fig. 3).

Table 3. Comparison of the period of organogenesis, birth weight and type of placenta in marsupials

Species	No. of days to birth from: Primitive streak	Early somite stage	Birth weight (g)	Type of placenta
A. stuartii	6 This study	4 This study	0·0164 Marlow 1961	–
D. virginiana	6 McCrady 1938	5 McCrady 1938	0·13 Hartman 1928	Not invasive McCrady 1938
P. nasuta	6 Lyne and Hollis 1977		0·237 Lyne 1964	Invasive Flynn 1923
T. vulpecula	7·5 Hughes 1974	5 Hughes 1974	0·20 Pilton and Sharman 1962	Not invasive Sharman 1961
S. brachyurus	–	1·5 Tyndale-Biscoe 1963	0·31 Tyndale-Biscoe 1963	Not invasive Sharman 1955*b*
M. eugenii	15 Renfree and Tyndale-Biscoe 1973	11 Tyndale-Biscoe 1979	*c.* 0·5 Tyndale-Biscoe 1973	Slightly invasive Renfree and Tyndale-Biscoe 1973

The time between the first appearance of the primitive streak and the development of the notochord (Fig. 3) is 2 days in both *A. stuartii* and *D. virginiana* (McCrady 1938). This information is not available for any other species of marsupial, but streak and notochord formation is also rapid in many eutherians (New 1966), where it takes approximately the same time.

Organogenesis, the period between the appearance of the notochord and parturition, occupied 5–6 days in *A. stuartii* (Figs 2, 3) compared with 5½ days in *D. virginiana* (McCrady 1938). Exact comparisons with other marsupial species is not yet possible as no aged embryos at this stage, of advanced primitive streak with notochord, have been described. However, one can get approximate values of the period of organogenesis for *P. nasuta, I. macrourus* (Lyne and Hollis 1977), *S. brachyurus* (Sharman 1955*b*), *M. eugenii* (Renfree and Tyndale-Biscoe 1973), and

T. vulpecula (Hughes 1974), starting from either the primitive streak stage (Fig. 3) or the early stages in somite formation (Table 3).

Table 3 shows that for the marsupial species which have been studied there is an apparent relationship between the length of organogenesis, the type of placenta and the weight of the newborn pouch young. Those species in which organogenesis is shortest either have a low birth weight (*A. stuartii*) or non-invasive placenta (*D. virginiana*), or have a slightly higher birth weight and an invasive placenta (*P. nasuta*). Those species in which organogenesis is longer have a higher birth weight and the placenta is not invasive (*T. vulpecula* and *S. brachyurus*), or an even higher birth weight and a slightly invasive placenta (*M. eugenii*). For the latter group the greater the period of organogenesis or the more invasive the placenta the higher the birth weight. Also, in *Setonix* and *Trichosurus* the shell membranes persists to very late in development (Sharman 1961), which must restrict the flow of nutrients to the embryo. *D. virginiana* doesn't quite fit into the general scheme, in that although the birth weight is ten times that of *A. stuartii* and the placenta is non-invasive, organogenesis takes approximately the same length of time. A reexamination of the placenta of *D. virginiana* by ultrastructural techniques may reveal that the embryo–maternal connections are more intimate than previously thought.

In contrast to the development of the opossum (McCrady 1938), that of *A. stuartii* is very slow up to the formation of the bilaminar blastocyst. The following evidence suggests that in this species development halts at the 4-cell and 1·0-mm unilaminar blastocyst stages. These two stages were collected more frequently than any other (Tables 1, 2). If all the 4-cell stages are matched on the one day in the timetable and all the 1·0-mm unilaminar blastocysts are matched on the one day, the rest of the data will not fit unless it is accepted that development proceeds at different rates during each stage of development for each animal studied, which seems unlikely and has never been demonstrated in either eutherians (New 1966) or marsupials (McCrady 1938). In addition, the provisional timetable which was prepared from the embryos collected in 1976, and which implied that development halts at the 4-cell stage and 1·0-mm unilaminar blastocyst, was used to collect embryos of a desired stage during 1978. It is possible that these apparent pauses are a result of the method of preparation of the timetable or a result of surgical intervention affecting the development of the embryos in the remaining uterus. This did not occur in Hartman's study of the opossum (1928) and did not appear to do so here, because the number of embryos removed from one uterus in the first sample was not significantly different to that from the other uterus in the second sample. While this study suggests that development is arrested for up to 4 days at the 4-cell stage and up to 3 days at the unilaminar blastocyst stage, more sampling of embryos at each end of these periods is needed to clarify the extent of developmental arrest. Because of the paucity of material collected at the 1- to 2-cell stages, it may be that the first and second divisions are irregular and slow, so that the 4-cell stage develops at widely different times in each individual.

It may be that the periods of slow or arrested development in *A. stuartii* can be related to the state of the corpus luteum. Woolley (1966*b*) has shown that in this species formation of the corpus luteum takes until 22–21 days before parturition, i.e. day 5–6 in the present timetable (Fig. 2). Therefore cleavage after the

4-cell stage took place only after the corpus luteum was fully formed; variations in the length of arrest at the 4-cell stage (Fig. 1) were presumably due in part to variations in the period of formation of the corpus luteum. In *D. virginiana,* where cleavage is fast, the corpus luteum is formed in 3 days (Hartman 1923) and in the latter half of the third day the embryos undergo the third and fourth cleavage leading to the 16-cell stage (McCrady 1938). In *A. stuartii* hypertrophy of the luteal cells precedes parturition by 20–18 days (Woolley 1966*b*); this is equivalent to day 7–9 in the present timetable, and the period of resumption of cleavage and first expansion of the blastocyst. Woolley (1966*b*) has shown that in *A. stuartii* the corpus luteum reaches its maximum size 11 days before parturition. This corresponds with day 16 of the timetable, the period of conspicuous endoderm formation.

These periods of apparent developmental arrest are true periods of embryonic dormancy (Tyndale-Biscoe 1963) in that no growth or mitotic divisions have been found in whole specimens or flat-mounted blastocysts (although examination of sectioned material is not complete). It is interesting to note the structural similarity between the dormant 1·0-mm unilaminar blastocyst of *A. stuartii* and that of some macropods (Clark 1966; Clark and Poole 1967; Sharman and Berger 1969). In *Cercartetus,* where the gestation period is relatively long, Clark (1967) has demonstrated, by comparison of head length of pouch young and diameter of embryos, that continuous growth and mitotic figures are present in the embryos formed at post-partum oestrus.

The dormant period at the 4-cell stage in *A. stuartii* is so far unique in that it is not found in eutherians, although short pauses have been reported during cleavage in some species (Austin 1961; New 1966). However, McLaren (in Tyndale-Biscoe 1979) reported that in the tammar cleavage stops at the 1-cell stage for 3 days. In dasyurids, all *Antechinus* species so far studied, *Dasycercus cristicauda, Dasyuroides byrnei* and *Sarcophilus harrisii* have relatively long gestation periods, but *Sminthopsis* sp. and some species of *Dasyurus* have relatively short ones (see Woolley 1973, review table). In macropods as well, gestation periods can vary widely between species which produce young of similar birth weight, e.g. *Bettongia* and *Potorous tridactylus* (Tyndale-Biscoe 1973). As information on the rate of development in other marsupials is relatively limited, it is possible that a period of slow development or developmental arrest during cleavage or blastocyst formation will be found in dasyurids and other marsupials where the gestation periods are relatively long.

On the basis of long gestation period and state of newborn young, Sharman (1963) proposed that *Dasyuroides byrnei* and *A. stuartii* may have discontinuous development. This study suggests that this may be so for *A. stuartii.*

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