Concentrations of oestradiol-17 β in plasma and corpora lutea throughout pregnancy in the tammar, *Macropus eugenii**

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Summary. Oestradiol-17 β concentrations were measured by radioimmunoassay in peripheral blood samples from 10 tammar wallabies after their pouch young were removed to terminate embryonic diapause. Oestradiol concentrations rose from 8.3 ± 1.2 pg/ml on Days 3 and 4 to a peak of 15.8 ± 2.9 pg/ml on Day 5, coincident with an increase in 'progesterone' concentrations, and then fell to 10.5 ± 2.7 pg/ml on Day 7. No changes in oestradiol concentrations were associated with parturition. Five females came into oestrus and mated 9.8 ± 6.1 h *post partum*; peak concentrations of plasma oestradiol (20.9 ± 2.1 pg/ml) occurred around the time of mating. None of the females that did not mate up to the end of the experiment at Day 30 had a rise in plasma oestradiol concentrations. Corpora lutea contained 20–100 pg oestradiol during pregnancy. The highest ovarian oestradiol content (> 1200 pg) was measured in whole ovaries containing Graafian follicles from full-term pregnant females. The rise in oestradiol concentrations at Day 5 may be important in the termination of diapause. The post-partum increase in plasma oestradiol concentrations coincides with oestrus. The source of this oestrogen appears to be the preovulatory follicle.

Introduction

Lactating female tammar wallabies carry a diapausing blastocyst formed at post-partum oestrus which will reactivate if the pouch young is removed between late January and May; birth occurs 27–28 days later. By June, seasonal factors prevent reactivation until around the time of the summer solstice in December when it occurs over a 10-day period (Sharman, 1955; Berger, 1966; Tyndale-Biscoe, Hearn & Renfree, 1974). Diapausing embryos can be reactivated by progesterone injections, but about 10 mg/day is needed for about 10 days, and there is a 50% loss, mostly at early stages of development (Renfree & Tyndale-Biscoe, 1973). This suggests that progesterone may not act alone to induce normal reactivation. Oestradiol may be one additional factor, since injections stimulated mitosis in diapausing tammar blastocysts (Smith & Sharman, 1969). Flint & Renfree (1982) reported a transient rise in plasma oestradiol concentrations which preceded embryonic expansion in wild-shot, seasonally reactivating tammars.

During lactation and the first 15 days of gestation the corpus luteum inhibits follicular growth (Tyndale-Biscoe & Hawkins, 1977). This inhibition can be mimicked after removal of the corpus luteum by injections of oestradiol but not progesterone, implicating luteal oestrogen production as a mediator (Evans, Tyndale-Biscoe & Sutherland, 1980; Renfree, Wallace & Young, 1982). Follicular growth occurs in late pregnancy, with oestrus 8–12 h *post partum* and ovulation about 1

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day later (Tyndale-Biscoe & Rodger, 1978). This follicular growth is associated with an increase in plasma oestradiol concentrations, suggesting that the preovulatory follicle may be the main source of oestrogen (Flint & Renfree, 1982). Certainly at this time there is a marked hypertrophy of the median and lateral vaginae (Tyndale-Biscoe, 1966) which can be induced at other times by giving oestradiol (Smith & Sharman, 1969). Oestradiol concentrations are also elevated before oestrus in another marsupial, the opossum *Didelphis virginiana* (Harder & Fleming, 1981).

Because seasonal reactivation occurs over about a 10-day period, it is difficult to relate the oestradiol values reported by Flint & Renfree (1982) precisely to the events of normal pregnancy. In this study, plasma oestradiol concentrations were measured in pregnancies following removal of pouch young during the breeding season. Progesterone content of the plasma extracts was also assayed to relate the changes in oestradiol to the changes in progesterone and to the peak in progesterone at Day 5 after reactivation reported by Hinds & Tyndale-Biscoe (1982). Since Flint & Renfree (1982) reported a peak of oestradiol 2 days before their first recovery of expanded embryos, frequent sampling was conducted for the 5-day period which precedes the start of embryo expansion, as well as in the peri-partum period.

Materials and Methods

Animals. The tammaras used in this study were part of a breeding colony established at Monash University with animals originally from Kangaroo Island, South Australia. Husbandry and handling were as previously described (Renfree & Tyndale-Biscoe, 1978). The females were housed with males throughout this study.

Pouch young were removed from 10 lactating females to terminate quiescence at 09:00–10:00 h on 1 March (i.e. during the breeding season). Blood samples were taken between 09:00 and 10:00 h before removal of pouch young (Day 0) and on Days 3, 4, 5, 6, 7, 15, 19, 23, 24 and 26. An additional sample was taken on Day 5 at 18:00 h. On Day 23, the animals were moved from an outdoor enclosure to smaller indoor observation pens. Pouches were checked for the presence of newborn young twice daily, and from 09:00 h on Days 26 and 27 the females were watched continuously for parturition. Blood samples were taken at 09:00 h on Days 27, 28 and 30, and more frequently if parturition or mating was observed (at 0, 4, 8 and 16 h after birth).

Collection of blood samples. Blood samples of 4–5 ml were taken from one of the lateral tail veins into heparinized syringes. Plasma was separated immediately and stored at -20°C until assayed. Blood haematocrit showed no significant changes over the duration of the experiment so it was not necessary to replace the red cells.

Oestradiol and progesterone assays. Duplicate samples of 1 ml plasma were diluted with 1 ml distilled water and heated in a water bath at 90°C , for 3 min. This heating was needed to denature an oestradiol-binding component which otherwise greatly reduced the extraction recovery to $<60\%$. This oestradiol binding does not change significantly during gestation (G. Shaw, unpublished observations). The samples were extracted by shaking vigorously for 3 min with 5 ml freshly opened diethyl ether (Analar, B.D.H. Australia, Port Fairy, Victoria) on a horizontal tube shaker at about four 3-cm excursions per second. The aqueous phase was frozen and the ether decanted into assay tubes, and dried at 50°C under dry nitrogen. After the ether had evaporated the temperature was increased to 90°C for a further 10 min. Without this heating the charcoal-stripped plasma blank was 15–20 pg/tube, and the standard curve in plasma flatter than the ethanol standard curve. With heating, the plasma blank dropped to 4–5 pg/tube, close to the values obtained for a buffer blank. The stripped plasma extract added to standard was also parallel to the ethanol standard curve. The extracts were redissolved in 300 μl assay buffer (0.1 M-phosphate buffer pH 7.1 containing 9 g NaCl/l, 1 g gelatin/l, 1 g NaN_3 /l). Oestradiol was assayed in 200 μl samples by adding 100 μl oestradiol antiserum (dilution 1:200 000 in assay buffer) and 100 μl assay

buffer containing 3 nCi [2,4,6,7-³H]oestradiol-17 β (Amersham International Ltd, Bucks, U.K.) were added.

The remaining 100 μ l of redissolved extract were assayed for progesterone by adding 100 μ l progesterone antiserum (dilution 1:9000 in assay buffer) and 100 μ l buffer containing 10 nCi [1,2,6,7-³H]progesterone (Amersham International Ltd). After an overnight incubation at 4°C, bound and free steroids were separated by adding dextran-coated charcoal (oestradiol assay: 300 μ l containing 0.625% w/v charcoal, 0.0625% dextran T70; progesterone assay: 400 μ l containing 0.25% charcoal, 0.025% dextran). After 10 min at 4°C the charcoal was sedimented by centrifugation. Radioactivity remaining in the supernatant was counted in a toluene-based scintillation fluid containing Teric X-10 using a Packard TriCarb scintillation counter.

Extraction recovery in each assay was determined in plasma samples which had been incubated with [³H]oestradiol or [³H]progesterone at 4°C for at least 2 h. Standards in the range 4–128 pg oestradiol or 10–640 pg progesterone were included in each assay as well as a variety of blanks and plasma samples with or without added oestradiol and progesterone.

Mean recovery of oestradiol was 85%, and of progesterone 79.2%. Buffer extraction blank was between 3.5 and 5.4 pg oestradiol and this was subtracted from all assay values. Extraction blank for the progesterone assay was <10 pg and was not subtracted. Charcoal-stripped plasma contained <2 pg oestradiol/ml and about 10 pg progesterone/ml. Plasma from a bilaterally ovariectomized tammar contained ~6 pg oestradiol/ml and ~160 pg progesterone/ml. These levels are similar to those previously reported for oestradiol by Flint & Renfree (1982) and for progesterone by Hinds & Tyndale-Biscoe (1982) and probably represent some adrenal contribution. All the samples from each of the serially bled females were assayed within a single assay. Intra- and inter-assay coefficients of variance were 10.5% and 7.3% for oestradiol and 14.6% and 10.9% for progesterone. Equilibration of 32 pg oestradiol/ml and 320 pg progesterone/ml with plasma increased the assayed concentration of oestradiol by 31.2 ± 1.1 (s.e.m.) pg ($n = 4$) and progesterone by 280 ± 27 pg ($n = 4$). An extract from a pool of plasma from late pregnancy containing [³H]oestradiol and [³H]progesterone to monitor recovery was applied to a 30 cm $\times$ 3.9 mm i.d., C₁₈ high performance liquid chromatography (HPLC) column (μ Bondapak: Waters Assoc., Milford, MA, U.S.A.), and eluted with 65% methanol in water. The eluate was collected in 1 ml fractions which were vacuum dried and assayed as described. Due to lack of surplus plasma, HPLC was not performed on a plasma pool from other stages of pregnancy.

The oestradiol antiserum (P6181), prepared by Dr R. I. Cox, CSIRO Division of Animal Production, Prospect, New South Wales, Australia, was raised against oestradiol-17 β -6-CMO-BSA, and is highly specific. Cross-reactions previously determined by 50% inhibition of binding of [³H]oestradiol were: oestradiol, 100%; oestrone, oestriol, and oestradiol-17 α , <1%; androstenedione, cortisol, and progesterone, <0.1%; and 17 α -hydroxyprogesterone, <0.01%. The progesterone antiserum (No. 230), also provided by Dr R. I. Cox, was raised against progesterone-6-BSA and has cross-reactivities of: progesterone, 100%; 11 β -hydroxyprogesterone, 6.7%; pregnenolone, 1.6%; 11-deoxycorticosterone, 1%, 5 β -pregnanedione, 3.5%; 17 α -hydroxyprogesterone, 20 α -dihydroprogesterone, 20 β -dihydroprogesterone, and corticosterone, 0.3%; and cortisol, <0.03%.

Extracts of ovarian tissue. Ovarian tissue was obtained from wild-shot seasonally reactivating tammaris in 1983 and stored frozen. The stage of pregnancy was estimated from embryonic growth curves (Renfree & Tyndale-Biscoe, 1973) and additional data from over 200 embryos measured by us in the course of other experiments. Extracts were made from homogenates of corpora lutea and from whole ovaries (containing numerous follicles at several stages of development and interstitial tissue) contralateral to the CL. Thawed tissues were homogenized in 1 ml distilled water containing ~1 nCi [³H]oestradiol to monitor recovery and extracted in 5 ml diethyl ether. The dried extracts were chromatographed through LH-20 in benzene-methanol (85:15, v/v). The oestradiol fraction was dried and resuspended in 600 μ l buffer. Recovery of [³H]oestradiol was determined in a 200 μ l aliquot and was $52 \pm 6\%$ (mean $\pm$ s.d.). The two remaining 200 μ l aliquots were assayed for oestradiol as described. To compensate for the contribution of the additional trace added (recovery

[^3H]oestradiol), a standard curve was prepared using an extra 0.15 nCi [^3H]oestradiol in each tube which corresponded to the average contribution of recovery of [^3H]oestradiol in the assay tubes.

Statistics. Data were compared by analysis of variance (Sokal & Rohlf, 1981).

Results

The HPLC eluate showed only a single peak of oestradiol immunoactivity which coincided with the elution of authentic oestradiol (Text-fig. 1). However, two peaks of progesterone immunoactivity were measured, one coinciding with authentic progesterone, and a smaller peak appearing between oestradiol and progesterone (i.e. more polar than progesterone) (Text-fig. 1). Therefore, the progesterone assay was measuring mostly progesterone, but some other substance(s) was contributing to the assayed value.

Text-fig. 1. HPLC elution profile of an extract of pooled plasma from late pregnant tammars. The dried extract was dissolved in 65% methanol in water and applied to a $\mu\text{Bondapac C}_{18}$ column. Elution was isocratic, at room temperature, with a flow rate of 1 ml/min; 1 ml fractions were collected, dried and redissolved in 500 μl assay buffer. Radioactivity in 200 μl was counted to monitor recovery of [^3H]oestradiol and [^3H]progesterone which had been added to the plasma before extraction. Total recovery was 85% for oestradiol and 82% for progesterone; the peak of oestradiol represents 81%, and for progesterone 58%. The scale is arbitrary (1 division = 50 c.p.m. in the fraction taken for counting).

Six females were seen to give birth, and 5 of these mated. In these 6 females, plasma oestradiol concentration was 10.5 ± 3.9 pg/ml (mean $\pm$ s.d.) before removal of pouch young, and 8.3 ± 1.2 pg/ml on Days 3 and 4, and rose significantly ($P < 0.01$) to a peak of 15.8 ± 2.9 pg/ml by 18:00 h on Day 5 (Text-fig. 2). By Day 7, concentrations had declined to 10.5 ± 2.7 pg/ml. The Day 5 peak coincided with the first signs of embryonic reactivation (Moore, 1978; Thorner, Renfree & Wallace, 1981), with our measured peak of progestagen (Text-figs 2a & 3) and with previously reported progesterone peak (Hinds & Tyndale-Biscoe, 1982). Progesterone on Day 5 was significantly higher ($P < 0.05$) than on Days 0–4 in our study.

The seventh female was transferred to another experiment on Day 27, and is believed to have been pregnant; a similar peak of oestradiol was seen on Day 5 in this female (Text-fig. 2a).

The other 3 females were not observed to give birth or to mate. The possibility that parturition was not observed and the pouch young lost cannot be excluded, but it seems most likely that they were not pregnant. In 2 of these animals oestradiol increased to a clear peak on Day 5 (Text-fig. 2b). The remaining female had consistently elevated oestradiol concentrations (≈ 15 pg/ml) which fluctuated erratically. In view of the consistent pattern displayed by the other 9 tammars this female was considered to have had an abnormal cycle and was not included for statistical analysis.

Text-fig. 2. Oestradiol concentrations in (a) pregnant tammar wallabies after removing pouch young, and (b) in tammar wallabies that were not seen to give birth and were probably not pregnant. In (a) one animal (●) gave birth on Day 28 but did not mate; the remaining 5 gave birth and mated on Days 26 (○ △ ◇ ★) and 28 (□). One animal (▲) was removed from the experiment on Day 27. Oestrus and mating occurred 9.8 ± 6.1 (s.d.) h *post partum*. In (b) the results joined by the broken line are thought to represent an abnormal cycle and have not been included in statistical analyses or in the data presented in Text-fig. 3.

Oestradiol concentrations at Days 15 and 19 were generally high (10–15 pg/ml), although there was considerable variation both between animals and days (Text-figs 2 & 3). No differences were evident when the pregnant and non-pregnant animals were compared.

In the 5 parturient females which came into oestrus, mating was first recorded 9.8 ± 6.1 (s.d.) h later. In these, oestradiol levels were elevated for about 2 days before parturition, reaching a peak 1–2 days *post partum*, then declining by Day 3 *post partum* (Text-fig. 4a). This increase was statistically significant when Day 23 and 24 (–2 to –5 days *prepartum*) concentrations were compared with the concentrations in samples taken after parturition and after mating ($P < 0.05$). No marked changes in oestradiol were noted coincident with parturition. In these 5 females progesterone assay values decreased at parturition. In 2 females parturition occurred before the decrease, in 1 parturition occurred while values were high but decreasing, and 1 gave birth after progesterone concentrations reached basal levels. Three of the 5 females that subsequently mated had very high concentrations of immunoreactive progesterone in the plasma in the sample taken immediately after copulation.

Text-fig. 3. Plasma oestradiol and 'progesterone' concentrations (means $\pm$ s.e.m.) in all 9 animals whether pregnant or not between Days 0 and 24. 'Progesterone' values are not all attributable to authentic progesterone due to the presence of a cross-reacting material (see Text-fig. 1). Data for the 10th animal were considered aberrant (Text-fig. 2b) and have not been included in the means presented here.

The parturient tammar that did not mate, and the 2 that were probably not pregnant and did not mate, had low oestradiol concentrations between Days 23 and 30, and no significant day-to-day variations were evident ($P < 0.20$) (Text-fig. 4b). 'Progesterone' fell between Days 24 and 28, but concentrations were highly variable in the 3 animals after this time (Text-fig. 4b).

Corpora lutea contained little oestradiol (20–100 pg/gland) at the stages examined (Table 1), and no obvious changes with stage of pregnancy were evident. However, no corpora lutea from the equivalent of Days 5–7 after removal of pouch young were assayed. Contralateral ovaries contained about 400–500 pg oestradiol during early to mid-pregnancy (Table 1). Over 1200 pg/ovary was measured at Day 27, when Graafian follicles were observed in the ovary. The 2 post-partum samples contained <200 pg: both animals had new corpora lutea although one still had a large unruptured follicle as well, and both had mated.

Text-fig. 4. Mean $\pm$ s.e.m. plasma oestradiol and progesterone concentrations in tammaris in the peri-partum period: (a) 5 tammaris that mated 9.8 ± 6.1 (s.d.) h post partum; and (b) 3 females that did not mate (see text). In (a), data have been synchronized about the time of parturition (arrow). When more than one sample was taken from a female on any day, the average of those samples was used to calculate the mean value presented.

Table 1. Oestradiol content of the corpus luteum and the contralateral ovary in tammaris

State of gestation	Corpus luteum			Contralateral ovary		
	No. of samples	Wet wt (mg)	pg/CL	No. of samples	pg/ovary	Follicle size
Quiescent	1	25	26.5	1	521	< 1 mm
Pre-attachment (Days 10–18)	9	43 ± 3	69 ± 38	4	392 ± 82	~ 3 mm
Late/full term (Days 24–27)	4	38 ± 6	24 ± 4	2	1230, 1280	> 4 mm
Post partum (2–3 days)	2	–	–	2	166, 191	New CL

Values are mean ± s.e.m.

Discussion

The oestradiol concentrations reported in this study are similar to those reported previously for tammaris (Renfree & Heap, 1977; Flint & Renfree, 1982), and in opossums (Harder & Fleming, 1981) and show relatively small increases during pregnancy and the oestrous cycle. The results confirm the oestrous oestradiol peak described previously and also show a peak of oestradiol at Day 5, coincident with embryonic reactivation and a peak of luteal progesterone secretion (Text-fig. 3) (Moore, 1978; Thornber *et al.*, 1981; Hinds & Tyndale-Biscoe, 1982; Hinds, Evans & Tyndale-Biscoe, 1983). This supports the evidence from wild-shot tammaris that an early rise in oestradiol concentration occurs just before the time of embryonic expansion (Flint & Renfree, 1982).

The 'progesterone' concentrations assayed follow a pattern qualitatively similar but somewhat higher than that described by Hinds & Tyndale-Biscoe (1982), reflecting the interference from the cross-reactant observed in the HPLC analysis. This substance, which was extracted together with progesterone and oestradiol-17 β by the diethyl ether used in this study, presumably does not normally contribute to the immunoactive progesterone measured after extraction with hexane (Hinds & Tyndale-Biscoe, 1982) or petroleum spirits (Renfree, Green & Young, 1979). It is unlikely to be 11 β -hydroxyprogesterone, 11-deoxycorticosterone, or 17 α -hydroxyprogesterone because these elute close to oestradiol, or pregnenolone or 20 α -dihydroprogesterone, which elute close to progesterone in the HPLC system used here.

The increase in plasma oestradiol coinciding with a peak of progesterone at Day 5 suggests a luteal origin for the oestrogens at this time. This has been proposed by Evans *et al.* (1980) and Renfree *et al.* (1982), who observed that oestradiol but not progesterone or androstenedione could mimic the inhibition of follicular growth by the quiescent corpus luteum in tammaris from which the corpus luteum had been removed. However, Renfree, Flint, Green & Heap (1984) could not detect aromatase in luteal tissue at Day 0 or between Days 11 and 25 after removal of pouch young, and corpora lutea contained only minute quantities of oestradiol. Likewise, in this study, the content of oestradiol in corpora lutea from mid- to late pregnant tammaris was very low and of a similar quantity to that found by Renfree *et al.* (1984), and much larger amounts were found in homogenates of whole ovary contralateral to the active corpus luteum and containing developing follicles. Interstitial tissue is unlikely to contribute to oestradiol levels because it too apparently lacks aromatase, although whole ovaries containing follicles are rich in this enzyme (Renfree *et al.*, 1984).

The first reported sign of embryonic reactivation is an increase in RNA metabolism at Day 5 after removal of pouch young (Moore, 1978; Thornber *et al.*, 1981; G. Shaw, unpublished observations). Hinds & Tyndale-Biscoe (1982) suggested that this reactivation was induced by the rise in progesterone at this time. Since oestradiol rises concurrently, it may be synergistic with progesterone, as originally suggested by Clark (1968).

Oestradiol administered to tammar with diapausing blastocysts causes uterine growth, and induces mitosis and early development of the blastocysts (Smith & Sharman, 1969; Renfree *et al.*, 1982). Blastocysts reactivated in this way would probably develop to term if given appropriate luteal support. Five of 11 lactating red kangaroos given 3 daily oestradiol injections and the pouch young removed on the 4th day produced young, and in 3 of the 5 the interval between removal of pouch young and birth was shorter than in non-injected animals (Days 23, 25 and 26 compared with 31–33 Days) (Clark, 1968). Bilateral ovariectomy of tammars on Day 8 after removal of pouch young does not affect the subsequent gestation (Tyndale-Biscoe, 1970), although plasma progesterone concentrations have been elevated for only 3–4 days (Hinds & Tyndale-Biscoe, 1982). Treatment of ovariectomized tammar with 1, 2 or 4 mg progesterone for 3 days reactivates most embryos, although most have degenerative changes (Berger & Sharman, 1969). Higher doses of 10 mg/day given over 10 days to intact tammar reactivates most embryos, but only about half of them develop normally, with the greatest incidence of abnormal development occurring before Day 11 after removal of pouch young (Renfree & Tyndale-Biscoe, 1973). Progesterone is therefore probably not the sole factor involved in embryo reactivation and maintenance of pregnancy, and the results of the present study suggest that oestradiol may be equally important.

In all 5 tammar that mated there was a clear rise in oestradiol at the time of oestrus. Since the ovarian content of oestradiol assayed at Day 27 was very high, but low by 2 days *post partum*, the peak of oestradiol measured in the peripheral plasma is apparently of follicular origin. The highest levels were seen within 1 day *post partum* and may be associated with the LH peak which occurs about 16 h *post partum* (Sutherland, Evans & Tyndale-Biscoe, 1980; Tyndale-Biscoe, Hinds, Horn & Jenkin, 1983). It is likely that the elevation of oestradiol in these females induced oestrous behaviour since none of the females that had low plasma oestradiol concentrations in the post-partum period mated. If sampling had continued beyond Day 30 in the non-pregnant animals, an oestradiol peak may have been detected as oestrus is known to occur later, around Day 30, in non-pregnant females after removal of pouch young (Merchant, 1979).

The data presented in this study confirm that plasma oestradiol concentrations are elevated at oestrus in tammar. The finding of a peak of oestradiol in plasma at Day 5 warrants further study to clarify whether it is of luteal origin and whether it is involved synergistically with progesterone in the termination of embryonic diapause.

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