Cleavage *in vitro* following destruction of some blastomeres in the marsupial *Antechinus stuartii* (Macleay)

LYNNE SELWOOD

Department of Zoology, La Trobe University, Plenty Road, Bundoora, 3083 Victoria, Australia

SUMMARY

The effect of destruction of some blastomeres on subsequent cleavage *in vitro* was investigated in the marsupial *Antechinus stuartii*. Blastomeres were killed by puncturing with a fine glass needle. The positions of the punctured and surviving blastomeres were recorded with respect to the yolk mass and defects on the shell membrane. Blastomeres were punctured in 2-cell, 4-cell, 14-cell, 16-cell and 22-cell embryos. Subsequent development of the embryos was followed *in vitro*. Embryos developed at a normal rate after destruction of some blastomeres. Puncturing of the shell, mucoid layer and zona pellucida, without damage to blastomeres, did not affect subsequent development. The embryos developed in accordance with the proportion of surviving blastomeres. Embryos with half of the blastomeres surviving developed as embryos with half the zona pellucida lined by blastomeres. Embryos with 1/4 of the blastomeres destroyed developed as 3/4 embryos. It is not known, without transfer into surrogate mothers, whether these fractional embryos were capable of developing into complete embryos.

INTRODUCTION

Despite the early studies of Selenka (1887), Hill (1910) and Hartman (1916, 1919) on cleavage and blastocyst formation in marsupials, no experimental analylysis of marsupial development has been attempted. The major reasons for this are the difficulty in obtaining material of a known stage and the lack of techniques for induced ovulation, superovulation and for culture of early marsupial embryos. While New & Mizell (1972) successfully cultured foetal stages of *Didelphis virginiana* and Renfree & Tyndale-Biscoe (1978) achieved maintenance *in vitro* of marsupial blastocysts, culture of early stages proved unsuccessful. However, Selwood & Young (1983) successfully cultured embryos of *Antechinus stuartii* during cleavage. This culture method and the timetable of normal development (Selwood, 1980) make experimental analysis a possibility in this species.

Cleavage and blastocyst formation in marsupials is quite different to that in eutherian mammals. In marsupials, yolk is always eliminated in the early cleavage divisions, either as a distinct yolk mass as in *Dasyurus viverrinus* (Hill, 1910) and *A. stuartii* (Selwood, 1980; Selwood & Young, 1983) or as many small particles as in species of *Didelphis* (Hartman, 1916, 1919; Hill, 1918). The amount and

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distribution of yolk appears to influence the pattern of cleavage in marsupials and two types of cleavage are found (Selwood & Young, 1983). With both types of cleavage pattern, the fourth division results in two tiers of eight cells. Each tier is temporarily distinct from the other with respect to size in *Didelphis* (Hartman, 1916, 1919; Hill, 1918) or histology in *D. viverrinus* (Hill, 1910) and *A. stuartii* (Selwood, 1980; Selwood & Young, 1983). Except when actually dividing, the blastomeres lie separately in the cleavage cavity until the 8-cell stage at the beginning of the fourth division.

Blastocyst formation in marsupials has some similarities to the events of compaction as described by Ducibella & Anderson (1975, 1979) and Ducibella, Ukena, Karnovsky & Anderson (1977) in the mouse embryo. In marsupials a marked change in blastomeric form and activity begins about the 8-cell stage (Hill, 1910; Hartman, 1916, 1919; Lyne & Hollis, 1976, 1977; Selwood, 1980; Selwood & Young, 1983) and this is accompanied by increasing amounts of contact between blastomeres (Lyne & Hollis, 1976, 1977). These changes occur *in vivo* and *in vitro* (Selwood & Young, 1983). However, the markedly different features of marsupial blastomeric activity at this time effectively exclude the formation of a morula and an inner cell mass and result in the unilaminar marsupial blastocyst. All blastomeres develop the capacity to flatten, but flatten against the zona pellucida, *never* against each other. As they flatten and spread against the zona, they make contact with their neighbours and eventually form a complete epithelium lining the zona. The cells which will later give rise to the embryo are at this stage indistinguishable from the remainder of the blastocyst. It is not known whether the initial differentiation of the blastocyst into embryonic and extraembryonic areas is because certain early cleavage divisions are determinate as was suggested by Hill (1910) and (Hartman, 1916, 1919). This study is an investigation of this problem. The capacity of the embryo to develop following the loss of blastomeres at the 2-cell, 4-cell and 16-cell stage is investigated *in vitro*.

MATERIALS AND METHODS

A. stuartii were trapped near Kinglake, Victoria, and maintained in the laboratory following the procedures outlined by Woolley (1973). A urine sample was collected daily during oestrus when females were mated. The urine was scored for the presence of sperm which is present following mating in dasyurids (Godfrey, 1969; Woolley, 1971; Selwood, 1982) and for a persistent reduction in the number of cornified epithelial cells which occurs within 24 h of ovulation in this species (Selwood, 1980, 1982). Embryos were sampled at timed intervals after ovulation using the timetable of development for this species (Selwood, 1980).

Anaesthesia was induced with an initial dose of 4·5 % halothane in a 1:1 mixture of oxygen and nitrous oxide for 2 to 3 min. The gas was administered at the rate of $200 \, cc \, min^{-1}$ using a Cyprane continuous flow TEC Vaporiser. Anaesthesia was maintained at 2·5 % halothane. In some cases, the uterus and oviduct from the right-hand side were removed and the animal was allowed to recover. After removal of both uteri, the animals were killed by exsanguination or terminal anaesthesia. The ovaries and uteri were placed in warm calcium- and magnesium-free phosphate-buffered saline (PBS^-), transferred to a laminar flow hood and washed in warm PBS^- to remove any blood. An estimate of the number of embryos in each uterus was obtained by counting the number of corpora lutea per ovary (Selwood, 1983). The embryos were removed by flushing or by slitting open and inverting the uterus into warm PBS^-, counted, and then

washed in the culture medium. The medium, which was changed every one or two days, was the mixture of antibiotics, glutamine and 10 % foetal calf serum in Dulbecco's Modified Eagle's Medium (DMEG) with high glucose (Gibco) used by Selwood & Young (1983) for culture of embryos of this species. Embryos were cultured in 2·0 ml of DMEG per well in a 24-well covered Linbro plate in an incubator maintained at 35 °C and 10 % carbon dioxide in air.

Embryos were held in DMEG in the incubator until needed for manipulation, or in the case of zygotes, until the 2-cell stage was reached. Manipulations were performed in DMEG, with added antibiotics and glutamine but without added foetal calf serum, at room temperature. Embryos were suspended in DMEG in a well slide on a Leitz Diavert during the manipulation. They were immobilized by the use of a sucking pipette with inner diameter of 120 μm prepared from plain microhaematocrit tubes (length 75 mm, O.D. 1·5 mm, I.D. 1·10 mm, Clay Adams). Sucking pressure was provided by an Agla syringe, filled with sterile paraffin and, at the tip of the syringe, a small amount of medium. Blastomeres were killed by puncturing, sometimes several times, with a fine glass needle with a tip diameter of 1 to 2 μm. The microinstruments were held in Albrecht micromanipulators.

As no techniques for superovulation or induced ovulation have been developed for marsupials and they are seasonal breeders, the number of available embryos for any experiment is low. The numbers were especially low in 1984 (Table 1) compared to previous years (Selwood, 1983), possibly because of severe drought conditions. For this reason it was decided to try and minimize the number of control embryos as much as possible. As normal development and cleavage times in culture have previously been described for *A. stuartii* (Selwood & Young, 1983) few unmanipulated embryos were cultured. The effect of manipulation and puncturing of the investments (shell, mucoid and zona layers) without damaging a blastomere was tested on the first batch of 2-cell embryos but not on subsequent embryos. In all females some embryos had imperfections in deposition of the shell layer. These imperfections in shell deposition have been found previously in embryos of this and other species of dasyurids and do not appear to affect subsequent development (Selwood, unpublished). In these specimens, the position of the puncture was recorded with respect to the imperfections in the shell as well as to the position of the yolk mass and other blastomeres. The following experiments were performed.

(1) *Experiments with 2-cell embryos*

The effect of damage to the egg investments alone on subsequent development was tested in four specimens (Table 2) by puncturing the investments adjacent to a blastomere in 2-cell embryos (Female No. 1, Table 1). One of these embryos had three small imperfections of the shell. After development *in vitro* to the 2-cell stage, three embryos from female No. 2 (Table 1) had one of the two blastomeres punctured (Table 2). One embryo had a large additional mass of shell deposited on the surface of the shell layer.

(2) *Experiments with 4-cell embryos*

Early 4-cell embryos were obtained from female No. 3 and late 4-cell embryos from female No. 2 (Table 1). Of the five early 4-cell embryos one was damaged during manipulation and was discarded and one was cultured without any manipulation (Table 2).

Destruction of one blastomere. Two early 4-cell embryos and three late 4-cell embryos each had one of the four blastomeres punctured (Table 2).

Destruction of two blastomeres. Two adjacent blastomeres were destroyed in an early 4-cell embryo and in a late 4-cell embryo (Table 2).

(3) *Experiments with 14- to 16-cell embryos*

These stages were obtained from females No. 4 and 5 (Table 1). Some of these specimens had defects in shell deposition. Three 16-cell embryos were cultured without manipulation (Table 2). During the non-synchronous fourth division, the eight blastomeres which lie stretched on the zona pellucida extending along the meridians of the egg from the yolky pole towards the non-yolky pole, are divided to form eight upper blastomeres in a ring around the yolk mass and eight lower blastomeres (Selwood & Young, 1983). In manipulation of embryos

with between 8 and 16 cells, the as yet undivided blastomeres from the 8-cell stage will be called 'long blastomeres', blastomeres of the upper ring will be called 'upper blastomeres' and those of the lower ring 'lower blastomeres'. In one 14-cell embryo, one of the upper blastomeres and one of the two remaining long blastomeres were punctured (Table 2). In another 14-cell embryo one of the two long blastomeres was destroyed (Table 2). In four embryos with 16 cells, one of the upper blastomeres was destroyed in each of three embryos and one of the lower blastomeres was destroyed in one embryo (Table 2).

(4) *Experiments with embryos with more than 16 cells*

One embryo, which was a complete blastocyst with about 22 cells, was obtained from female No. 5 (Table 1). One of the upper blastomeres near the yolk mass was destroyed in this embryo.

After manipulation and recording the position of puncture all embryos were washed briefly in warm complete medium, returned to the culture well, incubated and examined and photographed at daily intervals.

RESULTS

1-cell to early 4-cell stages were obtained in the first day and a half after the estimated time of ovulation (Table 1). Late 4- to 32-cell stages were obtained during days 4 and 5 after ovulation. The number of embryos per uterus (Table 1) was the same or approximately the same as the number of corpora lutea counted in the ovary and varied from nought to six with a mean of $3 \cdot 3 \pm 1 \cdot 7$ s.D. (n = 11).

A punctured blastomere extruded some of its cytoplasmic contents into the blastocoel. If large amounts of cytoplasm were extruded the blastomere became transparent. If relatively small amounts of cytoplasm were extruded the blastomere became shrunken and darkened. The punctured blastomeres never flattened against the zona pellucida or divided to form two daughter blastomeres. Punctured blastomeres which were already flattened on the zona usually lost contact with the zona pellucida and became rounded shrunken cells lying in the blastocoel. In one case (one from Female No. 2), the shrunken cell remained attached to the zona pellucida.

Table 1. *Stage of development at timed intervals after estimated ovulation in* Antechinus stuartii

Female number	Time after estimated ovulation (days:hours)	Stage of development	Number of embryos per uterus
1	—	not ovulated	0
	0:17	2-cell	4
2	1:05	1-cell	4
	4:05	late 4-cell	4
		unfertilized	2
3	0:21	early 4-cell	5
		unfertilized	1
	3:21	c. 32-cell	3
4	4:21	14- to 16-cell	5, 3
5	5:12	16- to 22-cell	5, 0

(1) *Experiments with 2-cell embryos*

Of the four embryos at the 2-cell stage (Fig. 1) which were punctured but had no destruction of blastomeres (Table 2), one did not develop past the 2-cell stage. The other three each had four rounded cells after 24 h in culture. This arrested stage (Selwood & Young, 1983) persisted for a further 48 h, then the four blastomeres flattened on the zona pellucida as a ring of lens-shaped cells around the yolk in preparation for the next division (Fig. 2). After this the embryos deteriorated. The three zygotes (Fig. 3) which were cultured to the 2-cell stage over 23 h, each had one of the two blastomeres punctured just prior to the second division (Table 2). They completed the division after a further 6 h in culture. The punctured blastomere shrivelled and lay free in the blastocoel and the remaining blastomere divided to form two apparently normal blastomeres. These daughter blastomeres rounded up and persisted in this arrested state for 48 h when they began flattening on the zona in preparation for the next division, so that they formed half of a ring of cells encircling the yolk mass (Fig. 4). The embryos began to disintegrate during the next 20 h in culture.

Embryos at a similar stage of development to these embryos developing *in vitro* (i.e. flattening 4-cell stage) were obtained within one hour of the *in vitro* observations from the left uterus of the same female (Female No. 2, Table 1) which provided the original 1-cell stages from the right uterus.

(2) *Experiments with 4-cell embryos*

Development is arrested at the 4-cell stage in *A. stuartii* (Selwood, 1980) and embryos which are collected early in this stage fail to complete successfully the next division in culture (Selwood & Young, 1983). One early 4-cell embryo which was not manipulated was cultured to the failing 8-cell stage (Table 2) over 72 h. Late 4-cell embryos can be successfully cultured and usually successfully complete the next two or three cell divisions (Selwood & Young, 1983).

Destruction of one blastomere. Two early 4-cell embryos which had one of the four blastomeres punctured each had three flattened blastomeres forming an incomplete ring of blastomeres around the yolk mass (Fig. 5) after 72 h *in vitro* (Table 2). No blastomeres occupied the space of the zona where the damaged blastomere would normally be found. In the three embryos which were at the late 4-cell stage when manipulated (Table 2), the surviving three blastomeres in each embryo were still flattened as an incomplete ring of blastomeres around the yolk mass after 20 h in culture. In one embryo during the next 20 h, the third division was completed to give six blastomeres. These six then flattened and started the fourth division to give seven blastomeres (Fig. 6), one of which was in the process of mitosis. After a further 24 h, this division was still incomplete and the embryo had nine blastomeres, three large and six small (Table 2). Another embryo had completed the third division to give six living blastomeres after 40 h of culture. One blastomere had started the next division (Fig. 7). The killed blastomere, although rounded and shrunken, remained stuck to the zona pellucida in this
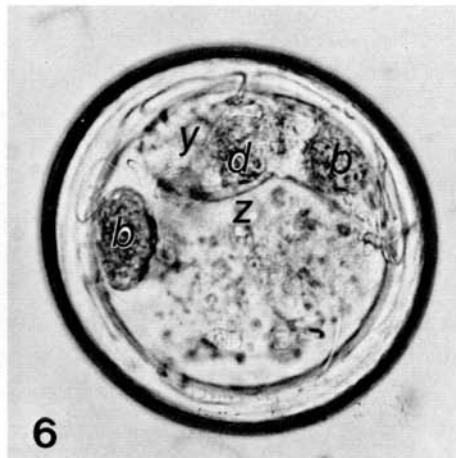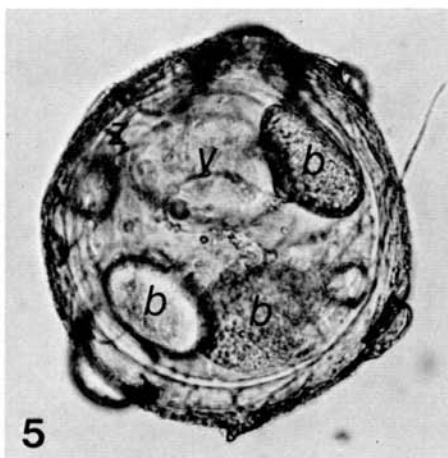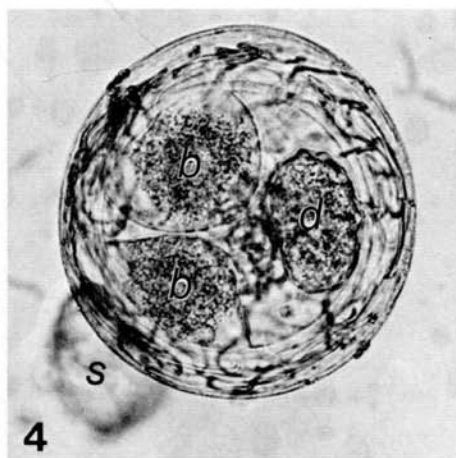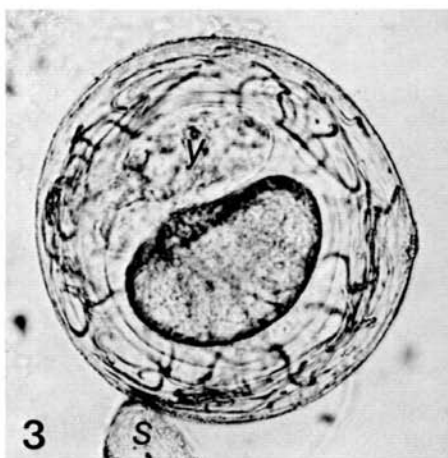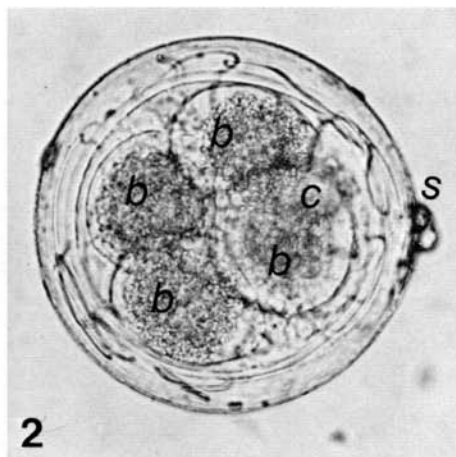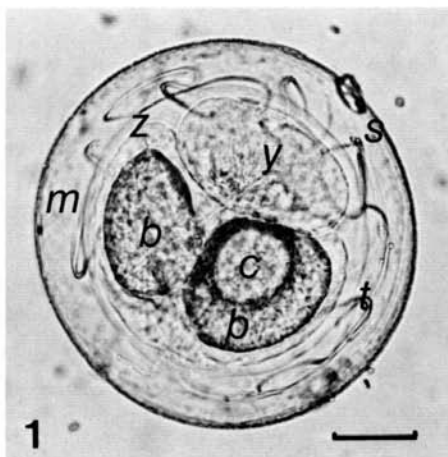
embryo. It was surrounded by cell-free zona occupying about a quarter of the inner surface of the zona pellucida. This embryo did not develop past seven cells (Table 2). In the other embryo the third division was incomplete after a total of 40 h in culture and it had five blastomeres (Table 2). Two of the original three surviving blastomeres had divided but the other had not. The zona pellucida was free of blastomeres in the space which would normally be occupied by the progeny of the blastomere which was killed and which now lay free in the blastocoel.

Destruction of two blastomeres. In the early 4-cell embryo after 72 h in culture, the two surviving blastomeres had flattened to form half a ring composed of two lens-shaped flattened blastomeres around the yolk (Table 2). The two damaged blastomeres lay free in the blastocoel. After 40 h in culture the two remaining blastomeres in the late 4-cell embryo had completed the third division to produce four blastomeres and started the next, giving five blastomeres (Fig. 8, Table 2). The two killed blastomeres which could be seen lying free in the blastocoel after 20 h of culture, had begun to fragment by this stage. No blastomeres were found

Fig. 1. Unmanipulated 2-cell stage (from Female No. 1) viewed from the side. The yolk mass (*y*) lies separately from the two blastomeres (*b*). One blastomere has an additional mass of cytoplasm (*c*) associated with it. The embryo is enclosed within a zona pellucida (*z*), mucoid coat (*m*), in which the tails of many spermatozoa (*t*) are visible and a shell (*s*) with an abnormal bleb in the shell coat. Scale bar Figs 1–6 equals 50 μm.

Fig. 2. The same embryo as in Fig. 1 after 72 h in culture and viewed from the non-yolky pole. The four blastomeres (*b*) have started to round up and deteriorate after flattening on the zona pellucida. The yolk mass which is not visible and the cytoplasmic mass (*c*) which is faintly visible lie beneath the blastomeres. The bleb of shell (*s*) seen in Fig. 1 lies slightly out of focus to one side.

Fig. 3. Unmanipulated 1-cell stage (from Female No. 1) just prior to the first division viewed from the side. The yolk mass (*y*) is separating from the remainder of the vitellus. A large bleb of shell material (*s*) is located on the shell.

Fig. 4. The same embryo as in Fig. 3 viewed from the yolky hemisphere after a total of 77 h in culture. The yolk mass is not visible at this depth of focus. After 23 h in culture the first division was completed and one of the two blastomeres was punctured. The surviving blastomere divided after a further 6 h in culture. During the next 48 h the blastomeres rounded up in the arrested state and then flattened against the zona pellucida as shown in this figure. In this figure the two flattened blastomeres (*b*) and the shrunken punctured blastomere (*d*) are visible and the defect in the shell coat (*s*) is out of focus on one side.

Fig. 5. Oblique view from the yolky pole of the late 4-cell stage after 72 h in culture (from Female No. 3). The three surviving blastomeres (*b*) are flattening to form an incomplete ring of cells about the yolk mass (*y*) and the part of the zona pellucida (*z*) which would normally be occupied by the fourth punctured blastomere is free of cells. The punctured blastomere has begun to disintegrate and is not visible. Many imperfections in deposition of the shell are present in this specimen.

Fig. 6. View from the side of an embryo from Female No. 2 during the fourth division. It shows the cell-free space on the zona pellucida (*z*) which would normally be occupied by the progeny of the one out of four blastomeres which was punctured. In this embryo the surviving three blastomeres had completed the third division to give six blastomeres, then started the fourth division and now has seven blastomeres. Two of these (*b*) are lying against the zona pellucida and the yolk mass (*y*) and a portion of the cytoplasm of the punctured blastomere (*d*) lies in the blastocoel.

Table 2. *Development* in vitro *following puncture of some blastomeres during cleavage in the marsupial* Antechinus stuartii

Stage of development	Experiment		Results	
	No. of embryos	Proportion of cells punctured	No. of embryos	Proportion of cells surviving
2-cell	4	0/2	3	4/4
			1	0/2
2-cell	3	1/2	3	2/4
early 4-cell	1	0/4	1	8/8
	2	1/4	2	3/4
late 4-cell	3	1/4	1	9/13
			1	7/11
			1	5/8
early 4-cell	1	2/4	1	2/4
late 4-cell	1	2/4	1	5/13
16-cell	3	0/16	3	32/32
14-cell	1	2(1U+1Lo)/14	1	26/32
	1	1Lo/14	1	28/32
16-cell	3	1U/16	3	26/28
	1	1Lo/16	1	26/28
22-cell	1	1U/22	1	32/32

In the column 'proportion of surviving cells', the left-hand number is the number of living cells in the embryo and the right-hand number is the possible number of living cells if the punctured cells had continued to divide. For embryos with between 9 and 16 cells: L, lower blastomere; Lo, long blastomere; U, upper blastomere.

lining the zona pellucida in the space which would normally be occupied by the progeny of the two blastomeres which were killed.

(3) *Experiments with 14- to 16-cell embryos*

The three 16-cell specimens, which were not manipulated, developed to an early expanding blastocyst of about 32 cells after 20 h in culture (Table 2). After a further 8 h in culture, the blastocysts showed rounding up of some of the cells.

In the 14-cell embryos, during 20 h of culture, the fourth and fifth cleavage divisions were finished to form blastocysts which were completely lined by cells except for a space on the zona which extended from the yolky pole to the non-yolky pole (Fig. 9, Table 2). These blastocysts had rounded cells and had started to fail after a further 24 h in culture.

In each of three embryos with 16 blastomeres with one of the upper blastomeres destroyed, the fifth division was almost completed during 20 h of culture. The resultant blastocysts were fully lined by cells except for a space on the zona lying above the yolk mass (Fig. 10) and a space on the zona of the non-yolky hemisphere which was not yet covered by cell processes (Table 2). In the 16-cell embryo with one of the lower blastomeres destroyed, the embryo had almost completed the fifth division after 20 h of culture but had a cell-free space on the zona pellucida in the non-yolky hemisphere (Fig. 11, Table 2). This space was much larger than the

cell-free space which is normally found at this stage. The blastomeres in this embryo had begun to round up.

### (4) *Experiments with embryos with more than 16-cells*

In this blastocyst with about 22 cells, the punctured cell, after losing some cell contents, rounded up, detached from the zona and lay free in the blastocoel. After 20 h in culture this blastocyst was an apparently normal blastocyst in early expansion with no cell-free spaces on the zona pellucida (Fig. 12, Table 2).

DISCUSSION

Developmental times *in vitro* in *A. stuartii* (Selwood & Young, 1983) are similar although slightly slower than those *in vivo* (Selwood, 1980). In this study, embryos obtained from one uterus developed *in vitro* at a similar rate (Female No. 2) or about 12 h slower to (Female No. 3) embryos developing *in vivo* in the remaining uterus. However, as the cleavage divisions *in·vitro* were not timed with time-lapse cinematography and getting exact developmental times *in utero* has some problems (Selwood, 1980) exact comparisons between the two were not possible. Developmental times in culture in both unmanipulated and manipulated embryos did not differ markedly from those obtained by Selwood & Young (1983) for this species. The behaviour of the remaining intact blastomeres following destruction of one or more of the population was similar to that in normal untouched embryos both in this and other studies (Selwood & Young, 1983).

The control experiments at the 2-cell stage where egg investments, but not one of the blastomeres, were punctured, established that the puncture did not seem to prevent blastomeres flattening on that part of the zona pellucida as three of the four 2-cell stages developed to normal flattened 4-cell embryos. As punctured blastomeres, except in one case, detached from the zona and lay free in the blastocoel, they did not prevent other blastomeres flattening on that part of the zona. It is· possible that the presence of the dead blastomeres influenced the development of the surviving blastomeres as has been suggested in similar experiments on rabbit and amphibian embryos (Denker, 1976). This possibility was not explored in this study because of the technical difficulties associated with removing such large blastomeres whilst leaving the egg investments relatively intact. However, a small proportion of blastomeres normally are detached from the zona into the blastocoel and die during early stages of blastocyst formation in *A. stuartii* (Selwood & Young, 1983). As this occurs in a number of other marsupials (Lyne & Hollis, 1977), the presence of dead blastomeres is probably not detrimental to further development.

Abnormalities in the deposition of the shell in some embryos made it possible to destroy a specific blastomere with respect to the yolk mass and blebs on the shell membrane. Results in these specimens confirmed that the yolk mass remains fixed during development as suggested by Hill (1910), Selwood (1980) and Selwood & Young (1983).
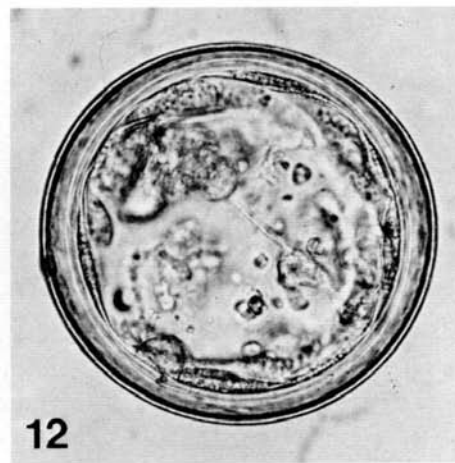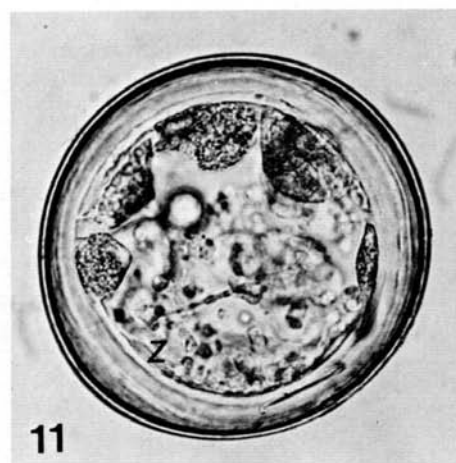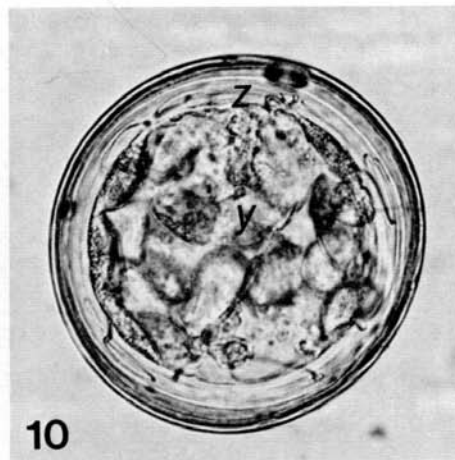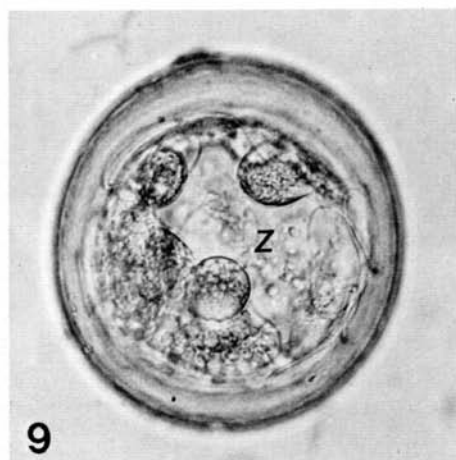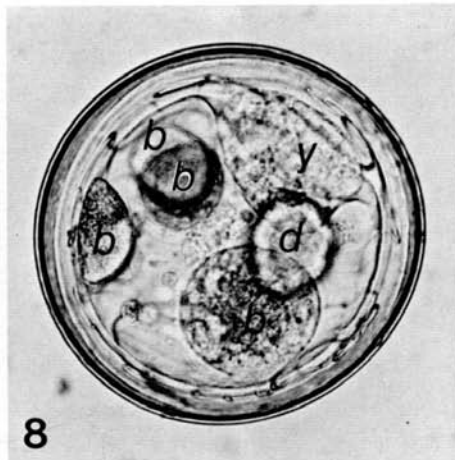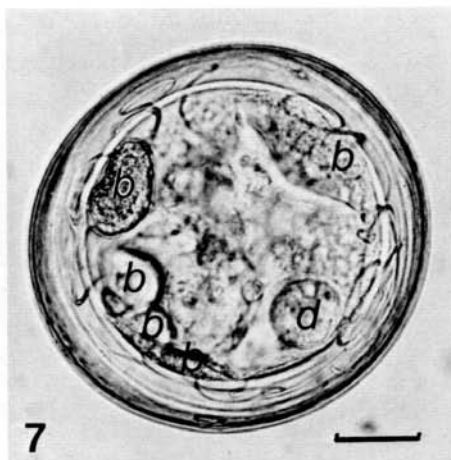
The development of blastomeres following destruction of one or more blastomeres is schematically summarized in Fig. 13. Normal development in unmanipulated embryos is included for comparison (Fig. 13A). Following destruction of one out of two, or two out of four blastomeres the remaining blastomeres and their progeny flattened to lie in their adjacent half of the zona pellucida (Fig. 13B,D). Where one out of four blastomeres was destroyed, the remaining three blastomeres and their progeny flattened to lie in their adjacent 3/4 of the zona pellucida (Fig. 13C). Destruction of one of the long blastomeres (undivided blastomere from the 8-cell stage) during the fourth division, resulted in a blastocyst which had a cell-free space along the length of a meridian in the wall of an otherwise complete blastocyst. Destruction of one of the blastomeres of the upper ring around the yolk at the 16-cell stage, produced an otherwise complete blastocyst with a space on the zona pellucida above the yolk mass (Fig. 13E). At the 16-cell stage, destruction of one of the blastomeres of the lower ring resulted in an otherwise complete blastocyst with a cell-free space on the zona pellucida in the lower hemisphere (Fig. 13F).

Blastomeres tended to attach to the area of zona pellucida nearest to them and they and their progeny were not capable, at least during the next few divisions, of occupying the space on the zona pellucida normally occupied by other

Fig. 7. Slightly oblique view from the non-yolky pole showing cell-free zona around the punctured blastomere (*d*) and five of the six living blastomeres (*b*). This four-cell embryo from Female No. 2 had one blastomere punctured. The remainder completed the third division to give six blastomeres which were flattening in preparation for the fourth division after 40 h *in vitro*. Scale bar Figs 7–12 equals 50 μm.

Fig. 8. Oblique view from the side of an embryo from Female No. 2. Two of the four blastomeres were punctured in this specimen which has completed the third division and started the fourth, giving five blastomeres (*b*), four of which are visible. Cytoplasm from one of the punctured blastomeres (*d*) is lying in the blastocoel near the yolk mass (*y*) which is obscuring the fifth blastomere.

Fig. 9. Slightly oblique view from the yolky pole of a blastocyst developed after 20 h *in vitro* from a manipulated 14-cell embryo from Female No. 4. This embryo had one long and one adjacent upper blastomere punctured. The punctured blastomeres became detached into the blastocoel leaving a cell-free space on the zona (*z*) extending from the yolky pole to the non-yolky pole.

Fig. 10. Side view of a blastocyst which has developed after 20 h *in vitro* from a 16-cell embryo from Female No. 4. One of the upper blastomeres was punctured in this embryo at the 16-cell stage. In addition to the normal cell-free zona in the non-yolky hemisphere, which has not yet been covered by blastomeres, cell-free zona (*z*) is also found in the yolky hemisphere above the yolk mass (*y*).

Fig. 11. Side view of a blastocyst which has developed after 20 h *in vitro* from a 16-cell embryo from Female No. 4. One of the lower blastomeres was punctured in this embryo at the 16-cell stage. A wide cell-free space is found on the zona (*z*) in the non-yolky hemisphere. This space is much wider than would normally be expected at this stage. Compare with Fig. 10.

Fig. 12. Complete blastocyst in early expansion which was developed after 20 h in culture from a 22-cell embryo from Female No. 5. One upper blastomere near the yolk mass was punctured in this embryo before culture. The zona pellucida is completely lined by flattened blastomeres and compression of the mucoid layer has begun as the blastocyst expands.
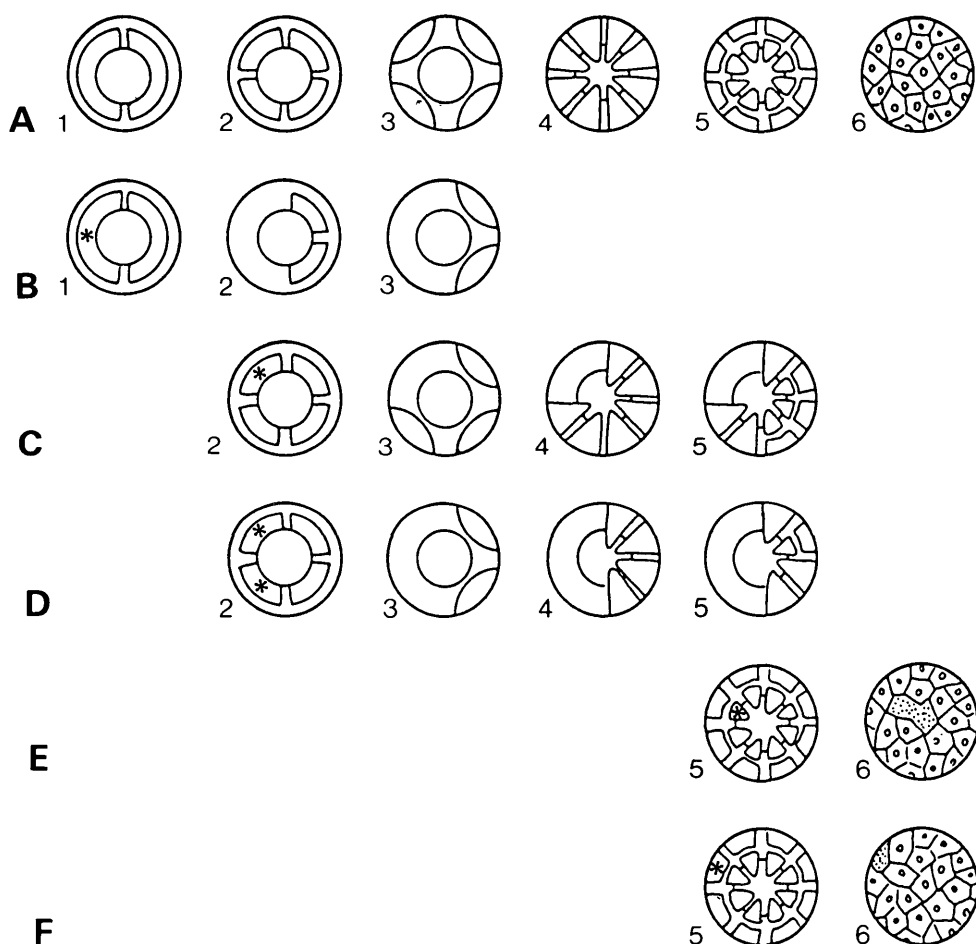
Fig. 13. Diagrammatic representation of cleavage *in vitro* following puncture of some blastomeres in *Antechinus stuartii*. Mucoid and shell layers are not shown. Not drawn to scale. The punctured blastomere is marked with a star. A, 1–6. Normal cleavage from the 2-cell stage to complete unilaminar blastocyst viewed from the yolky pole. By the 2-cell stage (A) the yolk mass has separated from the two blastomeres and lies as a spherical mass centrally above the two blastomeres. Based on information in Selwood & Young (1983). B, 1–3. Development following puncture of one of the two blastomeres. C, 2–5. Development following puncture of one out of four blastomeres. D, 2–5. Development following puncture of two out of four blastomeres. E, 5–6. Development following puncture of one upper blastomere out of 16 blastomeres. F, 5–6. Development following puncture of one lower blastomere out of 16 blastomeres.

blastomeres. This was very obvious in those specimens where blastomeres could be identified by their position relative to the yolk mass and blebs on the shell. Embryos with only a part of the blastocyst wall developed have been reported in *D. virginiana* Hartman (1919) and in *D. viverrinus* Hill (1910). These specimens had normally flattened blastocyst epithelium in one hemisphere, some rounded cells or degenerating cells in the blastocoel and cell-free zona pellucida in the other

hemisphere. Both authors gave this as supportive evidence for their suggestion that during cleavage, two cell lines, the formative (or embryonic) and the non-formative (or extraembryonic) are established.

   This study supports the suggestion of Hill (1910) and Hartman (1916, 1919) that the position of a blastomere during cleavage predicts the position of its progeny in the blastocyst. In *A. stuartii* at the 16-cell stage as in *D. viverrinus* and *D. virginiana*, the eight upper blastomeres contribute to one hemisphere of the blastocyst and the eight lower blastomeres to the other half. However, this study does not support or refute the second part of their hypothesis, namely that during cleavage (at the first division in *D. virginiana* and the fourth in *D. viverrinus*) two cell lines, the embryonic and extraembryonic, are established. In all marsupials, a unilaminar blastocyst with all the cells apparently identical develops at the end of cleavage before the appearance of the primary endoderm divides the embryo into presumptive embryonic and extraembryonic areas. Without the use of cell markers, the intervention of the uniform unilaminar blastocyst stage makes it impossible to relate the cleavage pattern to cell fate. In *A. stuartii* the differentiation of the blastocyst into embryonic and extraembryonic areas does not occur until day 16 of development when the blastocyst measures 1·3 to 1·5 mm in diameter (Selwood, 1980). The marsupial embryo is capable of regulation to accommodate for the loss of some cells during blastocyst formation and early expansion, as death of some cells is always a feature of this stage (Hill, 1910; Hartman, 1916, 1919; McCrady, 1938; Lyne & Hollis, 1976, 1977; Selwood & Young, 1983). However, the extent of its capacity to regulate is unknown. In order to follow the effect of destruction of one or more blastomeres during cleavage on differentiation into embryonic and extraembryonic areas in the blastocyst it will be necessary to transfer embryos back into surrogate mothers.

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