

DEVELOPMENT OF DORMANT BLASTOCYSTS INDUCED BY
OESTROGEN IN THE OVARIECTOMIZED MARSUPIAL,
MACROPUS EUGENII

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Summary

Ovariectomized and intact tammars (*M. eugenii*) were injected with oestrogen (oestradiol benzoate) for 3 days. Five developing blastocysts were recovered from six animals killed 7 days after first injection of a total of 150 µg of oestrogen. Four developing blastocysts were recovered from nine animals killed 7 days after first injection of a total of 300 µg of oestrogen. A single blastocyst, which had undergone some development, was recovered from one of seven intact animals which received 150 µg of oestrogen. Fewer blastocysts were recovered from ovariectomized animals receiving 300 µg of oestrogen, and from intact animals receiving 150 µg of oestrogen, than were recovered from ovariectomized non-injected animals and from ovariectomized animals receiving 150 µg of oestrogen.

The shell diameter of blastocysts recovered from injected ovariectomized animals was not significantly enlarged but protoderm diameter and number of contained cells were both significantly greater than in blastocysts from non-injected controls.

Systemic injection of oestrogen induced development in dormant blastocysts in tammars, but the developing blastocysts did not survive as well and were not retained as long as blastocysts in which other workers induced development by use of progesterone. It is possible that the uterus of macropodid marsupials imposes an inhibition to embryonic development during diapause and that this inhibition is removed by systemic injection of either progesterone or oestrogen.

I. INTRODUCTION

Many macropodid marsupials exhibit the phenomenon of embryonic diapause while a young is suckled within the pouch (Sharman, Calaby, and Poole 1966), or during an annual non-breeding season, as in the tamarin wallaby (Berger 1966). There is evidence that in these animals, a secretion from the corpus luteum is required for the termination of diapause. In the red kangaroo (*Megaleia rufa*) corpora lutea associated with diapause embryos were uniformly small in size, whereas corpora lutea associated with blastocysts that had resumed development often exceeded the rest of the ovarian tissue in size. Corpora lutea responded almost immediately to removal of the suckling stimulus and this suggested that suckling prevents completion of corpus luteum development and that embryonic development is arrested in the absence of a developed corpus luteum (Sharman 1963). After removal of pouch young in the quokka (*Setonix brachyurus*) corpus luteum and blastocyst resumed

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development at the same time (Tyndale-Biscoe 1963a). The corpus luteum was necessary for development of the embryo, and removal of the corpus luteum on days 0-2 after removal of pouch young prevented development of eight out of nine blastocysts (Tyndale-Biscoe 1963b). However, embryonic development occurred when animals bilaterally ovariectomized at removal of pouch young were injected with 70 mg of progesterone over a period of 7 days.

When progesterone was injected into red kangaroos carrying small pouch young, the blastocyst resumed development although the corpus luteum continued to be inhibited by the suckling stimulus (Clark 1968). Injected progesterone terminated embryonic diapause in intact and ovariectomized tammars whether given to females suckling pouch young during the breeding season or to females in the non-breeding condition (Berger and Sharman, unpublished results).

Injected oestrogen caused development of the dormant blastocyst of the red kangaroo suckling a young in the pouch, as did injection of progesterone (Clark 1968), but the corpus luteum was more vascular after oestrogen injection than during embryonic diapause (Smith, unpublished results). The histology and histochemistry of the corpus luteum was investigated in intact tammars to which oestrogen and progesterone were administered to terminate embryonic diapause (Sharman and Berger, unpublished results). The corpora lutea of hormone-treated animals, and saline-injected controls, did not differ in $\Delta^5\text{-}3\beta\text{-hydroxysteroid}$ dehydrogenase activity, but corpora lutea of animals injected with oestrogen were more vascular and had a much higher concentration of lipid than did those of progesterone-injected and control animals. These results suggested that the effects of progesterone and oestrogen on red kangaroos and tammars may have been mediated via different routes, injected progesterone acting directly on the uterus (or blastocyst) and oestrogen acting through the corpus luteum. This investigation was undertaken to see if oestrogen had an effect on the blastocyst in the absence of the corpus luteum.

Clark (1966) described the diapause blastocyst of the red kangaroo which, like the tammar dormant blastocyst, is about $300\ \mu$ in diameter and contains about 80 cells.

II. MATERIALS AND METHODS

Tammars (*Macropus eugenii* Desm.) trapped in early October on Kangaroo Island, S.A., were shipped to the mainland and accommodated in open pens at the University of Adelaide. They were fed dry lucerne hay and a mixture of wheat, oats, bran, and lucerne chaff.

The non-breeding season of the tammar extends from July to December, and during these months the blastocyst in the uterus of adult females does not develop, even though the pouch young or young-at-foot may be lost and the suckling stimulus terminated (Berger 1966). The animals used in this investigation were all adult females which had recently weaned a young and which could be expected to be carrying a dormant blastocyst.

Prior to surgery the animals were anaesthetized with pentobarbitone sodium (Nembutal, Abbott, 40 mg/kg), administered through one of the superficial tail veins. After the incision area had been clipped and sterilized, a further injection of 50-100 mg of Nembutal was given through an ear vein to maintain surgical anaesthesia. The reproductive system was exposed through a mid-ventral incision in the pouch region. The oviducts were held with artery forceps and the ovaries were cut from the bursae. The cut surface was cauterized to destroy any remnants of ovarian tissue. The body wall and the skin were sutured. Ovaries were fixed in alcohol-formalin-acetic acid, and the presence or absence of a corpus luteum of lactation was recorded.

Hormone injections were begun 7 days after ovariectomy. Oestrogen, administered as oestradiol benzoate in peanut oil (100 $\mu\text{g}/\text{ml}$), was injected intramuscularly at approximately 9.00 a.m. for 3 days, at the rate of 50 $\mu\text{g}/\text{day}$ (six animals) or 100 $\mu\text{g}/\text{day}$ (nine animals). Ovariectomized control animals (seven) were not injected and intact controls (seven animals) were injected with 50 $\mu\text{g}/\text{day}$ of oestradiol benzoate.

Seven days after the first injection, each animal was killed with chloroform, and the urogenital tract was removed. The uterus corresponding to the ovary bearing the corpus luteum of lactation was flushed with Ringer's solution (Cosol infusion fluid). Blastocysts located in the flushings under a binocular microscope were measured with a graduated eyepiece before being fixed in alcohol-formalin-acetic acid. The urogenital system was fixed in the same fluid.

Small pieces of corpora lutea, uteri, and lateral vaginae (taken near the point of confluence of the two lateral vaginae) were embedded in wax, sectioned at 6–8 μ , and stained with Ehrlich's haematoxylin and eosin.

Sections of uteri were stained with eosin-methylene blue to test for basophilia. Blastocysts were torn open with fine needles (Clark 1966), flattened, and stained with Weigert's haematoxylin (Fig. 5).

III. RESULTS

(a) Ovariectomized Control Animals

The uteri of animals killed 14 days after ovariectomy were like those of animals in the non-breeding condition (Figs. 1 and 2). The uterine glands were small, and in some the lumen was not patent. The epithelial cells of the glands were low columnar, with oval-shaped nuclei and basophilic cytoplasm. In the lateral vaginae the superficial cell layers of the epithelium were not cornified and few cells were being sloughed into the lumen.

Five blastocysts and one degenerate egg were recovered (Table 1). Shell diameters ranged from 270 to 320 μ , compared to diameters of 240–330 μ in normal blastocysts recovered from untreated animals (Berger 1966). The nuclei of protoderm cells were round or slightly elongate with granular chromatin (Fig. 6). No mitoses occurred in the cells but the blastocysts were normal and seemingly viable.

(b) Ovariectomized Animals Treated with Oestrogen

Seven days after the first oestrogen injection the uterus, seen in transverse section, resembled that of animals in the luteal phase of the oestrous cycle. The numerous uterine glands were irregularly scattered and many had a wide lumen. The epithelial cells were tall columnar, and the small, round nuclei were regularly basal (Fig. 3). Furthermore, the uteri of about half of the oestrogen-treated animals gave a negative reaction for basophilia when tested with the eosin-methylene blue reaction. In the brush possum, a characteristic of the luteal phase of the oestrous cycle is the negative reaction for basophilia in the peripheral portions of the uterine glands (Pilton and Sharman 1962), and a similar pattern of basophilia was seen in the uterus of an untreated tammar carrying a 3-mm blastocyst (Fig. 4). There were, however, differences between the uteri of animals injected with oestrogen and those of normal animals in the luteal phase. The staining differences between inner and peripheral uterine glands were not as marked in oestrogen-treated animals as during the luteal phase and the gland nuclei in oestrogen-treated animals were smaller and more strictly basal than in the luteal phase animal (cf. Figs. 3 and 4). No mitotic

activity occurred in the uterine epithelia of ovariectomized animals injected with oestrogen.

The lateral vaginae were enlarged, the stratified squamous epithelia of the posterior lateral vaginae were hypertrophied, and several layers of cells were cornified. The lumen was partially occluded with sloughed cornified cells.

Blastocysts were recovered from five out of six animals receiving 150 μg oestrogen and from four out of nine animals receiving 300 μg oestrogen (Table 1). The protoderm of one blastocyst was so degenerate that it was seen as an irregular white mass inside the shell. The shell of two others was dented although the protoderm was spherical. These three blastocysts were from animals that received a total of 300 μg oestrogen; all other blastocysts recovered were spherical.

The number of cells in each blastocyst ranged from 82 to 255 (Table 1). Mitotic figures were seen in nuclei of four blastocysts (Fig. 7) and, in general, dividing cells were seen in the blastocysts with fewer cells. Some of the nuclei in each blastocyst were round or slightly elongate, with granular chromatin, and resembled nuclei of blastocysts from ovariectomized controls, but many other nuclei were degenerating. Degeneration in some nuclei was seen as an involution of the nuclear membrane in several places, so that the outline of the nucleus was wavy (Fig. 8); other nuclei were shrunken and pycnotic. Together with degenerate nuclei there were many darkly stained droplets whose basophilic nature suggested that they may have been chromatin material released from broken-down nuclei.

(c) *Intact, Oestrogen-treated Control Animals*

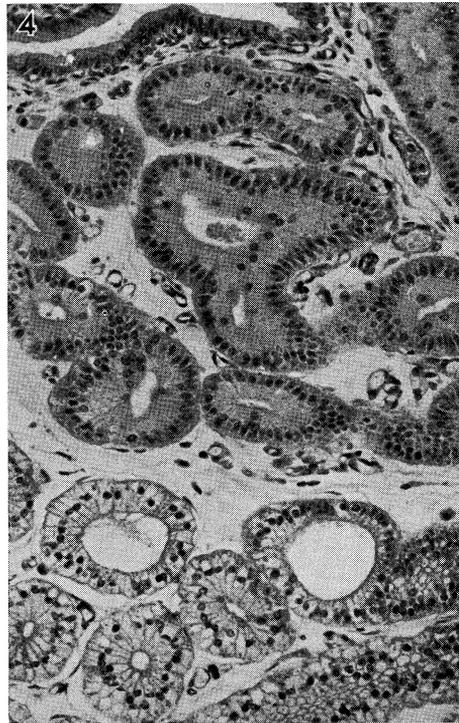
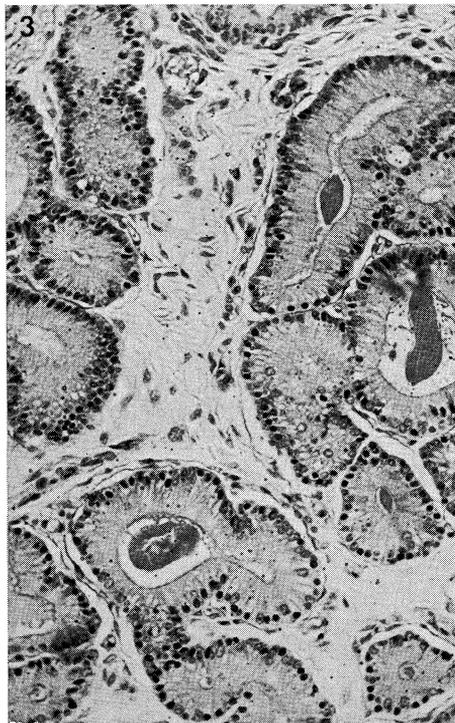
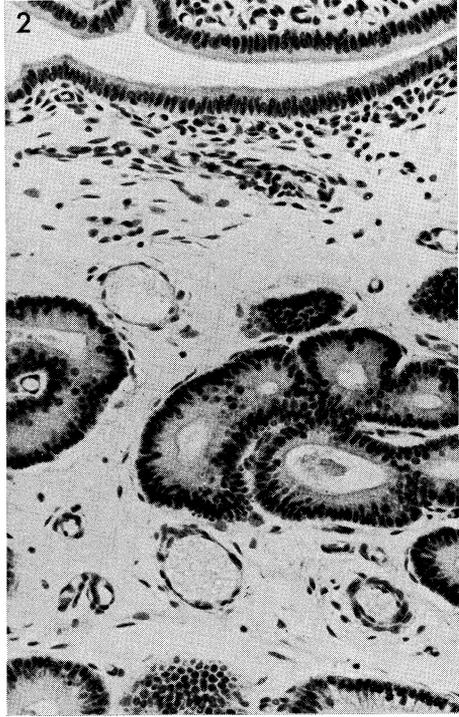
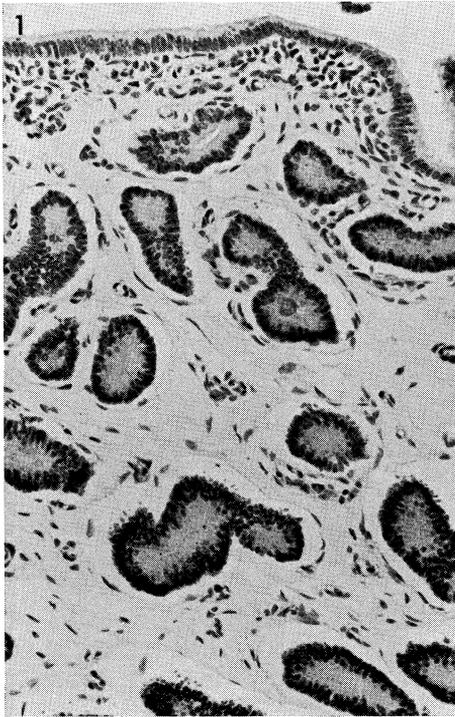
The microscopic appearance of uteri and lateral vaginae were similar to those of ovariectomized animals treated with oestrogen and the corpora lutea, stained with haematoxylin and eosin, did not differ appreciably from corpora lutea of lactation removed at ovariectomy.

Five of the seven animals failed to yield a blastocyst or egg from the uterus, and from one a degenerate egg was recovered. None of the cells were dividing in the single blastocyst recovered and many nuclei were degenerate. The higher number of cells in this blastocyst compared with those of uninjected animals suggests that some cell division may have occurred. A degenerating blastocyst was located in the median vagina of another animal.

IV. DISCUSSION

The shell diameters of blastocysts recovered from ovariectomized, injected animals (groups 2 and 3, Table 1) were not significantly enlarged, but both protoderm

Figs. 1-4.—Transverse sections of uteri. 1, Section taken 14 days after bilateral ovariectomy, uterine lumen epithelium at top. $\times 190$. 2, From an intact animal during the non-breeding season. Note general resemblance to uterus shown in Figure 1, and differences from uteri in Figures 3 and 4. $\times 190$. 3, From an animal bilaterally ovariectomized for 7 days then treated with 150 μg of oestrogen, cf. Figure 4. Small portion of uterine lumen epithelium at top, centre. $\times 160$. 4, From a pregnant animal which had a developing embryo 3 mm in diameter. The animal had a functional corpus luteum and was in the luteal phase of the oestrous cycle. Note difference in staining reaction between inner (top) and peripheral (bottom) glands. $\times 160$.



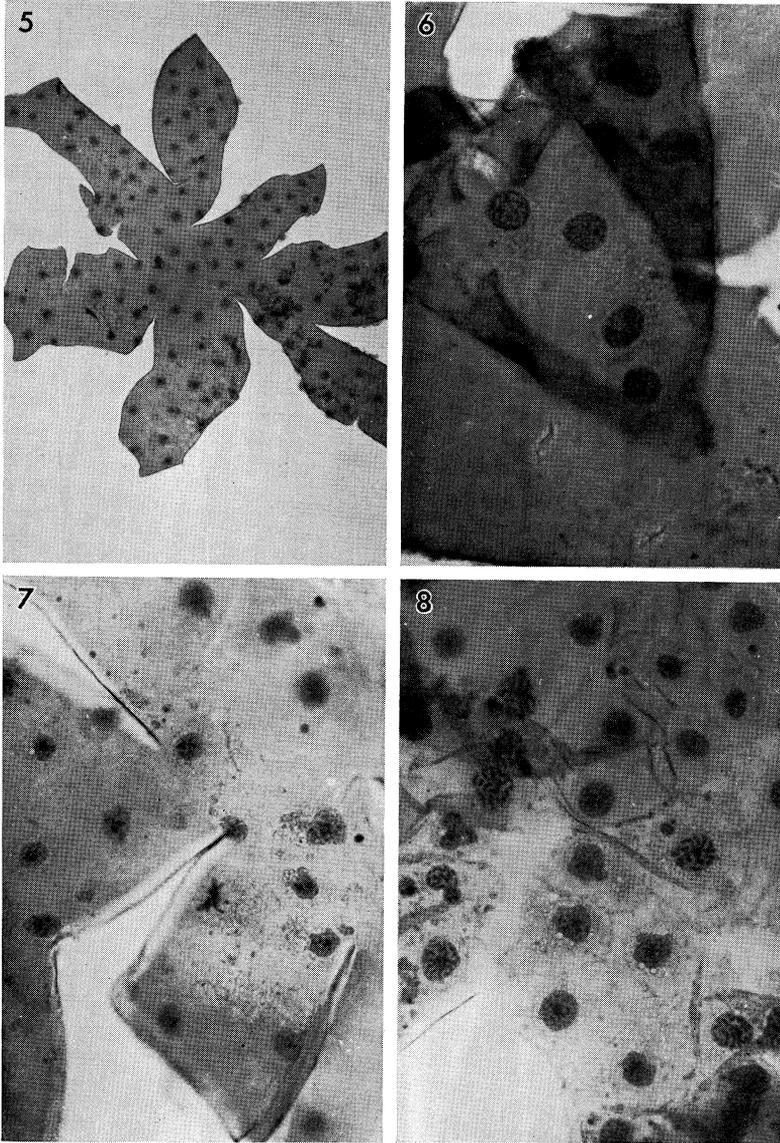


Fig. 5.—Flattened blastocyst from ovariectomized animal treated with 150 μ g of oestrogen. More nuclei are present than in the dormant blastocyst and many nuclei show signs of degeneration. $\times 85$.

Figs. 6–8.—Portions of protoderms of blastocysts. **6**, From bilaterally ovariectomized animal. Nuclei are round with granular chromatin. $\times 360$. **7**, From ovariectomized animal treated with 150 μ g of oestrogen. One nucleus is undergoing mitosis. $\times 360$. **8**, From intact animal treated with 150 μ g of oestrogen. Nuclei are degenerate. $\times 360$.

diameter and cell number were significantly increased ($P < 0.05$). The single uterine blastocyst recovered from one oestrogen-injected, intact animal had a greater protoderm diameter and more cells than had any blastocyst from non-injected animals. Increase in protoderm diameter and in number of cells present, coupled with the observation of mitotic activity in some blastocysts, demonstrated that oestrogen caused at least initial development of the dormant blastocyst in ovariectomized animals.

Five developing blastocysts were recovered from six ovariectomized animals which received a total of 150 μg of oestrogen — a slightly higher recovery than from ovariectomized control animals (cf. groups 1 and 2, Table 1). However, only four of the nine ovariectomized animals receiving a total of 300 μg of oestrogen (group 3, Table 1) yielded blastocysts. This suggests that the higher dose of oestrogen may have caused expulsion of developing blastocysts. The seven intact animals which received a total of 150 μg of oestrogen (group 4, Table 1) yielded only one blastocyst. It is assumed that the high loss of blastocysts from these animals may have been due to the combined effects of injected and intrinsic ovarian oestrogen. Expulsion of blastocysts, rather than their lysis in the uterus, appears to have occurred since a blastocyst was recovered from the vagina of one animal which did not have a uterine blastocyst. However, expulsion may have been a consequence of blastocyst death, since the ovariectomized animals receiving the highest dose of oestrogen had the most degenerate blastocysts [see Section III(b)].

Dormant blastocysts of the tammar resumed development when progesterone was injected 21 days after bilateral ovariectomy (Berger and Sharman, unpublished results). Seven developing blastocysts were recovered from eight animals 7 days after initial progesterone injection. A similar high yield of developing blastocysts was obtained from intact animals which were injected with progesterone. Clark (1968) terminated embryonic diapause in the intact red kangaroo with both oestrogen and progesterone used alone. Three days after initial injection the pouch young was removed and pregnancy was allowed to proceed to term. Of 33 progesterone-injected animals, 63% gave birth whereas only 45% of 11 oestrogen-injected animals gave birth.

It is thus evident that, although both oestrogen and progesterone, used alone, induced development of marsupial dormant blastocysts, a better recovery of blastocysts was obtained when development was induced with progesterone. This is different from the situation found amongst eutherian mammals which exhibit delayed implantation. Systemic injection of progesterone is without effect on the dormant blastocysts of rodents but Weichert (1942) and Krehbiel (1941) terminated delay in intact lactating rats with oestrogen. Whitten (1958) obtained implantation 26 hr after lactating mice were injected with oestrogen. Progesterone maintained delay of development of blastocysts in intact lactating rats until oestrogen was given in addition (Nutting and Meyer 1963). Canivenc and Bonnin-Laffargue (1963) failed to hasten the implantation of badger (*Meles meles*) blastocysts with progesterone and reported that, so far as they were aware, progesterone was without effect on the delayed implantation of all species in which it was tried. Yoshinaga (1961), however, induced development of the delayed blastocysts of rats by injecting minute amounts of progesterone into the adipose tissue surrounding the uterine arteries.

TABLE I
EFFECTS OF INJECTED OESTRADIOL BENZOATE ON DORMANT BLASTOCYSTS OF *MACROPUS EUGENII*

Group No.	Experimental Procedure	No. of Animals	No. of Blastocysts Recovered	Shell Diam. of Blastocysts (μ)			Protoderm Diam. of Blastocysts (μ)			No. of Cells in Blastocysts		
				Range	Mean	S.D.*	Range	Mean	S.D.*	Range	Mean	S.D.*
1	Ovariectomized, non-injected	7	5	265-320	294	21.1	176-240	202	31.1	60-97	79	15.9
2	Ovariectomized, injected 50 μ g/day for 3 days	6	5	245-400	328	50.2	200-360	276	46.9	82-255	150	49.5
3	Ovariectomized, injected 100 μ g/day for 3 days	9	4									
4	Intact, injected 50 μ g/day for 3 days	7	1	290			260			113		

* Standard deviation.

Progesterone induced development in the dormant blastocysts of three out of four quokkas bilaterally ovariectomized at removal of pouch young, and in one of five pregnant quokkas from which the ovary bearing the corpus luteum was removed on days 0-2 the embryo resumed development spontaneously (Tyndale-Biscoe 1963*b*). It is possible that this development was initiated by a release of oestrogen associated with the ovulation that occurs after removal of the corpus luteum, and that in the quokka, too, both oestrogen and progesterone are capable of terminating embryonic diapause.

Development of embryos in the rat may be delayed naturally, when the rat is suckling a large litter concurrent with pregnancy, or experimentally, when the ovaries are removed in early pregnancy. In the ovariectomized rat, progesterone is necessary for the maintenance of the blastocysts, but progesterone alone will not permit implantation if the ovaries have been removed before day 4 of pregnancy (Mayer 1963). A single injection of a small amount of oestrogen leads to implantation about 30 hr later. Both oestrogen and progesterone are required for development, and a synergism between the two hormones is indicated.

Rat blastocysts implant about a day after resuming development and oestrogen is necessary for uterine blastocysts to undergo pre-implantation changes (Yasukawa and Meyer 1966). Blastocysts maintained with progesterone alone were large and round. About 12 hr after the administration of oestrogen the blastocysts underwent a decrease in cross-sectional area associated with becoming elliptical in shape, and subsequent increases in size were by continued elongation until implantation occurred. When the tammar blastocyst resumes development, implantation is not imminent, and it is possible that the hormone requirements for growth are less stringent than in the rat.

When prepuberal mice were treated with gonadotropins to induce ovulation, corpora lutea did not form from the ruptured follicles, and the blastocysts remained dormant, but viable, in the uteri (Smithberg and Runner 1960). Progesterone and oestrogen are necessary for development of uterine blastocysts of rat and mouse, but blastocysts transferred to extra-uterine sites develop regardless of the sex or reproductive state of the host, and are independent of ovarian hormones (Kirby 1960, 1962). It seems that in the rodents, the uterus imposes the delay in embryonic development. Tyndale-Biscoe (1963*b*) believed that progesterone acted directly on the blastocyst of the quokka, for the blastocyst had increased in size before a luteal phase developed in the uteri. However, the uterus undergoes visible changes several days before it becomes fully luteal, and it is possible that in the macropodids the uterus imposes an inhibition to development that is removed by either progesterone or oestrogen. However, blastocyst development, after initial termination of diapause, proceeds further if progesterone rather than oestrogen is given.

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