Development *in vitro* of Marsupials: a comparative review of species and a timetable of cleavage and early blastocyst stages of development in *Monodelphis domestica*

LYNNE SELWOOD[1]*, EDWARD S. ROBINSON[2], ROGER A. PEDERSEN[3] and JOHN L. VANDEBERG[2]

[1]*School of Zoology, La Trobe University, Bundoora, Victoria, Australia,*
[2]*Department of Genetics, Southwest Foundation for Biomedical Research, San Antonio, Texas, USA and*
[3]*Laboratory of Radiobiology and Environmental Health, University of California, San Francisco, California, USA*

ABSTRACT The development of marsupial oocytes and embryos *in vitro* is reviewed. Most stages of development have been cultured successfully, usually in a complex medium with added fetal calf serum. Simpler media without added serum have been developed for fertilization and cleavage *in vitro*. Culture systems have been established for oocyte maturation and fertilization in the grey short-tailed opossum and for cleavage from the zygote to the early expanding unilaminar blastocyst in a number of other marsupials. Survival *in vitro* of the unilaminar and early bilaminar blastocyst stages is limited in all species examined. In contrast, late bilaminar, trilaminar, embryonic and fetal stages develop at rates approximating those *in vivo*. More stages have been cultured successfully in *Sminthopsis macroura* than in any other species. It has been cultured from the late bilaminar blastocyst to within 18 h of birth. Stages of cleavage and unilaminar blastocyst formation of *Monodelphis domestica* timed by videotaping mating animals, proceeded at similar rates *in vivo* and *in vitro*. As in other marsupials, cleavage in this opossum is characterized by a polarized conceptus. This polarity is expressed in the distribution of organelles in the zygote and the localization of secretion of the extracellular matrix material into the cleavage cavity and of the initial cell-zona attachment. Because cell-cell adhesion follows cell-zona adhesion, a unilaminar blastocyst forms without the development of an intervening morula stage.

KEY WORDS: *Marsupial, development, in vitro, Monodelphis domestica, Sminthopsis macroura*

Introduction

Marsupials are excellent animals for the study of mammalian development *in vitro* for a number of reasons. During cleavage, cell-zona adhesion occurs a number of divisions before cell-cell adhesion (Selwood, 1992). As a result a unilaminar blastocyst develops without an intervening morula stage. The blastocyst consists of pluriblast, a lineage with wide potential, which gives rise to the embryo and the extra embryonic membranes (Johnson and Selwood, 1996) and the trophoblast. The development of pluriblast, trophoblast, hypoblast and mesoderm can be studied in a relatively simple epithelium before implantation. Marsupials are also good models for the study of formation of the embryonic axes because these are formed well before implantation occurs. The pioneering study of New and Mizell (1972) recognized that because the embryos of the Virginia opossum *Didelphis virginiana* were not implanted until relatively late in development, the opossum would make an excellent model animal for the study of organogenesis in mammals. Similarly, in all marsupials studied implantation does not occur until the embryo has between 13 and 22 somites (Selwood, 1989a; Selwood and Woolley, 1991). Also, the late timing of implantation makes the extended preimplantation stages of marsupials suitable for studies to distinguish between embryo-trophic and utero-trophic signals during lineage formation and early organogenesis. Despite these advantages, progress in the study of marsupial embryos *in vitro* has been slight until the last 10 years or so. The major reasons for this have been the difficulty of determining the day of ovulation and hence getting access to accurately timed material in animals that are polyovular. These problems have now been solved in the brown antechinus, *Antechinus stuartii* (Selwood, 1980), stripe-faced dunnart, *Sminthopsis macroura* (Selwood, 1987; Selwood and Woolley, 1991) and the grey short-tailed opossum (Baggott and Moore, 1990; Mate *et al.*, 1994).

Abbreviations used in this paper: BSA, bovine serum albumin; CL, corpus luteum; DMEM, Dulbecco's Modified Eagle's Medium; HAF, human amniotic fluid; pc, post coitum.

***Address for reprints:** School of Zoology, La Trobe University, Plenty Rd, Bundoora, 3083 Vic, Australia. FAX: 61.3.94791551. e-mail: zools@zoom.latrobe.edu.au

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Unlike culture of the mouse conceptus, that of the marsupial has been developed against a background of very little information about the uterine environment. The studies of Renfree (1973, 1975) on the nature of the uterine protein secretions of the tammar wallaby, *Macropus eugenii* and the Virginia opossum have not been repeated for other marsupials. Most such vital information as the pH, osmotic pressure, protein profiles, nutrient concentrations and the type of growth factors present is unknown for all marsupials.

An unusual feature of the marsupial conceptus is the egg coats. The oocyte has a zona pellucida and is ovulated without a cumulus (Hill, 1910; McCrady, 1938). As it descends the tract it acquires a mucoid coat in the oviduct and a shell coat at the uterotubal junction or in the uterus (see Selwood, 1992; Roberts *et al.*, 1994 for review). These coats vary in thickness, particularly the mucoid coat. The mucoid coat is very thick in some didelphid opossums (>100 μm), thinner in the grey short-tailed opossum and the brush possum (ca. 50 μm) but is relatively thin in the dasyurids and the bandicoots (<25 μm). It is very susceptible to osmotic changes and the thickness presumably influences uptake of material. Hughes and Shorey (1973) showed that the molecular weight of substances affected their penetration of the coats. Because of its thickness and differential loss during blastocyst growth, Hartman (1919) suggested that the mucoid coat had a nutritive function in the Virginia opossum. Attempts, as outlined below, have been made to culture some marsupial embryos, or cells from them, without their egg coats.

The stages of marsupial embryonic development that have been cultured are shown in Figure 1 and the progress made for each stage is reviewed below. The special features and problems associated with the culture of marsupial embryos are examined in the discussion.

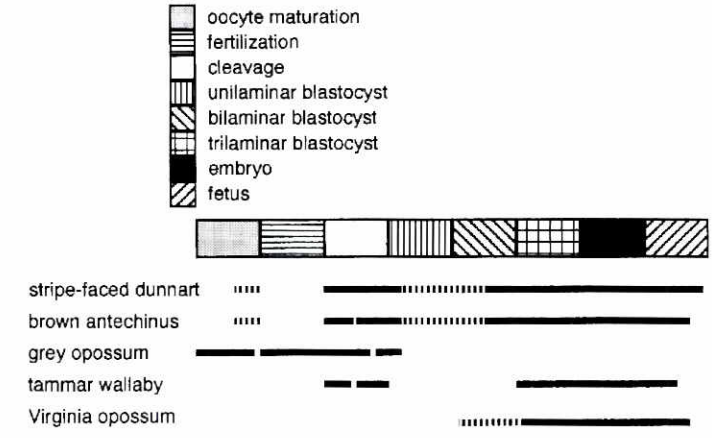

Fig. 1. Diagram illustrating the stages of development that have been cultured in different marsupials based on studies quoted in the text. *The solid line represents continuous culture of 12 or more h between the developmental stages indicated. The broken line represents periods of discontinuous culture between 6 and 12 h each.*

TABLE 1

SUMMARY OF CLEAVAGE *IN VITRO* FROM THE ZYGOTE TO THE BLASTOCYST WITH 32 CELLS OF A NUMBER OF SPECIES OF MARSUPIALS COMPARED WITH THE MOUSE

Species	Stage of culture start	Stage of culture end	Length of stage (h) 4-c	Length of stage (h) other	Medium	T (°C)	Ref.
S. macroura	zyg	bl	34	8-10	DMEM+10% FCS, glut. & lactate suppl.	35	1,2
	zyg	bl	-	-	mod. M16+EEAA	35	3
S. crassicaudata	zyg	late 4-c	48	12-18	DMEM+10% FCS, lactate suppl.	35	1
	late 4-c	bl					
M. domestica	zyg	bl	48	12-30	Ham's F10	37	4
	zyg	bl	6-25	8-25	DMEM+10%FCS	32.5	5,6
	zyg	1-c	13	20-24	mod.MEM+ BSA	32.5	7
	zyg	2-c	-	16	TALP-Hepes	37	8
M. eugenii	zyg	8-c	-	8-24	DMEM+10% FCS, lactate suppl.	37	9
A. stuartii	zyg	late 4-c	79	11-18	DMEM+ 10% FCS	35	2,10
	late 4-c	bl					
M. musculus	zyg	hatched bl	8-13	8-20	M16+BSA	37	11

Incubations were made in 5 or 10% CO_2 in air. bl, blastocyst; c, cell; EEAA, Eagle's essential amino acids; glut., glutamine; mod., modified; other, other cleavage stages, i.e. 1-cell, 2-cell, 8-cell, 16-cell, 32-cell; ref., references; suppl., supplements; T, temperature; zyg, zygote. List of references: 1) Selwood, 1987; 2) Selwood and Smith, 1990; 3) Gardner, *et al.*, 1996; 4) Baggott and Moore, 1990; 5) Selwood and VandeBerg, 1992; 6) This study; 7) Moore and Taggart, 1993; 8) Breed *et al.*, 1994a; 9) Renfree and Lewis, 1996; 10) Selwood and Young, 1983; 11) Pratt, 1987.

Oocyte maturation and parthenogenetic development

Recently, stages in oocyte maturation and early stages in parthenogenetic development of marsupials have been cultured successfully (Fig. 1). Cumulus oocyte complexes (COCs) from Graafian follicles taken at varying stages during estrus of the grey short-tailed opossum were cultured in Dulbecco's modified Eagle's Medium (DMEM) with 10% FCS (Selwood and VandeBerg, 1992) in 5% CO_2 in air. Cultures of released oocytes survived for between 24 and 96 h and they matured to formation of the first polar body. Some oocytes then underwent parthenogenetic activation and showed polarization of cytoplasmic vesicular structures, release of the second polar body and development to the two-cell stage. Maturing oocytes of the grey short-tailed opossum (Breed *et al.*, 1994a) that were removed from their Graafian follicles and freed of their cumulus cells were cultured to release of the first polar body in TALP-Hepes culture medium, pH 7.3. In the same study a double incubation with the calcium ionophore ionomycin resulted in extrusion of the second polar body and formation of the female pronucleus. It should be possible to culture COCs from culled animals from other species in the wild to improve techniques for oocyte maturation, and to analyze the requirements of oocyte maturation of marsupials *in vitro*.

Fertilization

Fertilization *in vitro* has been achieved in only one marsupial (Fig. 1), the grey short-tailed opossum, in which mature oocytes were collected from the Graafian follicle and fertilized with epididymal sperm (Moore and Taggart, 1993). The culture medium was a modified Minimal Essential Medium (MEM) supplemented with penicillin, streptomycin and bovine serum albumin (BSA) and culture was performed in 5% CO_2 in air. Fertilization, as defined by penetration of the zona pellucida and the presence of the sperm tail in the cytoplasm of the zygote occurred in more than half the cases and about one third developed to the 16-32 cell stage (Table 1). Zygotes (Fig. 2A) taken from either the oviduct or the uterus have

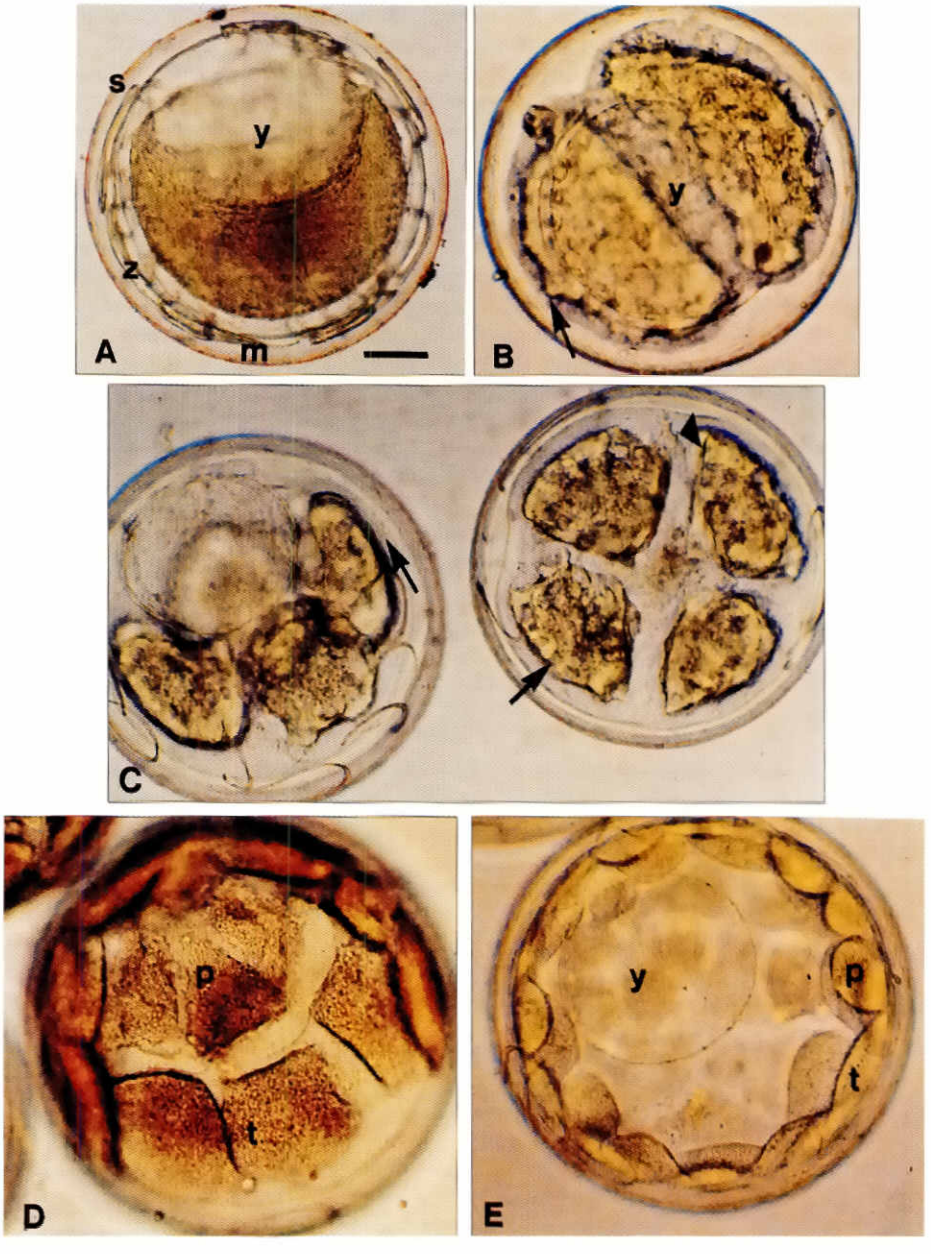

Fig. 2. Stages of cleavage in the stripe-faced dunnart. *Bar, 50 μm.* **(A)** *The polarized uterine zygote showing the transparent yolk mass (y) accumulating at the upper pole. The zygote is enclosed within a shell (s), mucoid (m) and zona (z) coat. Many sperm tails are visible in the mucoid coat. Day 0.5 of gestation.* **(B)** *The early 2-cell stage viewed from the yolk pole through the transparent yolk mass (y). The two cells are separating from each other but have numerous processes (arrow) contacting the zona pellucida. Day 1 of gestation.* **(C)** *Early 4-cell stages viewed from the side (left) and from the yolk-free pole (right). Vesicle secretion (arrows) is in progress and is associated with zona compression in the yolk free hemisphere. On the right the cells lie separately from each other but some processes (arrowhead) remain connecting to the zona. These are lost when the cells round up, then reappear at the end of the long four-cell stage. Day 1.5 of gestation.* **(D)** *An oblique view of the inside of the 16-cell stage showing the upper pluriblast (p) and lower trophoblast (t) cells flattened on the zona pellucida. All pluriblast cells are closely associated with each other, but cell-cell contact between trophoblast cells only occurs between sister cells from the third division. Day 2.5 of gestation.* **(E)** *An oblique view of the inside of a blastocyst with about 32 cells, in which the smaller, rounded pluriblast cells (p) in the hemisphere containing the yolk mass (y) are distinguishable from the larger, more flattened trophoblast cells (t) of the lower hemisphere. Day 3 of gestation.*

been cultured (Figs. 1 and 3) to between the two-cell stage and the early unilaminar blastocyst in a number of species (Selwood and Young, 1983; Selwood, 1987; Baggott and Moore, 1990; Selwood and VandeBerg, 1992; Renfree and Lewis, 1996).

The limited successful culture of stages during oocyte maturation and fertilization in marsupials is in marked contrast to the situation in eutherian mammals. In the latter group, these stages have been successfully cultured in a wide range of domestic and laboratory animals. Animals from such sources are readily available, thus increasing the chances of improving culture techniques. No marsupials have been domesticated and only three species, the fat-tailed and stripe-faced dunnarts and the grey short-tailed opossum, have been maintained as continuous colonies of laboratory animals for more than ten years. Most advances in culture have been made in these marsupials.

Cleavage stages

Although culture has been complicated by the occurrence of an arrested stage in some marsupials, most studies have been done on cleavage (Fig. 1, Table 1). The arrest, which usually occurs during the four-cell stage is found *in vivo* and *in vitro* and its length seems to be determined by the rate of development of the corpus luteum (CL) (Hinds and Selwood, 1990). In the brown antechinus, in which the arrest is for 3.5 days (Selwood, 1980; Selwood and Young, 1983), it develops slowly. In the stripe-faced dunnart, in which the arrest is for 34 h, it develops very quickly (Selwood and Woolley, 1991).

The first successful culture of marsupial cleavage stages was done using the conceptuses of the brown antechinus (Selwood and Young, 1983) (Table 1). During the cleavage arrest, which occurs *in vivo* and *in vitro* (Selwood, 1980), the four cells become rounded

and separate from each other. After 79 h they flatten on the zona pellucida in preparation for the next division. Continuous culture from the zygote to the 32-cell stage has not been achieved because of the four-cell arrest (Table 1). Expanding unilaminar blastocysts with between 32 and 64 cells do not survive for long in culture because blastocyst cells round up and pull away from the zona pellucida. A cumulative cleavage time of 152 h from the zygote to the end of the 32-cell stage *in vitro* is 32 h longer than *in vivo*. The cell cycle length of the cleavage stages, based on measurements made with time-lapse cinematography (Selwood and Smith, 1990) are shown in Table 1. Cleavage in the brown antechinus is relatively slow compared to that in some other marsupials. Except for the four-cell stage in marsupials, the length of the cell cycles during cleavage is similar in marsupials and eutherians (Table 1).

Cleavage stages from two other dasyurid marsupials belonging to the genus *Sminthopsis* (Table 1) have been cultured. Both species have much shorter gestation periods and shorter length of cleavage *in vivo* than the brown antechinus. The stripe-faced dunnart, *Sminthopsis macroura*, has the shortest gestation period of any mammal. It is a very suitable animal for embryological studies because a detailed timetable of development and a method for the detection of ovulation (Selwood and Woolley, 1991) allows collection of accurately timed stages at about 12 hour intervals over the gestation period of 10.5 days. The length of cleavage is short, being 80 h from the early uterine zygote to the end of the 32-cell stage *in vitro* and 72 h *in vivo*. The relatively shorter cell doubling time in the stripe-faced dunnart (Table 1) seems to account in part for its very short gestation period. In the fat-tailed dunnart, *Sminthopsis crassicaudata*, cleavage is less well documented but the rate of development *in vivo* is similar to that *in vitro*. The cleavage times are shown in Table 1. Four-cell stages did not develop further in culture, but the sample size was small. One study (Breed *et al.*, 1994b) has demonstrated that following freezing and thawing, a small number of one- to four-cell stages retain a relatively normal ultrastructure and the ability to undergo one or two cleavage divisions *in vitro*. These results are promising especially when one considers the relatively large size of marsupial zygotes and cleavage cells (Selwood, 1992).

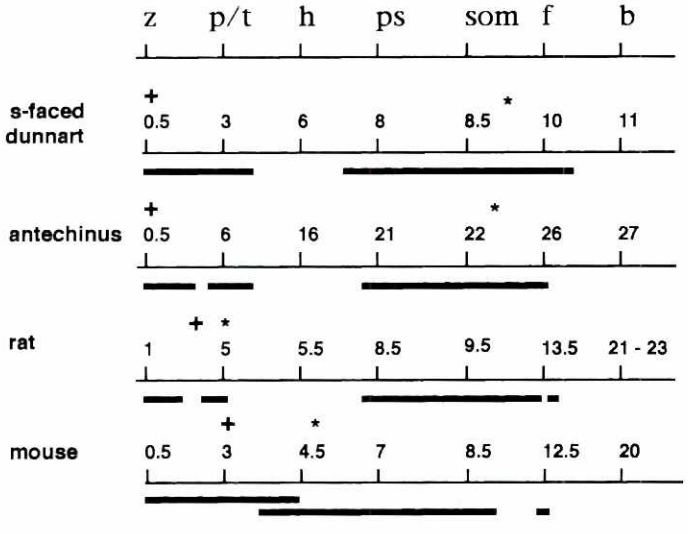

Fig. 3. Comparison of the stages successfully cultured of two dasyurid marsupials, the stripe-faced dunnart and the brown antechinus and two rodents, the mouse and the rat. *Particular events in development are indicated on the upper line as appearance of the zygotes (z), of the pluriblast and trophoblast cells (p/t), of the hypoblast cells (h), of the primitive streak (ps), of the first somites (som), of the fetus (f) and birth (b). For each animal the days of each of these events in vivo is indicated on the upper line. The day of entry into the uterus is marked with a + and the day of hatching and implantation with an *. Periods of continuous culture are recorded by solid bars on the lower level. Discontinuities in culture in both rodents and dasyurids are commonly associated with the time of hypoblast and mesoderm formation. The figure is based on information in the following references: stripe-faced dunnart (Selwood, 1987; Selwood and Smith, 1990; Selwood and Woolley, 1991; Yousef and Selwood, 1993); brown antechinus (Selwood, 1980; Selwood and Young, 1983; Yousef and Selwood, 1993); rat (Cockroft, 1973, 1976, 1990; New, 1990; Kaufman, 1990); mouse (Wiley and Pedersen, 1977; Chen and Hsu, 1982; Pratt, 1987; Kaufman, 1990).*

Renfree and Lewis (1996) have cultured tammar zygotes and two-cell stages to the eight-cell stage and four-cell stages to the unilaminar blastocyst stage (Table 1). Cleavage times *in vitro* are slower than the deduced cell doubling time of 8 h *in vivo*.

Cleavage stages of the grey short-tailed opossum, have been cultured in a number of different media (Table 1). All media support development but some arrests *in vitro* at the eight-cell stage (Baggott and Moore, 1990) and four-cell stage (Selwood and VandeBerg, 1992) have been reported. Mate *et al.*, (1994) found that the four-cell stage is the longest stage *in vivo* and lasts for 24 h or longer. Development from the uterine zygote *in vivo* took 104 h to the complete blastocyst (Mate *et al.*, 1994). Development *in vitro* takes longer, regardless of the culture medium employed. This opossum is also suitable for embryological studies because it is an induced ovulator (Fadem, 1985; Fadem and Rayve, 1985), video monitoring of paired animals allows the collection of timed stages of development (Baggott *et al.*, 1987), and a timetable of development *in vivo* has been prepared (Mate *et al.*, 1994).

Cleavage and blastocyst formation is dependent on intimate associations developing between the cleaving cells and the zona pellucida (Selwood and Young, 1983; Baggott and Moore, 1990; Selwood and Smith, 1990; Selwood, 1996). In the stripe-faced dunnart, this process begins at the two-cell stage (Fig. 2B), and becomes more enhanced at the four- (Fig. 2C) and eight-cell stage when blastomeres become closely adherent to the zona pellucida. Cell-cell contact is not seen until the eight-cell stage and becomes more obvious in later cleavage stages (Fig. 2D), initially mainly in the developing pluriblast (Fig. 2E). Extensive adherence by the cells of the developing blastocyst epithelium to the adjoining zona pellucida, such as that shown in Figure 2D and 2E, is an essential feature of normal marsupial blastocyst development. Rounded cells that do not adhere closely to the zona pellucida in conceptuses in late cleavage are an indication of eventual developmental failure *in vitro* and *in vivo*.

Arrests in cleavage leading to a block *in vitro* have also been found in eutherian mammals (Fig. 3) and these have been related to the onset of zygotic transcription (First and Barnes, 1989). In marsupials, temporary arrests occur *in vivo* at the same stages as *in vitro* but their relationship to the onset of transcription of the zygotic genome is unknown.

Marsupial cleavage appears to require a more complex media (Table 1) than that required for many eutherian mammals, possibly because cleavage in marsupials occurs in the uterus. As in

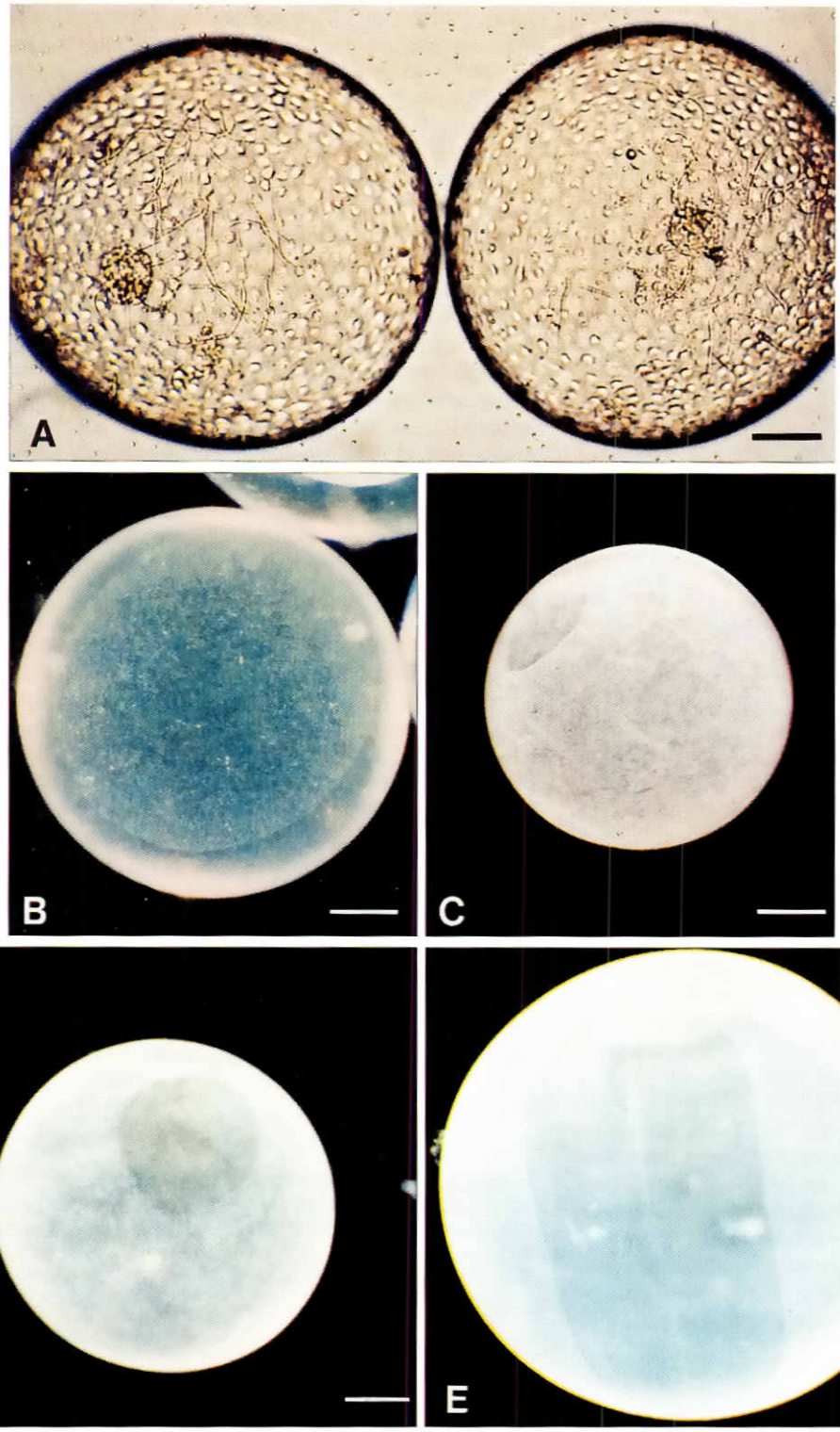

Fig. 4. Blastocyst stages of dasyurid marsupials. *Except for the unilaminar blastocyst, all stages have been cultured successfully for 24 h or more over several stages.* **(A)** *Unilaminar blastocysts of the stripe-faced dunnart. Blastocysts have an ovoid shape because of differential growth of the trophoblast and pluriblast. The yolk mass and numerous sperm tails can still be seen in both blastocysts. Day 4.5 of gestation. Bar, 100 μm.* **(B)** *Late bilaminar blastocyst of the stripe-faced dunnart, in which the pluriblast is visible as a more opaque region occupying about one third of the blastocyst. Day 7.5 of gestation. Bar, 500 μm.* **(C)** *Bilaminar blastocyst of the brown antechinus in which the pluriblast appears as a transparent window in the blastocyst wall. Day 19 of gestation. Bar, 500 μm.* **(D)** *Late bilaminar blastocyst of the brown antechinus just before the appearance of the primitive streak. Day 20 of gestation. Bar, 500 μm.* **(E)** *Late trilaminar blastocyst of the brown antechinus, with a well defined primitive streak, node and notochord. Day 22 of gestation. Bar, 500 μm.*

eutherian mammals, cleavage stages of several marsupial species have been cultured successfully to the expanding blastocyst stage. In both metatherians and eutherians, development of the first two cell lineages, trophoblast and pluriblast, occurs *in vitro* (Fig. 3) suggesting that this event is independent of uterine signals in both groups.

Unilaminar blastocyst

In many marsupials, the unilaminar blastocyst stage (Figs. 2E and 4A) is a relatively long stage during which cell volume does not increase (Selwood, 1989a), but blastocyst diameter does. Its duration can be considerably extended by embryonic diapause (Tyndale-Biscoe and Renfree, 1987). The unilaminar blastocysts

TABLE 2

NUMBER OF ANIMALS AND NUMBER OF CULTURED CONCEPTUSES USED TO DETERMINE THE NORMAL STAGES OF DEVELOPMENT *IN VITRO* OF THE GREY SHORT-TAILED OPOSSUM

stage	d:h pc	number animals	number concept.	max. h culture	stage at end of culture
video timed					
zygote	1:6 - 1:7	2	9	144	16-c
2-c	1:15	1	6	32	16-c
early 4-c	2:7	1	4	30	8-c
adv. 4-c	2:6	1	3	9	8-c
early 8-c	2:8 - 3:5	2	12	9	8-c
late 16-c	4:9	1	6	24	64-c
32-c	4:7 - 5:4	2	8	9	64-c
64-c	5:7 - 6:7	2	12	9	+exp.
128-c	6:10	1	7	9	+exp.
bb	8:8	1	3	9	+exp.
not video timed					
zygote	-	1	9	45	late 4-c
2-c	-	1	5	19	4-c
early 4-c	-	3	16	46	16-c
late 4-c	-	1	6	44	32-c
8-c	-	2	11	44	exp. 32-c
16-c	-	1	5	20	exp. 32-c
32-c	-	3	19	21	exp. 64-c
64-c	-	1	4	6	64-c

The time after observed mating (pc) is recorded in days and h for the animals examined after video recordings were made. The most advanced stage reached and the maximum time in culture is also noted. adv, advanced; bb, bilaminar blastocyst; c, cell; exp, expanding.

of marsupials have proved difficult to culture (Figs. 1 and 3). Blastocysts of macropodid and dasyurid marsupials have been cultured only for very short periods of 6 h or less without embryonic failure (Tyndale-Biscoe and Renfree, 1987; Yousef and Selwood, 1993; Renfree and Lewis, 1996). Media have included DMEM, Whittingham's T6 and Monomed medium (CSL Melbourne) in 5% CO_2 in air with FCS concentrations of 5 or 10%. Analyses of carbohydrate metabolism in tammar blastocysts (Pike, 1981; Spindler *et al.*, 1996) may eventually assist with the formulation of a suitable medium for culture of unilaminar blastocysts. It is known that the composition of uterine protein secretions change greatly during the unilaminar blastocyst stage (Tyndale-Biscoe and Renfree, 1987) and changes in osmotic pressure of the secretions may also occur. Further detailed studies of the nature of the uterine secretions and of their osmotic pressure might improve survival in culture during the stages when cell-zona adhesion is critical to blastocyst formation and survival.

Bilaminar blastocyst

The early bilaminar blastocyst is also difficult to culture (Fig. 3) but more success has been achieved with late bilaminar blastocysts (Fig. 4B and C) (Tyndale-Biscoe and Renfree, 1987; Yousef and Selwood, 1993). Bilaminar blastocysts of the stripe-faced dunnart, brown antechinus and the tammar wallaby have been cultured (Fig. 1) in DMEM, Whittingham's T6, or human amniotic fluid (HAF) with supplements of 5, 10, 12.5, 15 or 20% FCS and 0.1 or 0.2% BSA. The more mature blastocysts survived better in culture than younger ones. Late bilaminar blastocysts developed to trilaminar blastocysts and into various stages of somitogenesis and neurulation during 12 and 54 h in the best media, which were HAF and DMEM+10% FCS. Culture of bilaminar blastocysts of most marsupials has similar problems to the culture of unilaminar blastocysts in that little is known of the uterine environment.

In marsupials, it has not been possible to culture over the period of hypoblast formation and the transition to mesoderm formation is also difficult (Fig. 1). Only late bilaminar blastocysts develop a primitive streak and progress further in culture. These results suggest that development of hypoblast and mesoderm may require special uterine conditions or signals in marsupials. These are also difficult transitions to make *in vitro* in rodents (Fig. 3). These difficulties have been overcome in the mouse by Chen and Hsu (1982) who used a collagen substrate and MEM supplemented by a series of sera, and by Wiley and Pedersen (1977) who used BME supplemented by two sera and essential amino acids. A comparison of the rodent and marsupial results (Fig. 3) suggests that in both groups, hypoblast and mesoderm development may require uterine signals *in vivo* or substances in serum *in vitro* for initiation or proliferation and that this requirement is independent of the time of implantation.

Trilaminar blastocysts, embryonic and fetal stages

New and Mizell (1972) and New *et al.* (1977) were the first to culture any conceptuses of marsupials. They cultured stages of the Virginia opossum, including the advanced primitive streak/early neurula (day 7.5) to late fetal stages for up to 30 h. They used morphological assessment based on the normal stages of the opossum (McCrady, 1938) and a total fetal protein assay to estimate growth. Embryos undergoing neural tube and heart formation and explanted on days 8.5-9.0 of gestation developed best in roller tube culture through one or two stages. Media used were Ham's F10, Medium 199, opossum serum and Hank's and Tyrode's saline supplemented with opossum serum. Opossum serum was effective either pure or diluted and was necessary for good growth. Of the range of oxygen levels tested in the gas phase, 95% initially was found to support better growth but had long term deleterious effects. Such high levels are damaging to embryonic

TABLE 3

CHANGES IN THE DIAMETER OF THE CONCEPTUS AND THE THICKNESS OF ITS EGG COATS DURING CLEAVAGE AND BLASTOCYST EXPANSION IN THE GREY SHORT-TAILED OPOSSUM

stage	diameter (μm)			thickness (μm)	
	total	inside zona	zona	average mucoid	shell
1-cell	280 ±7.8	137±9.3	8.6±1.9	67±4.0	1.3±0.4
2-cell	278±19.4	148±12.0	5.5±0.8	60±12.9	1.8±0.3
4-cell	277±20.5	132±14.2	6.5±0.9	66±8.5	1.6±0
8-cell	292±17.5	126±12.3	5.7±0.8	79±13.5	1.6±0
16-cell	297±17.1	123±3.0	4.8±0	83±12.4	1.6±0
32-cell	286±17.6	123±7.2	5.0±0.7	76±12.8	1.6±0
64-cell	316±30.3	157±14.9	3.9±0.7	74±14.1	1.6±0
128-cell	299±18.2	181±13.7	5.8±1.1	55±5.6	-

The dimensions given are means±standard deviation. N= 10 for all stages except the 16-cell stage in which N= 5.

tissues (New and Coppola, 1970; New *et al.*, 1976). New *et al.* (1976) recommended that lower levels of oxygen, approximating to those in the uterine artery be tried in subsequent studies.

The Virginia opossum is a particularly suitable animal for studies of organogenesis because the opossum embryo does not implant until day 10 of a 12.75 day gestation period. Another marsupial, the stripe-faced dunnart, is even more suitable to use for these purposes. It has the shortest gestation period (10.5 days) of any mammal and does not implant until the 9th day of gestation when the embryo has 13-15 somites (Selwood and Woolley, 1991). This species ovulates a mean of 13.2 oocytes per ovary. The mean number of follicles stimulated to ovulate can be increased significantly to 19.6 by induction with PMSG (Merry *et al.*, 1995). Up to eight young are held in the pouch (Godfrey, 1969).

Stages between the late bilaminar blastocyst (Fig. 4B), early and late trilaminar blastocyst with neural plate of the stripe-faced dunnart have been cultured to fetal stages within 18 h of birth (Yousef and Selwood, 1993). The culture media used were DMEM plus 10% FCS, Whittingham's T6, and Human Amniotic Fluid (HAF). A range of concentrations of FCS between 10 and 20% were also tested. DMEM with 10% fetal calf serum and HAF allowed the most advanced development in culture. The most successful stages in culture were trilaminar blastocysts with a well defined primitive streak and trilaminar blastocysts with early stages of neural plate formation. Culture was performed as stationary culture in 5% CO_2 in air at 35°C. Successful culture was maintained for 24 to 48 h with morphologically normal embryos or fetuses developing. The authors considered that culture for a longer period, perhaps even to birth, may have been possible if roller culture was used for the fetal stages. Development over the 48 h period was 4 h behind that *in vivo*. It was possible that growth of the fetus, but not the yolk sac, was slightly retarded *in vitro* but variations have also been found in the size of the fetus during normal development *in vivo*.

Cultures of similar stages of the brown antechinus had similar results to the study of the stripe-faced dunnart with respect to the best medium. The late bilaminar blastocyst (Fig. 4C), early (Fig. 4D) and late trilaminar blastocyst stage with pronounced primitive streak (Fig. 4E) developed to the most advanced stages over 40 and 54 h. Brown antechinus embryos were cultured to within 2 to 3 days of birth. The brown antechinus has a gestation period of 27 days. Periods of slow development involving differential growth of pluriblast and trophoblast occur at the unilaminar blastocyst stage between days 7-13 (Selwood, 1996) and implantation does not occur until day 23 (Selwood, 1980). Development over the 54 h period *in vitro* was 6 h behind that *in vivo* in the brown antechinus during embryonic and fetal stages.

TABLE 4

COMPARISON OF THE CUMULATIVE TIME OF DEVELOPMENT *IN VITRO* WITH DEVELOPMENT *IN VIVO* POST COITUM (pc) OF THE GREY SHORT-TAILED OPOSSUM

Stage	*In vitro* d:h to end of stage	*In vivo* stage obtained d:h pc	
		this study	Mate *et al.*, 1994
zygote enters uterus	–	1:6-1:7	1:0
1-cell	1:22	1:6 -1:7	1:0
2-cell	2:8	1:15	–
4-cell	3:4	2:6-2:7	2:1-2:12
8-cell	4:0	2:8-3:5	3:2-3:5
16-cell	4:10	4:9	4:0
32-cell	4:22	4:7-5:4	4:4
64-cell	5:6	5:7-6:7	5:4-5:17
128-cell	6:0	6:10	–

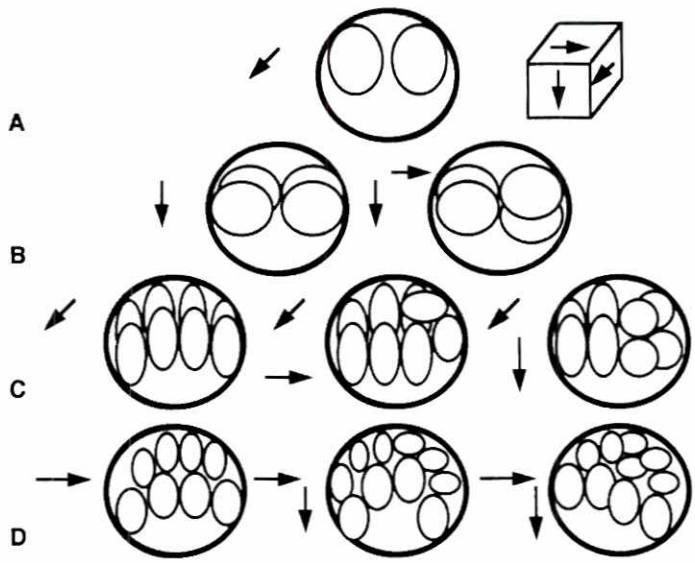

Fig. 5. Diagram of the cleavage pattern and planes of cleavage between the first and fourth division in the grey short-tailed opossum. (A) *Shows the meridional cleavage plane of the first division.* **(B)** *Shows that the second division occurs by meridional cleavage planes or by meridional and latitudinal planes.* **(C)** *Shows that the third division is achieved by four meridional divisions, three meridional and one latitudinal division or two meridional and two latitudinal divisions.* **(D)** *Shows that the fourth division is achieved by eight latitudinal divisions or a combination of latitudinal and meridional divisions.*

The relative ease with which marsupial conceptuses can be cultured over the early implantation period might be because those that have been cultured have a chorio-vitelline placenta. In the early but not the later stages of implantation, these conceptuses can be readily detached from the endometrium (see review of placental types in Tyndale-Biscoe and Renfree, 1987). Marsupials do not have a decidual reaction. Conceptuses of bandicoots, the marsupials with a chorio-allantoic placenta, have not been cultured.

Culture without the egg coats

Attempts have been made to culture marsupial conceptuses and their constituent cells without their egg coats. When cells of cleavage stages of the brown antechinus and the stripe-faced dunnart are cultured without their coats, they undergo the normal processes that occur in the intact embryos. But because of the mobility of the cells at the late four-cell and eight-cell stage they do not form an embryo (Selwood, 1989b). At the equivalent of the eight-cell to 64-cell stage *in vivo*, when cell-cell associations appear in intact embryos, cells *in vitro* form small clumps, but do not form a blastocyst. Selwood (1989b) suggested that the egg coats are necessary to confine the cells and to provide the framework in which the blastocyst is constructed. Time lapse analysis of embryos of these marsupials shows that the polarized secretion of

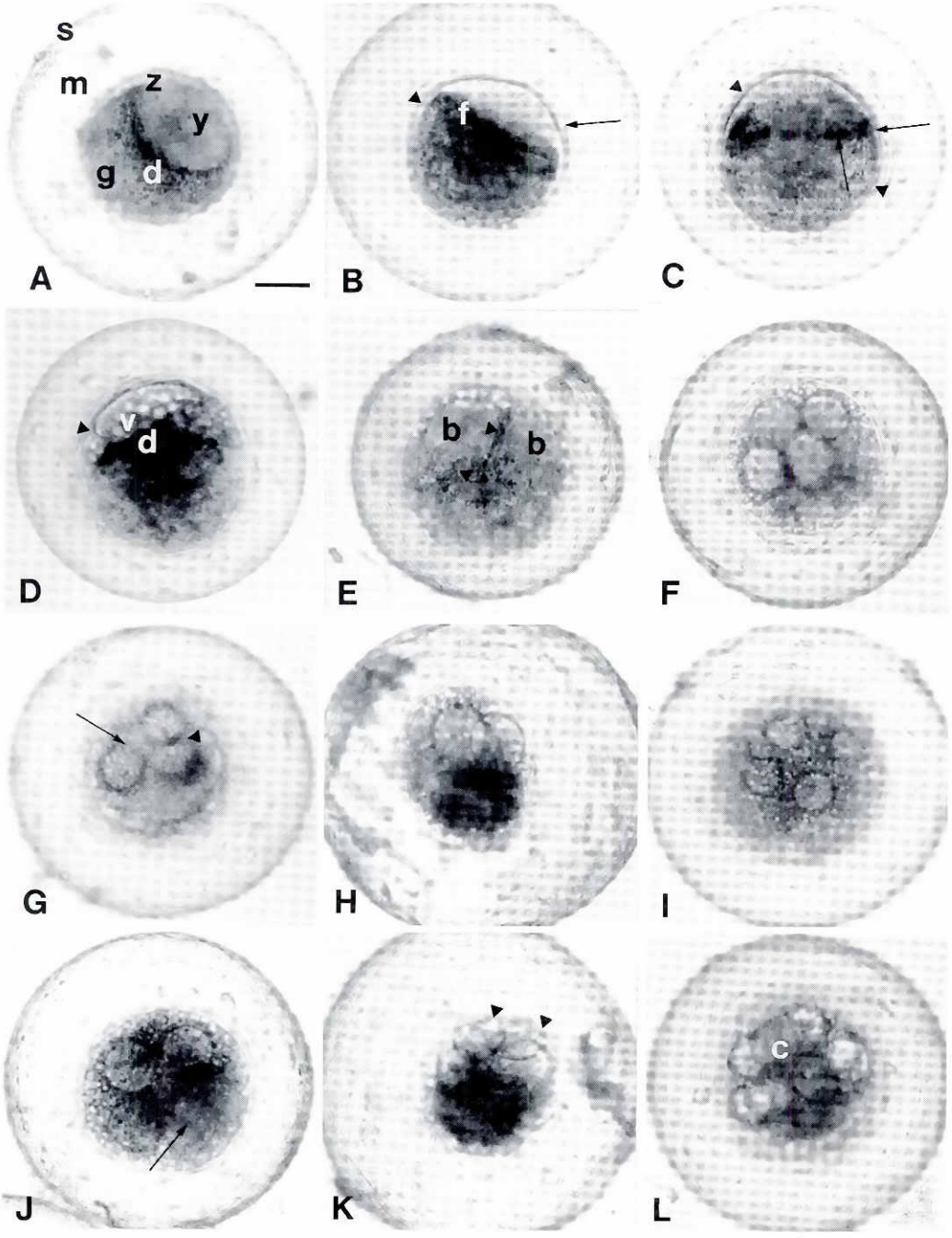

Fig. 6. Morphology of normal development *in vitro* of the grey short-tailed opossum from the early uterine zygote to the eight-cell stage. *For timings see text. Bar, 50 μm.* **(A)** *A view from the side of the polarized zygote. It has more transparent yellow cytoplasm (y) at the upper pole, a band of supra-equatorial granular, dark brown cytoplasm (d) and more opaque grey cytoplasm (g) at the lower pole. The zygote lies closely associated with the zona pellucida (z) at the upper pole. The mucoid coat (m) is turgid and the outer shell coat (s) is very narrow and transparent.* **(B)** *The zygote showing more marked polarity. The upper yellow cytoplasm has become more shiny and glassy. It is bounded by a furrow (f) that is more obvious on one side. The supra-equatorial granular cytoplasm has a darker more diffuse appearance and overlaps the furrow. A perivitelline space (arrowhead) is present and a polar body (arrow) lies in it at the upper pole.* **(C)** *The perivitelline space (arrowheads) has become wider and both it and the zona pellucida have a more irregular appearance. The supra-equatorial cytoplasm has separated into a series of brown granular associations (arrows) around the latitude of the zygote and a more amorphous, dark cytoplasm which has become more centrally located. Subcortical granules are present in the grey cytoplasm.* **(D)** *Early two-cell stage during cytokinesis, viewed from the side. While the upper cytoplasm is separated from the lower cytoplasm by constriction of the latitudinal furrow, numerous glassy yellow vesicles (v) are generated near it. A meridional cleavage furrow (arrowhead) bisects the upper cytoplasm. Most of the brown granular supra-equatorial cytoplasm is now located centrally (d) and near the furrow separating the upper and lower cytoplasm.* **(E)** *A side view of a two-cell stage in which the two blastomeres (b) are partially obscured by the many glassy vesicles in the perivitelline space of the upper hemisphere. The cells have moved apart from each other but appear to have retained continuity with the grey cytoplasm of the lower hemisphere. The central cytoplasm and the space between the two blastomeres is filled with a dark granular substance (arrowheads).* **(F)** *An oblique view from the upper pole of a late two-cell stage, that is undergoing cytokinesis in which both divisions are meridional. While cytokinesis is in progress, the blastomeres are pinching off from the underlying cytoplasm. This cytoplasm has an irregular lobed appearance.* **(G)** *A side view of a four-cell stage in which one division plane was latitudinal (arrowhead) and the other was meridional (arrow). The blastomeres are partially obscured by numerous glassy vesicles which have been eliminated into the perivitelline space during this division.* **(H)** *A side view of a four-cell stage in which vesicle emissions from the blastomeres at the upper pole and from the cytoplasmic mass at the lower pole have filled the perivitelline space and lie between the conceptus and the zona pellucida.* **(I)** *A view from the upper pole during the last half of the relatively long four-cell stage. The blastomeres have become smaller, rounded and separated from each other during a further wave of emission of small vesicles from the four blastomeres.* **(J)** *A side view of the late four-cell stage at the beginning of the third division. The division is asynchronous and a cleavage furrow is visible in one of the blastomeres (arrow). The polarization of the contents of the cleavage cavity is marked. The blastomeres become more visible as they begin to adhere to the zona pellucida.* **(K)** *A conceptus with five cells. The vesicular material in the upper hemisphere has disappeared and blastomere adhesion to the zona pellucida is more marked (arrowheads). The cytoplasmic mass in the lower hemisphere has transformed to a dark centrally located mass with numerous large vesicles at its lower margin.* **(L)** *An eight-cell stage in which three blastomeres at the four-cell stage have divided meridionally and one latitudinally to give a loose tier of cells around a more central cell (c) on the upper part of the dark cytoplasmic mass.*

extracellular matrix within the cleavage cavity, and subsequent polarization of the zona pellucida ensure that the blastocyst epithelium begins to form in one focal region (Fig. 2D) and spreads out from that region to eventually line the zona pellucida (Fig. 2E) (Selwood and Smith, 1990). This process is associated with allocation into pluriblast and trophoblast cells (Selwood, 1996).

If the egg coats are removed from unilaminar, bilaminar or trilaminar blastocysts (Selwood *et al.*, 1993) of the stripe-faced dunnart, only trilaminar blastocysts survive in culture. The embryos hatch from the shell *in vivo* at the late trilaminar blastocyst stage in both species (Yousef and Selwood, 1993). In contrast, if the blastocysts are dissociated into individual cells or the blastocyst is separated into smaller fragments, the pluriblast cells of the unilaminar and trilaminar blastocyst will survive *in vitro* for up to eight weeks without proliferating or differentiating (Yousef and Selwood, 1996). Two or three of the trophoblast cells in each culture transform to multinucleate giant cells. This is very different to the situation in the mouse and other eutherian mammals in which all trophoblast cells transform to the multinucleate state within a few days of initiation of culture (Sherman, 1975). The establishment of the conditions that allow for maintenance of coat-free marsupial embryonic cells *in vitro* is the first step in deriving marsupial embryonic stem cells.

In this study we describe the stages of development of the grey short-tailed opossum *in vitro* and compare them to development *in vivo*. At this time, a number of colonies of the grey short-tailed opossum are located around the world. The development of protocols for collection of timed stages by videotaping of mating animals (Baggott and Moore, 1990), *in vitro* fertilization (Moore and Taggart, 1993) and of a timetable of development *in vivo* (Mate *et al.*, 1994) make this an appropriate time for the provision of a timetable of stages of this animal during early embryonic development *in vitro*.

Results

Of 21 animals examined in the Laboratory of Radiobiology and Environmental Health, San Francisco in 1990, eight were not suitable because they were preovulatory (3), had unfertilized eggs (1) or had failed during early stages of unilaminar blastocyst formation (2) or expansion (2). Of the 15 examined in the Southwest Foundation for Biomedical Research, one had a very high proportion of unfertilized eggs and another had failed blastocysts. The number of animals and conceptuses used to determine the normal stages of development *in vitro* are shown in Table 2.

Normal stages in vitro

The pattern of cleavage and the cleavage planes observed in these specimens is shown in Figure 5 and described below.

The diameter of the conceptus and the thickness of the egg coats at each stage of development are shown in Table 3.

The duration of each stage *in vitro* is given following each major stage. The cumulative time *in vitro* is also given as days and h post coitum after each stage. The minimum period between mating and entry of the zygote into the uterus was 1 day 6 h (Table 4) and the cumulative h *in vitro* are added to this time.

Uterine zygote: duration, 16 h; cumulative time, 1 d 6 h - 1 d 22 h

1 d 6 h: the zygote was polarized (Fig. 6A) with transparent yellow cytoplasm at one pole (upper pole), grey, granular cytoplasm at the other (lower pole) and a supra-equatorial band of granular brown cytoplasm lying between the two. The terms "upper" and "lower" are used for convenience. Many specimens (Figs. 6 and 7) lay on their "side" with the upper and lower poles visible. Planes passing from pole to pole will be termed meridional and planes passing across a meridian at right angles will be termed latitudinal. The plasma membrane of the conceptus was closely applied to the zona pellucida, in which cross striations were sometimes seen, presumably because of microvilli. The mucoid layer had a turgid appearance and the shell was extremely thin and soft.

1 d 11 h: polarization was more marked (Fig. 6B) and the edge of the yellow cytoplasm formed a latitudinal furrow into the surface of the zygote. This cytoplasm had a distinctive shiny, glassy appearance. The supra-equatorial band of brown cytoplasm overlapped the furrow. In some specimens the yellow cytoplasm was wider on one side or was broken up into two masses. Those in the latter condition did not compete the first division. A polar body was sometimes visible at the upper pole in the developing perivitelline space. The shell was tougher.

1 d 13 h: the furrow at the edge of the glassy yellow cytoplasm became more marked. The brown cytoplasm segregated into a band comprised of dense clusters of granules and a more amorphous darker cytoplasm. A sub-cortical accumulation of large granules occurred in the grey cytoplasm (Fig. 6C). A perivitelline space was obvious and the zona was markedly irregular in outline.

1 d 20 h: the meridional constriction furrow of the first cleavage (Figs. 5 and 6D) bisected the yellow cytoplasm of the upper pole which also was progressively pinched off from the remainder of the cytoplasm by deepening of the latitudinal furrow. Prior to and during cytokinesis much of the yellow cytoplasm was segregated from the remainder of the cytoplasm as numerous glassy yellow vesicles which then lay around the forming cells and in the perivitelline space. Vesicle formation occurred first at the boundary between the yellow cytoplasm and the remainder of the lower cytoplasm. Once extruded into the perivitelline space, vesicles were not confined to the upper pole but the majority of them occurred there.

Two-cell stage: duration, 10 h; cumulative time, 1 d 22 h - 2 d 8 h

1 d 22 h: at the early two cell stage a cytoplasmic bridge was sometimes visible between the two cells and occasionally one cell was twisted in relation to the other. The cytoplasm of the two cells was transparent and did not have the glassy yellow appearance of the cytoplasm of the upper pole of the zygote. The wide perivitelline space contained many vesicles of yellow glassy cytoplasm especially in the upper hemisphere (Fig. 6E). The grey cytoplasm was somewhat irregular in outline but appeared to be membrane-bound and closely associated with the two blastomeres.

2 d 5 h: late in this stage, the two cells moved apart and appeared to be fully separated from each other with no interconnecting cytoplasmic bridge remaining. The cells lay flat on the grey cytoplasmic mass.

2 d 7 h: cytokinesis of the second cleavage division was in progress (Fig. 6F). Two, equally common, patterns of cleavage were found (Fig. 5). In one pattern both cells were bisected by a meridional cleavage plane (Fig. 6F) and in the other, one cell was divided by a meridional plane and the other by an equatorial plane (Fig. 6G). Cytokinesis was preceded and accompanied by further emission of

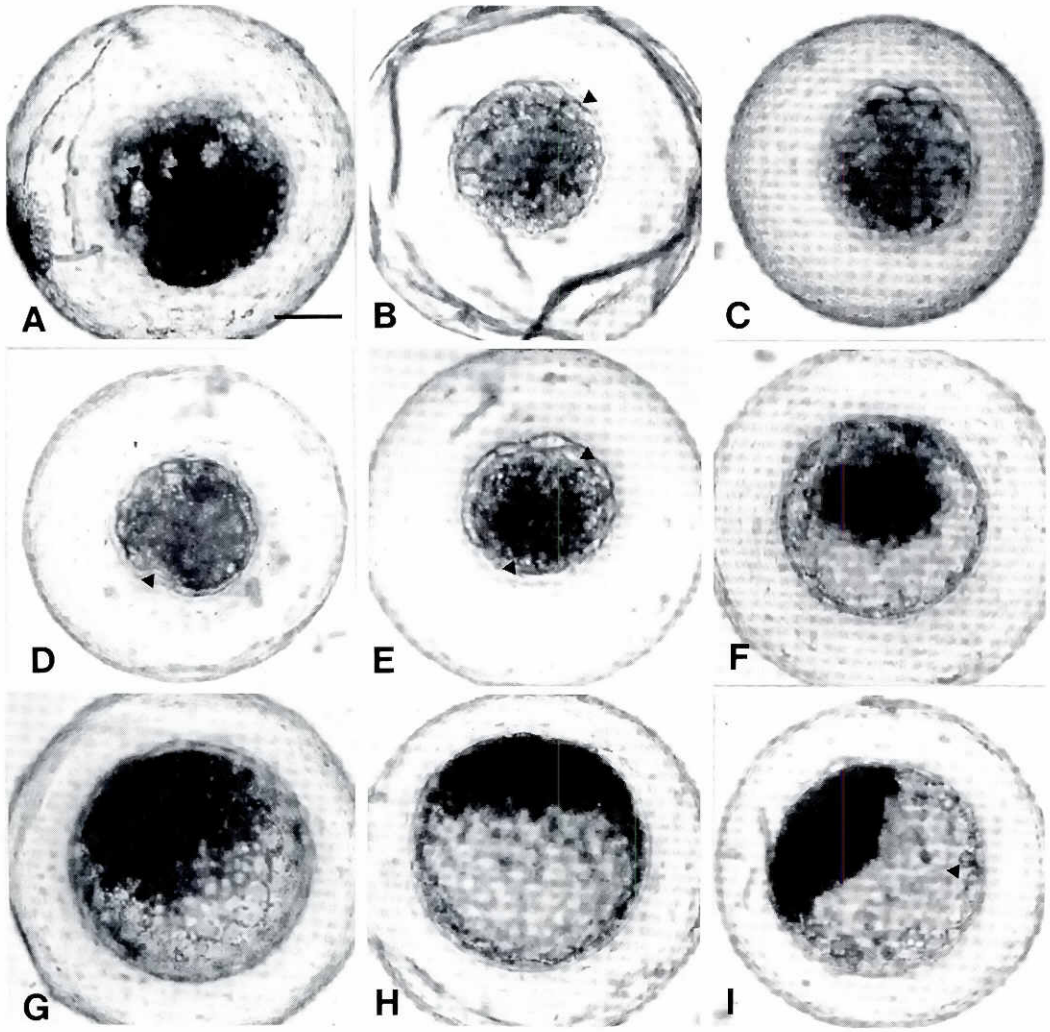

Fig. 7. The morphology of normal development *in vitro* of the grey short-tailed opossum from the eight-cell stage to the expanding unilaminar blastocyst with about 128 cells. *For timings see text. Bar, 50 μm.* **(A)** *An eight-cell stage in which two of the four blastomeres have divided latitudinally (arrowheads) and two have divided meridionally. Two blastomeres are obscured by the dark cytoplasmic mass. A series of shiny vesicles are found in the perivitelline space and in the cortex of the cytoplasmic mass in the hemisphere opposite the blastomeres. The zona has a beaded appearance.* **(B)** *A view from the side of a 16-cell stage in which the blastomeres form two tiers. Cell-cell adhesion is more marked. Blastomeres of the upper tier are more rounded and those of the lower tier are more flattened on the zona pellucida (arrowhead) and are progressively enclosing the cytoplasmic mass and vesicles of the lower hemisphere. This blastocyst has wrinkled egg coats associated with lesser turgidity in the mucoid coat.* **(C)** *A late 16-cell stage in which progressive cell-cell and cell-zona adhesion have formed a more obvious epithelium. The edge of the expanding epithelium is marked (arrowhead).* **(D)** *A 32-cell stage in which the blastomeres of the lower tiers (arrowhead) have totally lined the zona and enclosed the cytoplasmic material of the lower hemisphere. The blastomeres of the upper tiers have a more rounded appearance.* **(E)** *A 32-cell stage in the early stages of blastocyst expansion. The cells of the upper tier are more rounded than the cells in the remainder of the blastocyst epithelium. Small spaces (arrowheads) are beginning to appear between the cytoplasmic material in the cleavage cavity and the blastocyst epithelium.* **(F)** *An early expanding unilaminar blastocyst with slightly more than 32 cells. The dark cytoplasmic mass has remained associated with the more rounded cells of the upper hemisphere. Clear vesicular structures are being assembled at the lower boundary of this mass.* **(G)** *An expanding unilaminar blastocyst with about 64 cells. The cytoplasmic mass is somewhat dispersed and transparent vesicles are found at its lower margin. The difference between the cells of the upper and lower hemisphere is not obvious. Blastocyst expansion is associated with narrowing of the zona pellucida and the mucoid coat but with little or no change in the outer dimensions of the shell coat.* **(H)** *An expanding unilaminar blastocyst with about 128 cells. The cytoplasmic mass has become liquefied, very dark and adherent to the cells of the upper hemisphere.* **(I)** *An expanding unilaminar blastocyst with about 128 cells. Expansion is not as marked as in G and H. Cells or parts of cells have detached from the blastocyst epithelium and have moved into the blastocoel (arrowhead).*

shiny vesicles into the perivitelline space and constriction of the blastomeres off the underlying cytoplasmic mass.

Four-cell stage: duration, 20 h; cumulative time, 2 d 8 h-3 d 4 h

2 d 9 h: cytokinesis of the second division was completed (Fig. 6G). The cells separated from each other and rounded up as further vesicle emissions occurred but they remained in close contact with the underlying cytoplasmic mass (Fig. 6H).

2 d 20 h: vesicles were very numerous and in some specimens obscured some of the cells. Towards the middle of the four-cell stage the cells became rounded and smaller and lay well apart from each other (Fig. 6I).

3 d 3 h: cells were moving towards the zona pellucida and they became more readily visible as they did so (Fig. 6J) and displaced the vesicles lying near it. Cytokinesis of the next division began. As the division progressed, the vesicles disappeared and the cells adhered to the zona (Fig. 6K). The division was not synchronous. Three patterns of cytokinesis were found (Fig. 5); all planes meridional in about half the conceptuses, two meridional and two latitudinal planes or three meridional and one latitudinal plane in the other half.

Eight-cell stage: duration, 20 h; cumulative time, 3 d 4 h - 4 d

3 d 5 h: when the third division was completed the eight cells

rounded up slightly so that they were less flattened on the zona pellucida (Fig. 6L). They formed a tier of cells lying on top of the grey cytoplasmic mass. The exact form of the tier varied with the division planes of the third division (Fig. 5).

3 d 13 h: the blastomeres were slightly rounded and few or no vesicles remained in the perivitelline space.

3 d 23 h: at the end of this stage the blastomeres began to flatten further on the zona pellucida. Shiny vesicles appeared in the lower cortex of the grey cytoplasmic mass which darkened in appearance (Fig. 7A). In some specimens the outer egg coats had a wrinkled appearance. The division planes are shown in Figure 4.

16-cell stage: duration, 10 h; cumulative time, 4 d-4 d 10 h

4 d 1 h: the sixteen-cell stage had two cell types, eight larger more rounded cells in the upper hemisphere that formed the centre of the developing blastocyst epithelium and eight more flattened cells that extended from these cells towards the opposite pole. The latter were extremely flattened and adherent to the zona pellucida (Fig. 6B) and enclosed the grey cytoplasmic material in the lower hemisphere. The zona pellucida was wrinkled and the mucoid coat was less turgid giving the egg coats a crumpled appearance in many specimens.

4 d 9 h: prior to the fourth division the cells became more flattened on the zona and cell-cell adhesion increased as the developing blastocyst epithelium spread inside the zona (Fig. 6C).

32-cell stage: duration, 12 h; cumulative time, 4 d 10 h-4 d 22 h

4 d 11 h: a complete blastocyst epithelium formed by the 32-cell stage (Fig. 7D) from a combination of cell divisions and further spreading of the blastomeres on the zona pellucida. The division planes of the fifth cleavage were oblique or latitudinal. The epithelium completely lined the zona and enclosed the mass of grey cytoplasm. The epithelium consisted of larger more protuberant cells and flatter cells in approximately equal numbers.

4 d 20 h: after formation of the complete blastocyst epithelium, spaces appeared between the blastocyst epithelium and the cytoplasmic material occupying the cleavage cavity (Fig. 7E). Spaces appeared beneath both the larger and the more flattened cells, suggesting that both cell types were contributing fluid to the blastocoel. These spaces gradually coalesced into a single cavity with the cytoplasmic material pressed against the upper pole. Blastocyst epithelial cells became extremely flattened as the blastocyst began to expand prior to the next division.

Blastocyst undergoing primary expansion at about the 64-cell stage: duration, 8 h; cumulative time, 4 d 22 h - 5 d 6 h

4 d 23 h: the blastocyst continued to expand within the egg coats which had a crumpled appearance in many specimens. This primary expansion initially had little effect on the overall diameter of the conceptus (Table 3). The mucoid coat was soft and readily compressed at this time. The cytoplasmic material in the blastocoel formed a dark mass bounded by vesicular structures. This mass was closely associated with the blastocyst wall, most frequently with the part consisting of the more protuberant cells (Fig. 7F). The coats remained crumpled in appearance and the uterine cavity contained much mucoid material when conceptuses were collected at this stage.

5 d 6 h: expansion continued at a variable rate in different specimens. As the blastocyst expanded the inner cytoplasmic material broke up into vesicles and dark granular material (Fig. 7G).

Blastocyst undergoing primary expansion at about the 128-cell stage: duration, 18 h; cumulative time, 5 d 6 h - 6 d

5 d 15 h: during the division from the 64 to about 128 cells, the cells of the blastocyst epithelium became more uniformly flattened as the blastocyst expanded within the egg coats. As this happened, the coats lost their crumpled appearance, the mucoid coat thinned (Table 3) and the blastocyst became more turgid. The cytoplasmic material in the blastocoel became more irregular in appearance and formed a dark liquefied material on one side of the blastocoel (Fig. 7H).

6 d: blastocyst dimensions varied during expansion. Occasional large rounded cells or cell fragments lay in the cavity of the blastocoel (Fig. 7I). The dark liquefied material remained closely adherent to the blastocyst wall.

Discussion

The time from mating to collection of the various stages in this study varied little from that found in other studies (Baggott and Moore, 1990; Mate *et al.*, 1994). Some minor differences may be expected to occur between studies. Videotaping is usually done in the middle of the dark period and the mating that induced ovulation may have occurred before that time. In this study, collection time for stages to the two-cell stage varied by up to 6 h, but from the four-cell stage collection times for the same stage varied by up to 24 h, presumably because of variation in the length of the four-cell stage. This variation may be due to differences in rate of formation and development of the corpora lutea, which were in a more advanced morphological state in the specimens collected at the shortest time post coitum for the 8-, 16- and 32-cell stages collected. Faster development of the corpora lutea has been associated with faster embryonic development in the stripe-faced dunnart (Selwood and Woolley, 1991).

The duration of each stage *in vitro* in this study was shorter than those found by Baggott and Moore (1990), and similar to the times found by Selwood and VandeBerg (1992) perhaps because the same medium was used in this and the latter study. With the exception of the 64-cell stage, the cumulative times *in vitro* were similar to times post coitum *in vivo* (Table 4) in this study and in the study by Mate *et al.* (1994).

The simplest cleavage pattern (i.e. cleavage planes meridional, meridional, meridional and latitudinal) that was found in about half the specimens, has been described by Baggott and Moore (1990). The variations to this pattern found in this study and by Selwood and VandeBerg (1992) may be due to the differences in the amount of extruded cytoplasmic material in the cleavage cavity. The variations suggest that cleavage is not determinate, at least for the cells affected by the variations.

Difficulties encountered in culture of marsupial embryos

In the dasyurids and the grey short-tailed opossum the zygote becomes polarized (Figs. 2 and 6) and some cytoplasmic material is eliminated in a polarized fashion into the perivitelline space

during early cleavage. Thus a polarized conceptus is found in a polarized cleavage cavity in both animals. This polarization of the zygote and elimination of cytoplasm, which are essential for normal cleavage to proceed, are very susceptible to low temperatures (Selwood, unpublished). Handling at less than body temperature may be the reason for some of the early developmental failures *in vitro*.

The body temperature of many marsupials is less than that of comparable eutherians (Dawson and Hulbert, 1969; McNab, 1978) so that a temperature of 37°C may not be suitable for culture if long term viability of the embryos is desired. Studies that have investigated some aspects of the effects of temperatures higher than the body temperature (Selwood and VandeBerg, 1992; Moore and Taggart, 1993; Breed *et al.*, 1994a) have shown that some events during maturation, fertilization and early cleavage are not facilitated by higher temperatures although others are. Until studies with a reasonably large sample size are done a conservative approach would be to culture at the mean body temperature of the particular animal.

Information on determination of what constitutes a normal conceptus is scattered throughout the literature. Appreciation of what is normal is essential for selection of appropriate conceptuses to culture. As discussed above, for zygotes and early cleavage stages the development or presence of a polarized state is essential for normal development. Some definitions of normality that apply to dasyurid marsupials are given in a study assessing prenatal failure in the brown antechinus (Selwood, 1983). Two features of early development are relevant to assessment of normality of cleavage stages in all polytocous marsupials. Unfertilized eggs sometimes mimic the appearance of normal cleavage stages when the cytoplasm of the oocyte breaks up into smaller fragments. These specimens can be recognized as unfertilized by the absence of supernumerary sperm in the egg coats, by staining to show the number of nuclei or by comparing the more irregular morphology of the stage with a normal specimen. Secondly, marsupial oocytes are fertilized one at a time as the oocytes descend the oviduct over a 7 h to 15 h period dependent on the species (Selwood, 1992). This means, for example in the stripe-faced dunnart, that the stages of development can be 7 h apart. Because most cleavage divisions in this animal are 8-10 h long, two sequential stages can be found in the animal and still represent normal specimens. In cases where three cleavages stages are represented in the sample, the specimens with the smallest numbers of cells would be regarded as retarded abnormal stages. In later blastocyst stages (unilaminar, bilaminar and early trilaminar), rounding up of epithelial cells, detachment of the blastocyst epithelium from the overlying egg coats or darkening of the conceptus are signs of embryonic failure.

In general, marsupial cleavage and other later stages proceed in relatively complex media at slower rates to that *in vivo*. With complex media, analysis of conceptus requirements *in vitro* is more difficult. As a result, few studies have examined the effect of additions to the media or embryo requirements in culture. In the stripe-faced and fat-tailed dunnarts maintenance of early blastocyst expansion was improved when sodium lactate was added to the medium but progesterone did not have a direct effect on cell division (Selwood, 1987) as it has been shown to do in the mouse (Roblero and Izquierdo, 1976). Early blastocysts of the stripe-faced dunnart will continue to expand for a further two days if blastocysts are co-cultured with a combined stromal and epithelial, or epithelial monolayer of uterine endometrium (Selwood, 1989a). One study by Gardner *et al.* (1996) using a simpler medium has shown that glucose and pyruvate consumption by conceptuses of the stripe-faced dunnart during cleavage and early blastocyst expansion are much higher than those of the mouse, possibly because of the different mode of cleavage and blastocyst formation in the marsupial. Similar results would be expected in the grey short-tailed opossum in which the cleaving blastomeres have similar secretory behavior and cell-zona adhesiveness. The findings in the stripe-faced dunnart may explain why successful cleavage has been achieved *in vitro* in the brown antechinus, fat-tailed dunnart, grey short-tailed opossum and the tammar in DMEM with high glucose. Glucose consumption drops during the long four-cell stage. Development from the early uterine zygote to the complete unilaminar blastocyst was slower than development *in vivo* by about 12 h per day or 35 h over the culture period. This lag was reflected in the profiles of nutrient uptake. Although this defined, serum-free medium has not solved the problem of slower development *in vitro,* its application to marsupial conceptuses is a promising start for further analysis of the nutrient requirements of the marsupial embryo in culture. The simpler medium developed by Moore and Taggart (1993) for *in vitro* fertilization is also promising as a media for cleavage stages.

Conclusions

The establishment of methods to culture the marsupial oocyte and conceptus has given new insights into our knowledge of marsupial oocyte maturation, fertilization and embryonic and fetal development. The ability to culture these embryos allows more detailed investigation to proceed. This has particular implications for experimental studies of the mechanisms of lineage formation, evolution of the mammalian embryo, organogenesis and for preservation of some endangered marsupial species. Two animals, the stripe-faced dunnart and the grey short-tailed opossum should prove to be important experimental models, for reasons outlined earlier. With the appropriate culture systems developed, marsupial models have particular experimental advantages. The separation of trophoblast and pluriblast lineages occurs during the formation of the unilaminar blastocyst epithelium, not within a compacted structure as in the mouse. It is possible to study this process *in vitro* outside the egg coats in the dunnart. The requirements for formation and proliferation of the germ layers can be readily studied in marsupials because the pluriblast, and later the epiblast, are superficial in position and the conceptus does not implant until late. Finally, organogenesis to within 18 h of birth can now be studied *in vitro* in the stripe-faced dunnart using a relatively simple culture system.

Materials and Methods

This study is based on conceptuses from 21 grey short-tailed opossums examined at the Laboratory of Radiobiology and Environmental Health, University of California, San Francisco in 1990 and from 15 animals examined at Southwest Foundation, San Antonio in 1995. Maintenance and animal husbandry of the animals were as described by VandeBerg (1990) with the following exception in 1995. For mating in 1995, animals were paired in polycarbonate cages measuring 43x22x13 cm with a glass nest box 7 cm in diameter and 15 cm in length. Animals were filmed during the middle of the dark period on days 6-8 after pairing, the days when most matings occurred. Animals were filmed under red fluorescent light by a

Panasonic (Mitsubishi Electric Corp., Secaucus, NJ, USA) WVD-500 camera and a AG1250 recorder. Each video tape recording was examined the following morning on a BT-S1 monitor and the time of mating recorded. Fadem (1985) established that exposure to a male induces ovulation and that a fairly stereotyped behavior precedes mating. Baggott *et al.* (1987) used this to develop the video detection system and determined that estrus and mating occurred 6-14 days after pairing.

In 1990, 21 females that had been paired with males were shipped to San Francisco and examined on days 9-12 after pairing. In these animals the times post coitum were unknown. In 1995, 15 mated females were examined after video monitoring. These were examined on days 7 to 13 after pairing and 2 to 9 post coitum. Animals were killed by CO_2 inhalation. The uteri, oviducts and ovaries were held in sterile, warmed phosphate buffered saline (PBS) and the corpora lutea (CL) or follicles were examined and counted in each ovary. The CL count gives an estimate of the number of conceptuses to be expected in the uterus or oviduct. The uteri were opened by a midline-incision and the conceptuses everted into the PBS. They were counted, the best ones collected for culture (Selwood and VandeBerg, 1992), photographed and set up in culture in 1.0 ml of medium in wells of a 24-well Linbro plate. Examination and photography were done using a Nikon inverted TMS-F microscope and camera N2000. Half the medium was replaced at 24 hour intervals. The cultures were examined at 6 and 12 hour intervals or at lesser times. The medium used was DMEM plus 10% FCS with supplements of penicillin, streptomycin and glutamine (Selwood and Young, 1983). Culture was done at 32.6°C, the mean basal body temperature of the opossum, in 5% CO_2 in air.

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References

BAGGOTT, L.M. and MOORE, H.D.M. (1990). Early embryonic development of the grey short-tailed opossum *Monodelphis domestica: in vivo* and *in vitro*. *J. Zool. 222:* 623-639.

BAGGOTT, L.M., DAVIS-BUTLER, S. and MOORE, H.D.M. (1987). Characterisation of oestrus and timed collection of oocytes in the grey short-tailed opossum, *Monodelphis domestica*. *J. Reprod. Fertil. 79:* 105-114.

BREED, W.G., SIMERLY, C., NAVARA, C.S., VANDEBERG, J.L. and SCHATTEN, G. (1994a). Microtubule configurations in oocytes, zygotes and early embryos of a marsupial, *Monodelphis domestica*. *Dev. Biol. 164:* 230-240.

BREED, W.G., TAGGART, D.A., BRADTKE, V., LEIGH, C.M., GAMEAU, L. and CARROLL, J. (1994b). Effect of cryopreservation on development and ultrastructure of preimplantation embryos from the dasyurid marsupial *Sminthopsis crassicaudata*. *J. Reprod. Fertil. 100:* 429-438.

CHEN, L.T. and HSU, Y.C. (1982). Development of mouse embryos *in vitro*. Preimplantation to the limb bud stage. *Science 216:* 66.

COCKROFT, D.L. (1973). Development in culture of rat foetuses explanted at 12.5 and 13.5 days of gestation. *J. Embryol. Exp. Morphol. 29:* 473-83.

COCKROFT, D.L. (1976). Comparison of *in vitro* and *in vivo* development of rat foetuses. *Dev. Biol. 48:* 163-172.

COCKROFT, D.L. (1990). Dissection and culture of postimplantation embryos. In *Postimplantation Mammalian Embryos. A Practical Approach* (Eds. A.J. Copp and D.L. Cockroft). IRL Press, Oxford, pp. 15-40.

DAWSON, T.J. and HULBERT, A.J. (1969). Standard energy metabolism of marsupials. *Nature 22:* 383.

FADEM, B.H. (1985). Evidence for the activation of female reproduction by males in a marsupial, the gray short-tailed opossum *(Monodelphis domestica). Biol. Reprod. 33:* 112-116.

FADEM, B.H. and RAYVE, R. (1985). Characteristics of the oestrus cycle and influence of social factors in grey short-tailed opossums (*Monodelphis domestica*). *J. Reprod. Fertil. 73:* 337-42.

FIRST, N.L. and BARNES, F.L. (1989). Development of preimplantation mammalian embryos. In *Development of Preimplantation Embryos and Their Environment* (Eds. K. Yoshinaga and T. Mori). A. Liss, New York, pp. 151-170.

GARDNER, D.K., SELWOOD, L. and LANE, M. (1996). Nutrient uptake and culture of *Sminthopsis macroura* (Stripe-faced dunnart) embryos. *Reprod Fertil. Dev. 8:* 685-690.

GODFREY, G.K. (1969). Reproduction in a laboratory colony of the marsupial mouse *Sminthopsis larapinta* (Marsupialia: Dasyuridae). *Aust. J. Zool. 17:* 637-654.

HARTMAN, C.G. (1919). Studies in the development of the opossum *Didelphys virginiana*. III. Description of new material on maturation, cleavage and endoderm formation. IV. The bilaminar blastocyst. *J. Morphol. 32:* 1-139.

HILL, J.P. (1910). The early development of the marsupialia, with special reference to the native cat *(Dasyurus viverrinus).* Contributions to the embryology of the marsupialia, IV. *Q. J. Microsc. Sci. 56:* 1-134.

HINDS, L.A. and SELWOOD, L. (1990). Plasma progesterone concentrations during pregnancy in the dasyurid marsupial, *Antechinus stuartii*: relationship with differentiation of the embryo. *Reprod. Fertil. Dev. 2:* 61-70.

HUGHES, R.L. and SHOREY, C.D. (1973). Observations on the permeability properties of the egg membranes of the marsupial, *Trichosurus vulpecula*. *J. Reprod. Fertil. 32:* 25-32.

JOHNSON, M.H. and SELWOOD, L. (1996). A new terminology for early mammalian development. *Reprod. Fertil. Dev. 8:* 759-764.

KAUFMAN, M.H. (1990). Morphological stages of postimplantation embryonic development. In *Postimplantation Mammalian Embryos. A Practical Approach* (Eds. A.J. Copp and D.L. Cockroft). IRL Press, Oxford, pp. 81-92.

MATE, K.E., ROBINSON, E.S., VANDEBERG, J.L. and PEDERSEN, R.A. (1994). Timetable of *in vivo* embryonic development in the grey short-tailed opossum *(Monodelphis domestica). Mol. Reprod. Dev. 39:* 365-374.

McCRADY Jr, E. (1938). The embryology of the opossum. *Am. Anat. Mem. 16:* 1-233.

McNAB, B.K. (1978). The comparative energetics of neotropical marsupials. *J. Comp. Physiol. 125:* 115-28.

MERRY, N.E., JOHNSON, M.H., GEHRING, C.A. and SELWOOD, L. (1995). Cytoskeletal organization in the oocyte, zygote, and early cleaving embryo of the stripe-faced dunnart *(Sminthopsis macroura). Mol. Reprod. Dev. 41:* 212-224.

MOORE, H.D.M. and TAGGART, D.A. (1993). *In vitro* fertilization and embryo culture in the grey short-tailed opossum, *Monodelphis domestica*. *J. Reprod. Fertil. 98:* 267-274.

NEW, D.A.T. (1990). Introduction. In *Postimplantation Mammalian Embryos. A Practical Approach* (Eds. A.J. Copp and D.L. Cockroft). IRL Press, Oxford, pp. 1-14.

NEW, D.A.T. and COPPOLA, P.T. (1970). Effects of different oxygen concentrations on the development of rat embryos in culture. *J. Reprod. Fertil. 21:* 109-118.

NEW, D.A.T. and MIZELL, M. (1972). Opossum fetuses grown in culture. *Science 175:* 533-536.

NEW, D.A.T., COPPOLA, P.T. and COCKROFT, D.L. (1976). Comparison of growth *in vitro* and *in vivo* of postimplantation rat embryos. *J. Embryol. Exp. Morphol. 36:* 133-144.

NEW, D.A.T., MIZELL, M. and COCKROFT, D.L. (1977). Growth of opossum embryos *in vitro* during organogenesis. *J. Exp. Morphol. 41:* 111-123.

PIKE, I.L. (1981). Comparative studies of embryo metabolism in early pregnancy. *J. Reprod. Fertil. 29 (Suppl.):* 203-213.

PRATT, H.P.M. (1987). Isolation, culture and manipulation of preimplantation mouse embryos. In *Mammalian Development. A Practical Approach* (Ed. M. Monk). IRL Press, Oxford, pp. 13-42.

RENFREE, M.B. (1973). Proteins in the uterine secretions of the marsupial *Macropus eugenii*. *Dev. Biol. 32:* 41-49.

RENFREE, M.B. (1975). Uterine proteins in the marsupial, *Didelphis marsupialis virginiana* during gestation. *J. Reprod. Fertil. 42:* 163-166.

RENFREE, M.B. and LEWIS, A.McD. (1996). Cleavage and blastocyst formation in the tammar wallaby. *Reprod. Fertil. Dev. 8:* 725-42.

ROBERTS, C.T., BREED, W.G. and MAYRHOFER, G. (1994). The origin of the oocyte shell membrane of a dasyurid marsupial: an immunohistochemical study. *J. Exp. Zool. 270:* 321-331.

ROBLERO, L.S. and IZQUIERDO, L. (1976). Effect of progesterone on the cleavage rate of mouse embryos *in vitro*. *J. Reprod. Fertil. 46:* 475-476.

SELWOOD, L. (1980). A timetable of embryonic development of the dasyurid marsupial *Antechinus stuartii* (Macleay). *Aust. J. Zool. 28:* 649-668.

SELWOOD, L. (1983). Factors influencing pre-natal fertility in the brown marsupial mouse *Antechinus stuartii*. *J. Reprod. Fertil. 68:* 317-324.

SELWOOD, L. (1987). Embryonic development in culture of two dasyurid marsupials, *Sminthopsis crassicaudata* (Gould) and *Sminthopsis macroura* (Spencer) during cleavage and blastocyst formation. *Gamete Res. 16:* 355-370.

SELWOOD, L. (1989a). Marsupial preimplantation embryos *in vivo* and *in vitro*. In *Development of Preimplantation Embryos and Their Environment* (Eds. K. Yoshinaga and T. Mori). A. Liss, New York, pp. 225-236.

SELWOOD, L. (1989b). Development *in vitro* of investment-free marsupial embryos during cleavage and early blastocyst formation. *Gamete Res. 23:* 399-413.

SELWOOD, L. (1992). Mechanisms underlying the development of pattern in marsupial embryos. In *Current Topics in Developmental Biology*, Vol. 27 (Ed. R.A. Pedersen). Academic Press, New York, pp. 175-233.

SELWOOD, L. (1996). The blastocyst epithelium is not a protoderm in dasyurid marsupials: a review of the evidence. *Reprod. Fertil. Dev. 8:* 711-723.

SELWOOD, L. and SMITH, D. (1990). Time-lapse analysis and normal stages of development of cleavage and blastocyst formation in the marsupials the brown antechinus and the stripe-faced dunnart. *Mol. Reprod. Dev. 26:* 53-62.

SELWOOD, L. and VANDEBERG, J.L. (1992). The influence of incubation temperature on oocyte maturation, parthenogenetic and embryonic development *in vitro* of the marsupial *Monodelphis domestica*. *Anim. Reprod. Sci. 29:* 99-116.

SELWOOD, L. and WOOLLEY, P. (1991). A timetable of embryonic development and ovarian and uterine changes during pregnancy, in the stripe-faced dunnart, *Sminthopsis macroura* (Marsupialia: Dasyuridae). *J. Reprod. Fertil. 91:* 213-227.

SELWOOD, L. and YOUNG, G.J. (1983). Cleavage *in vivo* and in culture in the dasyurid marsupial *Antechinus stuartii* (Macleay). *J. Morphol. 176:* 43-60.

SELWOOD, L., SATHANANTHAN, H. and YOUSEF, A. (1993). The role of the egg coats in marsupial development. *J. Reprod. Dev. 39 (Suppl.):* 94-95.

SHERMAN, M.I. (1975). The culture of cells derived from mouse blastocysts. *Cell 5:* 343-349.

SPINDLER, R.E., RENFREE, M.B. and GARDNER, D.K. (1996). Metabolic assessment of wallaby blastocysts during embryonic diapause and subsequent reactivation. *Reprod. Fertil. Dev. 7:* 1157-1162.

TYNDALE-BISCOE, C.H. and RENFREE, M.B. (1987). *Reproductive Physiology of Marsupials*. Cambridge University Press, Cambridge.

VANDEBERG, J.L. (1990). The gray short-tailed opossum *(Monodelphis domestica)* as a model didelphid species for genetic research. *Aust. J. Zool. 37:* 235-247.

WILEY, L.M. and PEDERSEN, R.A. (1977). Morphology of mouse egg cylinder development *in vitro*. A light and electron microscope study. *J. Exp. Zool. 200:* 389-402.

YOUSEF, A. and SELWOOD, L. (1993). Embryonic development during gastrulation in *Antechinus stuartii* (MacLeay) and *Sminthopsis macroura* (Spencer) in culture. *Reprod. Fertil. Dev. 5:* 445-458.

YOUSEF, A. and SELWOOD, L. (1996). The type and differentiation of cells *in vitro* from unilaminar and bilaminar blastocysts of two marsupials, *Antechinus stuartii* and *Sminthopsis macroura*. *Reprod. Fertil. Dev. 8:* 743-752.