PROGESTERONE AND PROLACTIN IN MARSUPIAL REPRODUCTION

by

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of

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This thesis is my own work, except where specifically acknowledged.

L.A. Hinds

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ABSTRACT

In this thesis aspects of the endocrinology of marsupial reproduction and lactation have been examined. The main species used was the tammar wallaby, *Macropus eugenii*, but some comparative results for the possum, *Trichosurus vulpecula*, and the native cat, *Dasyurus viverrinus* are also presented.

Radioimmunoassays for plasma progesterone and prolactin were developed and validated, and were used to examine luteal function during pregnancy and the oestrous cycle in the three species, as well as hypophysial-ovarian interaction in the tammar and possum. In addition the changes in plasma prolactin during lactation were examined in the latter two species.

In tammars the pattern and concentration of plasma progesterone was very similar during pregnancy and the oestrous cycle. The pattern of progesterone comprised an early pulse on Day 5, 6 or 7 post-oestrus which was followed by another rise in the second half of the cycle or pregnancy. The early peak was also observed in cycles delayed by lactation. Basal concentrations of progesterone were less than 200 pg/ml, and the maximum levels reached in the early peak and at the end of the cycle were around 500 pg/ml. At the end of pregnancy the decline in progesterone was more rapid than at the end of an infertile cycle. Plasma prolactin levels were low (20 ng/ml) throughout the oestrous cycle and were similar during pregnancy except for a sharp surge (> 50 ng/ml) just before parturition. The role of the surge of prolactin is unknown. However, the differences in the patterns of plasma progesterone and prolactin at the end of pregnancy imply that the foetus influences its time of birth.
In the possum, during the oestrous cycle the plasma progesterone pattern was unimodal, with maximum concentrations (8-15 ng/ml) between Days 12 to 16 after oestrus. Pregnancy did not affect either the profile or concentration of progesterone. A surge of prolactin occurred before oestrus and at the end of the luteal phase in both reproductive states.

In the native cat there was a small but significant pro-oestrus rise (1-2 ng/ml) as well as a second major rise (8-15 ng/ml) in plasma progesterone between Days 12 to 17 of the cycle. A similar pattern was observed during pregnancy.

A transient peak of progesterone early in the cycle was not observed in either the native cat or possum. It was concluded that for these three species there was no evidence of maternal recognition of early pregnancy.

The interaction between the ovary and pituitary was examined in the tammar and possum. In the tammar, hypophysectomy induces reactivation of the corpus luteum (CL) and blastocyst. Plasma progesterone profiles and concentrations in hypophysectomised tammars were similar to intact cycling animals implying that the CL is independent of the pituitary for growth and production of progesterone.

In the possum, hypophysectomy on Days 1, 4, 8 or 12 after oestrus did not prevent the embryo developing to term but parturition failed. Plasma progesterone profiles were not significantly different from sham-operated females except on Day 14 when levels were lower. Thus in the absence of the pituitary the possum CL can grow and produce enough progesterone to support pregnancy to term. The pituitary, however, may be essential for successful parturition. It was concluded that the tammar and possum
CL are autonomous with respect to progesterone production for the maintenance of pregnancy.

In tammars the pattern of plasma prolactin during lactation differed markedly from the eutherian pattern. Levels were low (< 40 ng/ml) for the first 140 days of lactation, then fluctuated upwards to reach maximum levels (> 100 ng/ml) by 180-200 days. These high levels were maintained until the young permanently vacated the pouch (≈ 250 days). Thereafter levels declined markedly and remained low even though lactation continued for at least another 40 to 60 days. Removal of the pouch young between 200 and 250 days resulted in a rapid decrease in plasma prolactin which was reversed when the young was returned to the pouch. This sucking-induced response declined once the young vacated the pouch. Thus the second half of lactation (> 180 days) can be equated with lactation in eutherians. No eutherian equivalent for the first half of lactation in the tammar is known.

The pattern of plasma prolactin during lactation in the possum closely resembled that of the tammar. Therefore it is suggested that the pattern is typical of diprotodont marsupials, and may allow concurrent asynchronous lactation to occur.
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<td>corpus luteum</td>
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<td>RPY</td>
<td>removal of pouch young</td>
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<tr>
<td>p.c.</td>
<td>post-coitum</td>
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<tr>
<td>LH</td>
<td>luteinising hormone</td>
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<tr>
<td>FSH</td>
<td>follicle-stimulating hormone</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine 3',5'-monophosphate</td>
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<tr>
<td>PGFM</td>
<td>13,14-dihydro-15-keto-prostaglandin F\textsubscript{2\alpha}</td>
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<tr>
<td>CB154</td>
<td>bromocriptine</td>
</tr>
<tr>
<td>IU</td>
<td>International Units</td>
</tr>
<tr>
<td>RIAD</td>
<td>radioimmunoassay diluent</td>
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<tr>
<td>NGPS</td>
<td>normal guinea-pig serum</td>
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<tr>
<td>NSB</td>
<td>non-specific binding</td>
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<td>i.v.</td>
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<tr>
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<tr>
<td>Plate 7</td>
<td>Transverse section of sella turcica region after incomplete hypophysectomy (possum).</td>
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CHAPTER 1.

CORPUS LUTEUM FUNCTION AND REGULATION IN EUTHERIANS

The endocrine regulation of the mammalian corpus luteum (CL) has been of considerable interest to reproductive biologists for many years. The CL plays a central role during both the oestrous cycle and pregnancy, and its life-span can be influenced by numerous factors which may be of either intrinsic, hypophysial and/or placental origin (Heap, 1972).

Progesterone, the major steroid secreted by the CL, is essential for the establishment and maintenance of early embryonic development in all mammalian species although the subsequent importance of the CL and its progesterone secretion differs between the species (Heap, Perry and Challis, 1973). Some species such as the goat and rabbit depend on the CL throughout gestation, while others such as the sheep and guinea pig can dispense with the CL once the placenta begins producing progesterone (Parkes and Deanesly, 1966; Heap et al., 1973). The maintenance of progesterone secretion by the CL and/or placenta is largely regulated by the release of luteotrophic hormones from the pituitary, and so removal of the pituitary can also disrupt pregnancy. For example in some species (rabbit, pig, goat) hypophysectomy at any stage of pregnancy results in abortion while in others (sheep, guinea pig, rat) luteotrophic support from the pituitary is required only during early pregnancy, as subsequently the placenta produces luteotrophins (Heap et al., 1973).
After ovulation the cells of the ruptured follicle luteinise to form the CL and the progesterone produced conditions the uterus to accept and nourish the developing embryo. The progestational changes in the uterus, which are marked by growth of the uterine glands and proliferation of the uterine stroma, occur in most species regardless of whether the mating was fertile (Parkes and Deanesly, 1966). The secretory activity of the CL has been determined for many species by measuring the concentration of progesterone in the peripheral circulation. Such studies have shown that peripheral progesterone concentrations differ between species and also within a single species at different stages of the oestrous cycle, pregnancy, pseudopregnancy or diapause. The CL of pregnancy may grow larger, survive longer and produce more progesterone than during the oestrous cycle or pseudopregnancy as in the rodents (rat, mouse, guinea pig). In contrast, there may be no change in the size or secretory activity of the CL but the life span may be prolonged as occurs in the artiodactyls (goat, pig, sheep, cow, white-tailed deer). This prolongation of the functional life-span of the CL in response to the presence of an embryo is referred to as the maternal recognition of pregnancy (Short, 1969; Heap, Flint and Jenkin, 1978). Alternatively the life-span, growth and function of the CL of pregnancy may be indistinguishable from either a CL of pseudopregnancy or of an infertile cycle as in the monoestrous carnivores (ferret, blue fox, red fox, dog) (for list of references see below).

<table>
<thead>
<tr>
<th>Species</th>
<th>Effect of pregnancy on CL life-span</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Rodents</td>
<td></td>
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<tr>
<td>mouse</td>
<td>longer and produce</td>
<td>Murr, Bradford and Geschwind, 1974.</td>
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<tr>
<td>guinea-pig</td>
<td>more progesterone</td>
<td>Challis, Heap and Illingworth, 1971.</td>
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<td>Artiodactyls</td>
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<tr>
<td>goat</td>
<td>No change in size or secretory</td>
<td>Thorburn and Schneider, 1972.</td>
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<td></td>
<td>activity but live</td>
<td>Guthrie, Hendricks and Handin, 1972;</td>
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<td></td>
<td></td>
<td>Ash and Heap, 1975.</td>
</tr>
<tr>
<td>pig</td>
<td>longer</td>
<td>Thorburn, Basset and Smith, 1969;</td>
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<tr>
<td>sheep</td>
<td></td>
<td>Basset, Oxborrow, Smith and Thorburn, 1969.</td>
</tr>
<tr>
<td>white-tailed deer</td>
<td></td>
<td>Plotka, Seal, Schmoller, Karns and Kleenlyne, 1977.</td>
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<tr>
<td>Monoestrous carnivores</td>
<td>Life-span, growth and function</td>
<td>Heap and Hammond, 1974.</td>
</tr>
<tr>
<td>ferret</td>
<td>and function</td>
<td>Möller, 1973a.</td>
</tr>
<tr>
<td>red fox</td>
<td></td>
<td>Concannon, Hansel and Visek, 1975.</td>
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<td>dog</td>
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</table>

In eutherian species capable of exhibiting embryonic diapause, progesterone levels during diapause are generally low and not significantly different from levels in either non-pregnant, lactating or anoestrous females. However, at the time of implantation there is a marked increase in progesterone concentration which is maintained until near parturition (roe deer - Hoffmann, Barth and Karg, 1978; badger - Bonnin, Canivenc and

There is also wide variation in absolute concentrations of progesterone during the cycles. For example, in the artiodactyls peak levels are low, at less than 20 ng/ml, while in the rodents they tend to be high, 60-130 ng/ml (grey squirrel - Tait, Pope and Johnson, 1981; rat and mouse) with values in the guinea pig ranging up to 500 ng/ml. In the carnivores, levels are also variable ranging from 5 ng/ml to greater than 100 ng/ml (dog, fox, ferret, mink, spotted skunk). The level of plasma progesterone in the African elephant, *Loxodonta africana*, however, is very low compared to other eutherian species. Concentrations in non-pregnant cycling females range from 26 to 215 pg/ml and in pregnant animals increase to 482 pg/ml (Plotka, Seal, Schobert and Schmoller, 1975). It should be noted here that the progesterone produced throughout pregnancy is not always solely of luteal origin as in some species it is supplemented or replaced by placental progesterone (Heap et al., 1973).

After ovulation the subsequent persistence and function of the CL depends upon interaction between the ovary, pituitary, uterus and/or conceptus although the relative importance of each differs with the species (Hilliard, 1973; Hansel, Concannon and Lukasewska, 1973). The role of the pituitary and the importance of the adenohypophysial hormones in the regulation of the secretory activity of the CL during pregnancy has been assessed for several species. Hypophysectomy before mid-pregnancy often leads to foetal death and resorption due to the loss of anterior lobe secretions (rabbit, rat, mouse, guinea pig, rhesus monkey), although after
implantation hypophysectomy has different effects in different species depending on whether the placenta assumes a luteotrophic role (Parkes and Deanesly, 1966; Heap et al., 1973). In the rat, mouse, sheep, ferret, hamster, mink and goat, prolactin, either alone, or in conjunction with luteinizing hormone (LH) and/or follicle stimulating hormone (FSH), forms a luteotrophic complex which is essential for the support and maintenance of the CL at least in the early stages of pregnancy and/or the oestrous cycle (Morishige and Rothchild, 1974; Ford and Yoshinaga, 1975a,b; Barkley, Bradford and Geschwind, 1978; Denamur, Martinet and Short, 1973; Kann and Denamur, 1974; Murphy, 1979; Greenwald, 1973; Papke, Concannon, Travis and Hansel, 1980; Mohini, Raj and Chapeker, 1980). Nevertheless, during the later stages of pregnancy in the rat, mouse, guinea pig and sheep the placenta assumes a luteotrophic role by producing a placental luteotrophin which maintains progesterone secretion either by the CL or by the placenta (Heap et al., 1973).

In most mammals the production of progesterone by the existing CL must cease before a new cycle can be initiated. This luteolytic process depends on both pituitary and uterine factors. In the large domestic animals (cow, sheep, pig, mare), rodents (mouse, rat, hamster, guinea pig) and rabbits, luteal regression during the oestrous cycle depends on the presence of the non-gravid uterus which produces a luteolytic factor, prostaglandin (Hansel et al., 1973; Hilliard, 1973). Prolactin is also involved in the regression of the CL of the cycle in the rat and mouse. In both species, if the surge of prolactin at oestrus is blocked, the CL of the previous cycle accumulate on the ovary although the function of the next batch of CL is unaffected (Gelato, Lu and Meites, 1972;
Grandison and Meites, 1972). Although a surge of prolactin is present at oestrus in the ewe (Davis, Reichert and Niswender, 1971) it is not an essential component of the events leading to either regression of the last CL or successful ovulation and subsequent formation and function of the next CL (Niswender, 1974; Kann and Denamur, 1974). In the hamster (Bast and Greenwald, 1974) and pig (Bevers, Willemsen and Krup, 1978) a surge also occurs but its function is unknown, while in the vole the role of the peak at oestrus, after mating, is luteotrophic and not luteolytic (Milligan and Mackinnon, 1976).

In several species (guinea pig, hamster, rat, mouse, rabbit) the conceptus is anti-luteolytic during the initial phases of pregnancy and this allows the CL to continue to secrete progesterone beyond the end of the cycle (Hilliard, 1973). Similarly in the sheep, cow and pig embryonic secretions appear to be involved in the maintenance of the CL of early gestation (Niswender and Fitz, 1980). The production and secretion of uterine prostaglandins is suppressed until near parturition at which time they are implicated in the events leading to delivery of neonates. Parturition is preceded by a sequence of endocrine changes of both foetal and maternal origin which may include activation of the foetal pituitary-adrenal axis, withdrawal of progesterone from the maternal circulation and an increase in the concentration of prostaglandins in the utero-ovarian vein. In those species (e.g. cow, pig, goat) in which progesterone production during pregnancy depends on secretion from the CL, prostaglandins may be luteolytic affecting CL regression and thus progesterone withdrawal at term. In these and other species, prostaglandins then act on the uterine myometrium to induce contractions (Thorburn, Challis and Currie, 1977; Thorburn
and Challis, 1979). Many other factors, such as increased levels of oxytocin, glucocorticoid and oestrogen, are also involved, but the importance of any one hormone and its influence on subsequent events leading to parturition varies between the different species.

Thus, although there is a wide diversity in the regulation of the activity of the CL during the oestrous cycle and pregnancy, in all species, independent of the reproductive state, the pattern of progesterone secretion during the life-span of the CL consists of a rising phase, plateau phase and a regressing phase. The length of each phase may vary between species and may also depend on whether the animal is undergoing a fertile or infertile cycle. There is no universal trophic agent which maintains progesterone production - in some it is prolactin; in others LH or combinations of prolactin, LH and/or FSH; as well there may be a changeover in the emphasis of which hormone(s) is necessary. There are also different mechanisms which induce luteolysis although prostaglandins of either luteal or uterine origin are generally implicated.

The various reproductive mechanisms which have led to an increase in the dependence of the embryo on the endocrine secretions of the maternal CL and placenta in most eutherians may be regarded as adaptations for viviparity. Among the four cohorts of eutherian mammals (Unguiculata, Ferungulata, Glires and Mutica), however, there are many instances of parallel evolution in different orders and divergent evolution in closely related species (Sharman, 1976), and so it is difficult to propose a universal model for the evolution of the function and regulation of the mammalian CL. Rothchild (1981) has attempted to do this and has proposed that the CL evolved from an autonomous self-regulating body capable of producing progesterone and prostaglandins. Then, in the eutherian
species, with the exception of the carnivores, the evolution of viviparity was accompanied by a decrease in the degree of autonomy of the CL as it became responsive to extrinsic luteotrophins, firstly of hypophysial, then of placental, origin. In most species the CL has become dependent on these extrinsic influences which have modified at least the plateau phase of the CL life-cycle.

PROLACTIN DURING GESTATION, AT PARTURITION AND IN LACTATION IN EUTHERIANS

The patterns of maternal peripheral prolactin levels seen during pregnancy are very similar in the rabbit, mouse, rat, sheep, goat, cow, dog, monkey and human (McNeilly and Friesen, 1978a; Murr et al., 1974; Amenomori, Chen and Meites, 1970; Morishige et al., 1973; Davis et al., 1971; Kann and Denamur, 1974; Buttle, Forsyth and Knaggs, 1972; Hoffmann, Schams, Giménez, Ender, Hermann and Karg, 1973; Ingalls, Convey and Hafs, 1973; Concannon, Butler, Hansel, Knight and Hamilton, 1978; Weiss, Butler, Hotchkiss, Dierschke and Knobil 1976; McNeilly, 1979). In these species there is an increase in prolactin levels in late pregnancy and a surge of prolactin immediately before delivery. The general increase in prolactin levels in the second half of gestation appears to be essential for the development of the mammary gland and initiation of lactogenesis (Ensor, 1978; Cowie, Forsyth and Hart, 1980). For example in the ewe, if prolactin levels are suppressed using bromocriptine (CB154) before or during parturition, although normal delivery proceeds, milk yield but not milk composition is affected (Karg and Schams, 1974). The factor responsible for the prolactin surge in the ewe is not known but in the rat it is derived from the
ovary; ovariectomy on Day 17 or Day 21 of pregnancy prevents the dramatic rise in prolactin concentration observed in sham-ovariectomised animals between Days 21 and 23 (Bridges and Goldman, 1975). However, as the animals were killed on Day 23 shortly before normal parturition, it is not known whether successful delivery of pups would have occurred in the absence of the surge.

After parturition prolactin levels remain elevated only if the female lactates. The sucking stimulus of the young, or mechanical stimulation of the mammary gland, induces a release of prolactin which, in the rat (Amenomori et al., 1970), sheep (Lamming, Moseley and McNeilly, 1974; Kann and Denamur, 1974), cow (Koprowski and Tucker, 1973) and goat (Buttle et al., 1972) is important for the maintenance of milk production. As lactation proceeds however, the release of prolactin in response to the sucking stimulus decreases in magnitude as does the peripheral concentration of prolactin.

During lactation there is a period of either partial or complete inhibition of ovarian activity which is of variable duration and which is known as lactational anoestrus (Lamming, 1978). Many workers have found that elevated prolactin levels may be directly implicated in the maintenance of lactational anoestrus in several species (sheep - Lamming et al., 1974; man - McNeilly, 1979; cow - Lamming, 1978; rat - Maneckjee and Moudgal, 1975; monkey - Maneckjee, Srinath and Moudgal, 1976), although whether prolactin exerts its antigonadal effect on the hypothalamus, pituitary or ovary is often unresolved. In the ewe, for example, the release of prolactin following sucking is abolished by either injection of bromocriptine or denervation of the suckled gland. Either of these treatments results in an earlier return to oestrus
than in control ewes (Kann and Martinet, 1975), and so the surges of prolactin specifically induced by sucking are in some way responsible for the suppression of ovarian activity during lactation. Subsequent work indicates that hyperprolactinaemia in ewes may impair the positive feedback effect of oestrogen on the hypothalamus (Kann, Martinet and Schirar, 1976).

In women the number of sucking episodes per day is important for maintaining high prolactin concentrations and these two factors together determine the period of lactational amenorrhoea (McNeilly, 1979; Howie and McNeilly, 1982; McNeilly, Glasier, Jonassen and Howie, 1982). Clinically-induced high levels of prolactin result in the loss of pulsatile LH secretion by the pituitary and in the loss of positive feedback effects of ovarian oestrogens on LH and FSH release. In addition hyperprolactinaemia causes an increase in the sensitivity of the pituitary to the negative feedback effects of oestrogen, thereby reducing LH release (McNeilly, 1979). If ovulation occurs, while lactation continues, it is invariably associated with prolonged follicular phases and deficient CL secreting inadequate amounts of progesterone. Normal ovarian activity eventually resumes as a consequence of a decrease in the sucking stimulus which results in a fall of basal prolactin concentrations and an increase in both the basal level of LH and its pulsatile release (Howie and McNeilly, 1982).

**PATTERNS OF REPRODUCTION IN MARSUPIALS**

Most marsupials are polyoestrous, and after spontaneous ovulation the CL formed will grow and induce a secretory 'luteal' phase in the uterus regardless of whether fertilisation occurred.
The gestation length, which in the majority of species varies from 12-38 days, is generally shorter than the oestrous cycle (22-46 days), and after parturition subsequent cycles are suppressed by lactation (Tyndale-Biscoe, 1973; Tyndale-Biscoe, Hearn and Renfree, 1974). Among the marsupials there is a wide diversity of reproductive patterns, even amongst closely related species. Sharman, Calaby and Poole (1966) recognised four patterns of reproduction in the diprotodont marsupials but since this time further studies have shown that most marsupials (Diprotodonta and Polyprotodonta) exhibit one of two basic patterns (Stewart and Tyndale-Biscoe, 1983).

The first pattern, the most common, is regarded as the primitive form from which the remainder have evolved. Group 1 is characterised by polytocos or monotocos polyoestrous species in which the gestation period is considerably shorter than the oestrous cycle such that pregnancy occupies only the luteal phase and parturition occurs at the time the CL begin to regress. Lactation and/or the sucking stimulus suppress subsequent follicular growth, pro-oestrus, oestrous and ovulation, although follicular growth resumes if the young is lost or removed during the breeding season. The pattern is common to the Phalangeridae, Petauridae and Didelphidae, the best known examples being the monotocos possum, Trichosurus vulpecula (Pilton and Sharman, 1962), and the polytocos opossum, Didelphis virginiana (Hartman, 1923). The Peramelidae also fit this general pattern although pregnancy is shorter than the luteal phase; plasma progesterone declines to basal concentrations by 10 to 19 days after parturition, but the CL remain large and do not involute until after 42 days of lactation (Hughes, 1962; Lyne, 1974, 1976; Lyne and Hollis, 1979; Gemmell, 1981).
In Group 2 the species are monotocous and polyoestrous with a gestation period almost the same length as the oestrous cycle. Post-partum oestrus and ovulation occur but further development is arrested by lactation. The CL remains quiescent and the embryo is retained in the uterus as a dormant unilaminar blastocyst unless the young is lost or removed, or until near the end of lactation when development resumes. This pattern of lactational quiescence, or facultative diapause, is typical of the majority of macropodid species, the best known example being the red kangaroo, *Macropus rufus* (Tyndale-Biscoe et al., 1974). The quokka, *Setonix brachyurus*, and the tammar, *Macropus eugenii*, also belong to this group, although lactational quiescence is usually followed in the second half of the year by seasonal anoestrus (Sharman, 1955a,b) or obligate seasonal quiescence respectively (Berger, 1966). Four macropodid species do not fit this Group 2 pattern (Group 4 of Sharman et al., 1966). Although all have a lengthened gestation period typical of other macropodids, post-partum oestrus does not occur. For three species, the parma wallaby, *M. parma* (Maynes, 1973a), the whiptail wallaby, *M. parryi* (Maynes, 1973b) and the eastern grey kangaroo, *M. giganteus* (Poole and Catling, 1974; Poole, 1975) ovulation occurs near the end of lactation and if the egg is fertilised diapause follows. In the fourth species, the western grey kangaroo, *M. fuliginosus* ovulation occurs at the end of lactation but there is no delay (Poole and Catling, 1974). The pattern shown by these species is essentially another variation of the Group 1 type and Maynes (1973a) suggested that despite the lengthening of the gestation period they have retained some aspects of the more primitive reproductive pattern and so provide evidence of the way diapause may have evolved in the majority of macropodids.
displaying the Group 2 pattern.

Two other patterns are recognised. Group 3 is represented by a single species, the swamp wallaby, Wallabia bicolor. It is polypoestrous and monotocous and exhibits lactational delay of diapause, but unlike the species in Group 2, gestation exceeds the length of the oestrous cycle which is not affected by the pregnancy and so a pre-partum oestrus occurs (Sharman et al., 1966). The remaining pattern is represented by some members of the families Burramyidae (Cercatetus concinnus, Acrobates pygmaeus) and Tarsipedidae (Tarsipes spencerae) (Renfree, 1981a). They are polytocous, polypoestrous species with long gestation periods and slowly growing or totally delayed blastocysts during lactation. In Tarsipes spencerae, for example, the gestation period is between 60 and 80 days (Renfree, 1981a) the longest reported for any marsupial.

It is clear that there is a wide diversity of patterns in the marsupials with even closely related species showing considerable differences in their breeding cycles. Do these patterns reflect differences in the mechanisms regulating hypophysial-ovarian interactions, the function of the CL and its progesterone production?

**FORMATION AND FUNCTION OF THE MARSUPIAL CL**

The CL of marsupials is formed predominantly from the granulosa cells and, as in eutherians, mitoses are present in the luteal cells for the first 3 to 4 days but thereafter growth to the maximum size is due to hypertrophy (Tyndale-Biscoe, 1983). No further mitoses occur except in species exhibiting embryonic diapause; when development of the CL resumes after delay a short
period of cell division occurs 4 to 6 days after reactivation (quokka - Tyndale-Biscoe, 1963c; red kangaroo and tammar - Sharman and Berger, 1969; potoroo, Potorous tridactylus - Shaw and Rose, 1979; red-necked wallaby, Macropus rufogriseus banksianus - Walker, Gemmell and Hughes, 1983). Histologically the formation of the CL of pregnancy and the oestrous cycle is similar in all species examined (native cat, Dasyurus viverrinus - Sandes, 1903; O'Donoghue, 1912; opossum - Hartman, 1923; quokka - Sharman, 1955a,b; possum - Pilton and Sharman, 1962; Shorey and Hughes, 1973b), and it will grow and induce a secretory 'luteal' phase in the uterus regardless of whether fertilisation occurs. However, in contrast to many eutherians, hysterectomy has no effect on the life span of the CL of the opossum (Hartman, 1925) and the possum (Clark and Sharman, 1965). Indeed the life span and function of the CL is thought to be similar in each state (Sharman, 1970).

Sandes (1903) was the first to recognise that the CL of the marsupials may have an endocrine role. Nevertheless until recently few studies have been done, and then only in a few species - the opossum, possum and bandicoot (Group 1 species) and among the macropodids the red kangaroo, quokka and tammar (Group 2 species). These studies have shown that the CL has at least four distinct functions. Early in the cycle it suppresses follicular growth and ovulation and stimulates the onset of the luteal phase in the uterus, while later it induces mammary gland growth and development and may also be involved in the preparation of the birth canal for parturition (Tyndale-Biscoe et al., 1974).

In the above species the importance of the CL and its secretions during pregnancy and parturition has been determined. In the absence of suitably sensitive techniques for measuring plasma
steroid concentrations the main approach used was ablation or removal of the CL (or CL-bearing ovary) at various times during pregnancy to examine the effects on the development of the embryo and the condition of the uterus.

The 'luteal' uterus is established by Day 7 to 8 in the possum (Pilton and Sharman, 1962), Day 6 in the opossum (Hartman, 1923) and quokka (Tyndale-Biscoe, 1963a) and by Day 7 to 8 in the tammar (Sharman and Berger, 1969; Tyndale-Biscoe, 1970). In the quokka and tammar removal of the CL or ovaries before Day 2 post-coitum (p.c.) does not affect the development of the embryo to the unilaminar blastocyst stage (Tyndale-Biscoe, 1963a; Sharman and Berger, 1969) and the uterus does not become luteal, while luteectomy or ovariectomy between Days 2 to 6 after removal of pouch young (RPY) results in the subsequent collapse of the developing embryo. However after Day 6 in the quokka (Tyndale-Biscoe, 1963a) and by Day 8 in the tammar (Tyndale-Biscoe, 1970) the uterus is competent to maintain development of the embryo to term in the absence of CL secretions, although parturition does not occur. Successful parturition and establishment of lactation occurs in the tammar luteectomised on Day 23 or Day 25 after RPY but not when luteectomy is performed on Day 17 after RPY (Young and Renfree, 1979). The CL of the tammar and the quokka, therefore, is needed at the beginning of pregnancy to establish the luteal phase in the uterus and again at the end of the cycle for the development of the mammary gland and parturition. Similarly in the opossum, embryos collapse if the ovaries are removed before Day 6, but develop to term if ovariectomy is performed after Day 6 of the 13 day gestation period (Hartman, 1925; Renfree, 1974), and as in the macropodids, parturition is inhibited. In the possum, the CL can be dispensed with after Day 11
of the 17.5 day gestation period and parturition can occur (Sharman, 1965). In each of these species the critical factors allowing embryo development to continue appear to be the stimulation of the luteal condition in the uteri by secretions from the CL and the ability of the endometrium to remain in a secretory condition without further ovarian stimulation once it has been primed.

As in eutherian species, progesterone has been identified by paper- and gas-liquid chromatography as the main steroid produced by active luteal tissue (red kangaroo - Lindner and Sharman, 1966; opossum - Cook and Nalbandov, 1968; possum - Curlewis, Stone, Poulton and Axelson, 1981; tammar - Renfree and Heap, 1977; Renfree, Flint, Green and Heap, 1983). Other steroids, possibly oestrogens, were also found in the tammar CL (Renfree and Heap, 1977; Renfree et al., 1983). Injection of progesterone (10 mg/day for 3 days) induces the luteal phase in the uterus of the quokka (Tyndale-Biscoe, 1963a), red kangaroo (Clark, 1968) and tammar (Berger and Sharman, 1969; Renfree and Tyndale-Biscoe, 1973), and stimulates the resumption of blastocyst development to term in each of these species whether administered to intact or ovariectomised females. Estradiol-17β benzoate injections will also induce development of the blastocyst but only 46% continue to term compared with 64% after progesterone injection (red kangaroo - Clark, 1968; tammar - Smith and Sharman, 1969). These experiments indicate that the secretions of the CL may have a direct effect on the blastocyst, but an action mediated via the uterus can not be excluded.

The latter possibility was examined by transferring quiescent blastocysts to the uteri of tammars ovariectomised on Day 8 after RPY. Development resumed but most embryos collapsed and were recovered at various stages of development, ranging from undeveloped
blastocysts to advanced embryos (Tyndale-Biscoe, 1970). The results suggested that resumption depended solely on changes in the uterine environment, a change initially signalled by the secretions of the CL. Nevertheless the first signs of expansion (quokka - Tyndale-Biscoe, 1963c) and RNA polymerase activity (tammar - Moore, 1978) in the blastocyst appear before the secretory condition was first detected in the uteri of these species, supporting the view that the CL may directly signal the blastocyst in anticipation of improved uterine conditions.

The CL is also involved in the development of the mammary gland. During pregnancy and the oestrous cycle the enlargement of the mammary glands correlates closely with the growth of the CL (native cat - O'Donoghue, 1912; opossum - Hartman, 1923; brush possum - Sharman, 1962; red kangaroo - Sharman and Calaby, 1964). However, the transfer of new born young to the teats of non-pregnan
t females demonstrated that pregnancy was not necessary for the establishment of normal lactation (possum - Sharman, 1962; red kangaroo - Sharman and Calaby, 1964; Merchant and Sharman, 1966). These several studies have provided evidence which strongly indicates that in marsupials pregnancy does not influence either the life-span or function of the CL. In Group 1 and 2 species removal of young at birth does not affect the return to oestrus; in Group 2 species the lengths of oestrous cycles and pregnancies are very similar; there is a close resemblance of anatomical and histological appearances of the CL and uteri in both states for all species; blastocysts transferred to the uteri of non-pregnant synchronous females develop and new born young transferred to oestrous females successfully establish normal lactation. These close similarities led Sharman (1970) to suggest that the oestrous cycle might
also be hormonally equivalent. However in the absence of sensitive hormone assay techniques the hypothesis has not been rigorously tested.

A recent review (McDonald and Waring, 1979) of the hormones of marsupials and monotremes clearly demonstrates how little information is available on either the structure, secretion or peripheral concentrations of the reproductive hormones, despite the considerable literature on marsupial reproduction. Indeed before this study began, plasma progesterone concentrations had been reported for only two species of marsupial. Thorburn, Cox and Shorey (1971), using a competitive protein binding technique, reported levels ranging from 0.5 to 5 ng/ml for the brush possum through the oestrous cycle. These results in the possum were similar to those found in several eutherian species including the sheep (Thorburn et al., 1969), guinea-pig (Feder, Resko and Goy, 1968), monkey (Neill, Johansson and Knobil, 1967), spotted skunk (Mead and Eik-Nes, 1969) and cow (Donaldson et al., 1970). In 1972 Lemon measured peripheral progesterone in female tammar wallabies through pregnancies and oestrous cycles, also using the competitive protein binding technique, but found the levels (125-1000 pg/ml) to be much lower than those reported for the possum and other eutherian species.

Since these two studies, the technique of radioimmunoassay has been developed and used for the measurement of many hormones. The technique has many advantages compared with the previous methods particularly with respect to the marked increase in specificity, sensitivity and ease of performance. Furthermore, less plasma is required for each estimation which means that samples can be collected more frequently and/or over longer periods without
significantly affecting the haematocrit of the donor. With the knowledge that progesterone levels in the tammar were very low it was essential that any assay for progesterone in this species be both accurate and sensitive, and so the radioimmunoassay technique was adopted, and has been validated for three species, the tammar, possum and native cat. They are representatives of three marsupial families, each having different patterns of reproduction. The tammar is a diprotodont and exhibits embryonic diapause. It is monotocous and shows the Group 2 type of reproductive pattern. The possum, also a diprotodont, is monotocous and displays the Group 1 pattern of reproduction, while the native cat, a polyprotodont, also displays the Group 1 pattern but it is polycous. Neither the possum nor the native cat exhibit embryonic diapause, but all three species are strict seasonal breeders. These three species were chosen for investigation, not only because of their different patterns of reproduction, but also because many of the physiological aspects of their reproduction are known, and all are amenable to captivity.

Since 1970 some evidence has indicated that the hormonal equivalence hypothesis may not hold for all marsupials, particularly the macropodid species of the Group 2 pattern of reproduction. Lemon (1972) reported that levels of peripheral progesterone in the tammar were higher in late pregnancy than at the end of the oestrous cycle, although sequential samples were not taken from these animals through both cycles. Merchant (1976, 1979) and Merchant and Calaby (1981) provided the first unequivocal evidence that the oestrous cycle are not the same in macropodid marsupials by showing that the length of the delayed oestrous cycle was significantly shorter when pregnancy intervened in the agile wallaby, *Macropus agilis*, the
tammar and Macropus rufogriseus. More recently, Cake, Owen and Bradshaw (1980) reported differences between pregnant and non-pregnant quokkas at the beginning of the cycle initiated by RPY. They observed a transient peak of progesterone on Day 4 after RPY, but the rise occurred only in the pregnant animals. This was of considerable interest as it was the first evidence for maternal recognition of pregnancy in any marsupial.

THE REPRODUCTIVE CYCLE OF THE TAMMAR, MACROPUS EUGENII (DESMAREST)

The tammar, Macropus eugenii (Desmarest, 1817) (Order Diprotodontia, Family Macropodidae) has the distinction of being the first recorded Australian marsupial seen by Europeans. It was described by Francisco Pelsaert from the Dutch ship Batavia which was wrecked in the Wallabi Group of islands off Western Australia in 1629. The first type specimen for M. eugenii, however, was collected from St. Peter's Island, Nuyts Archipelago, South Australia by Desmarest in 1817 (Iredale and Troughton, 1934). During the 1800's the tammar was widely distributed on the mainland of South and Western Australia as well as on various islands off these coastlines (Recherche group, Kangaroo Island, S.A.; Garden Island and Houtman's Abrolhos, W.A.). Today, although populations still exist on most of these islands and in isolated pockets on mainland Western Australia the largest numbers are found on Kangaroo Island, South Australia (Calaby, 1971). This macropodid species exhibits embryonic diapause (Sharman, 1955c) and has a Group 2 type pattern of reproduction. It is a strictly seasonal breeder (Berger, 1966) with most females either entering oestrus or giving birth and undergoing post-partum oestrus in late January to early February.
Ovulation occurs between 30 and 40 h post-coitum (Tyndale-Biscoe and Rodger, 1978; Sutherland, Evans and Tyndale-Biscoe, 1980) and the CL forms within 3 to 4 days (Sharman and Berger, 1969). If fertilisation occurs, by Day 8 the embryo has developed to a unilaminar blastocyst comprising between 80 and 160 cells (Tyndale-Biscoe, 1979). Further growth of the CL and blastocyst is arrested during lactation. However if the pouch young is either removed or lost between February and June development of the quiescent CL and dormant blastocyst resumes. The CL increases in size from about 10 mg to 60 mg by Day 15 and remains large until just before birth (Renfree and Tyndale-Biscoe, 1973; Renfree, Green and Young, 1979). Tissue progesterone concentration is relatively constant until Day 13, but thereafter increases, reaching maximum concentrations around Days 22 to 24 and declining before birth. The total progesterone content of the CL reflects the changes in mass (Renfree et al., 1979). The earliest indication of reactivation of the blastocyst is the increase in RNA polymerase activity on Day 5 after RPY (Moore, 1978). Expansion begins between Days 8 to 10, the primitive streak appears on vesicles of 4 to 5 mm diameter (about Day 14 RPY), and embryogenesis is well advanced by Day 21 (Renfree and Tyndale-Biscoe, 1973; Tyndale-Biscoe, 1979). The intervals from RPY to birth and to post-partum oestrus are 26.2 ± 0.2 days and 26.4 ± 0.18 days (Mean ± SEM) respectively. If the female was not carrying a diapausing blastocyst the interval from RPY to oestrus is significantly longer (30.4 ± 0.3 days, P < 0.0005) (Merchant, 1979), indicating that there is an influence of the conceptus on the oestrous cycle. When the cycles are not delayed by lactation the interval between oestrous periods is 30.6 ± 0.37 days, and if pregnancy intervenes it is 29.4 ± 0.34 days (Merchant, 1979).
A period of facultative diapause, when sucking is the proximate stimulus arresting ovarian activity, is typical of all macropodid species exhibiting diapause (Group 2 pattern) and is termed lactational quiescence. In the quokka, lactational quiescence is followed by a period of seasonal anoestrus (August to December). During this period the quiescent CL and blastocyst formed after post-partum oestrus in lactational quiescence degenerate and disappear, the uteri are small and pale, and the uterine glands are few and poorly developed compared to the uteri of lactationally quiescent females. Thus at the beginning of the following breeding season (January) all females must enter oestrus before undergoing a pregnancy (Sharman 1955a,b). In the tammar, however, lactational quiescence is followed in the second half of the year by a period of seasonal quiescence. During this time, between June and December, seasonal factors predominate and RPY does not induce development of either the CL or blastocyst. It is termed seasonal quiescence rather than seasonal anoestrus as in the quokka (Sharman, 1955a,b), since in the tammar a viable blastocyst remains in the uterus which is histologically similar to that of lactational quiescence (Sharman and Berger, 1969). Hence a blastocyst formed at a post-partum fertilisation early in one breeding season may remain in the uterus for up to 11 months, until after the following summer solstice when reactivation of the CL and blastocyst will occur (Berger, 1966).

In the tammar, therefore, the activity of the CL is inhibited during both periods of quiescence. However, removal of the pituitary during either period of quiescence induces reactivation of the quiescent CL and dormant blastocyst - the CL increases in size and the embryo develops to term though parturition fails, while
follicular growth, oestrus and ovulation are abolished (Hearn, 1973, 1974, 1975). Subsequently Tyndale-Biscoe and Hawkins (1977) demonstrated that prolactin was probably the main agent of inhibition. They found that injection of prolactin or oxytocin 3 times daily for 7 days after RPY during lactational quiescence delayed reactivation for this interval. Since these animals were intact the results did not discriminate between a direct effect of the injected materials and an effect mediated via the pituitary itself. In a subsequent experiment therefore, the whole pituitary or only the anterior lobe was removed from non-lactating females in seasonal quiescence and each was treated with saline, prolactin or oxytocin as before. Reactivation was delayed in animals given prolactin but not in those given either oxytocin or saline. Thus it appears that prolactin is the most likely pituitary agent inhibiting the CL, a most unusual role for this hormone in mammals.

Subsequently Sernia and Tyndale-Biscoe (1979) found that there were many highly specific prolactin receptors present in luteal tissue, and so it became imperative to be able to measure peripheral prolactin concentrations.

As with the steroid hormones, few measurements of plasma protein hormone concentrations have been made in marsupials despite the refinement of the radioimmunoassay technique over the last ten years. For the protein hormones this is mainly due to the lack of purified marsupial preparations for use in an homologous system. In an attempt to measure prolactin in the tammar, Dr Jenny Hawkins, in 1975, prepared an antibody against purified tammar prolactin and, using an homologous system, demonstrated cross-reactivity with pituitary extracts, but not with plasma. Subsequently, Farmer, Licht, Gallo, Mercado-Simmen, Delisle and Papkoff (1981) developed
I homologous radioimmunoassay using grey kangaroo prolactin, but also were unable to demonstrate any cross-reaction in plasma. So, because of these problems encountered by other workers, a heterologous assay system was adopted and validated for the tammar. Once the assay was established, the patterns of plasma prolactin during pregnancy and the oestrous cycle and throughout lactation were determined. Comparison of the profiles during the cycles and the temporal relationships of prolactin and progesterone should provide further understanding of the role of prolactin, and may also reflect whether pituitary release of this hormone affects the life span or function of the CL. Since the CL can reactivate 7 days after hypophysectomy or adeno-hypophysectomy it cannot be dependent on a luteotrophic stimulus from the pituitary in order to develop full secretory activity and support a pregnancy. Thus to establish whether the CL of the tammar is autonomous after reactivation plasma progesterone levels will be monitored after hypophysectomy.

THE REPRODUCTIVE CYCLE OF THE POSSUM, TRICHOSURUS VULPECULA (KERR)

The common brush tail possum, Trichosurus vulpecula (Order Diprotodontia, Family Phalangeridae), first described by Kerr in 1792 (Iredale and Troughton, 1934), is an arboreal, nocturnal marsupial abundant in eastern Australian and Tasmania. Since its introduction to New Zealand it has become abundant there also. Female possums are seasonal breeders with the majority of young being born between April and June. A second minor breeding season often occurs between September and November so it is possible for females to raise two young to independence each year (Smith, Brown and Frith, 1969).
The adult female is monovular and polyoestrous, and the gestation period occupies about 17.5 days of the 26 day oestrous cycle. There is no post-partum oestrus and lactation suppresses further ovarian activity (Group 1 pattern). However, if the young is removed immediately after birth the cycle continues uninterrupted, and oestrus will occur 26 days after the last oestrus. During the breeding season follicular development resumes following removal of the sucking young and the female returns to oestrus 7 to 8 days later (Pilton and Sharman, 1962). Hence the 17.5 day gestation period occupies only the luteal phase and not the follicular phase which comprises the last 8 days of the cycle. Pilton and Sharman (1962) observed considerable variation in the length of the oestrous cycle (22 to 32 days) but did not know if this was due to lengthening of the luteal or follicular phases.

After ovulation the single CL formed on one ovary has completed mitotic proliferation by 4 days after oestrus and reaches its maximum size by 7 to 10 days post-oestrus (Pilton and Sharman, 1962; Shorey and Hughes, 1973b). There is no detectable difference in the size, structure or rate of formation of the CL between the pregnant and non-pregnant females. The uterine luteal phase present by 7 to 8 days after oestrus disappears at about 15 days in both states (Pilton and Sharman, 1962; Shorey and Hughes, 1973a), suggesting that pregnancy has no effect on the life span of the CL.

Peripheral progesterone was measured by a competitive protein binding assay in 1971. Levels were highest during the luteal phase of the cycle, increasing after Day 8 to reach maximum levels (5 ng/ml) between Days 11 and 15, and decreasing by Day 18 to 20 to low levels (Thorburn et al., 1971; Shorey and Hughes, 1973a). The increased peripheral levels were due mainly to an increase in the
progesterone secretion rate by the CL after it has reached its maximum size (Shorey and Hughes, 1973b). In these studies, however, only a single reading was obtained from each animal so no direct comparisons could be made between pregnancy and the oestrous cycle. To determine whether pregnancy influences the secretory activity of the CL, daily changes in the concentration of plasma progesterone throughout pregnancy and the oestrous cycle have been monitored. The pattern of plasma prolactin has been determined also so that the profiles of the two hormones can be compared with the pattern described for the tammar. In addition, the role of the pituitary in the maintenance of the possum CL during the cycle will be determined by measuring plasma progesterone concentrations after hypophysectomy.

THE REPRODUCTIVE CYCLE OF THE NATIVE CAT, DASYURUS VIVERRINUS (SHAW)

During the 1800's the native cat, Dasyurus viverrinus (Shaw, 1800) (Order Polyprotodonta, Family Dasyuridae) was common on mainland eastern Australia and in Tasmania. Today the eastern native cat, or quoll, is rare on the mainland but is still common in Tasmania (Archer, 1979).

In the early part of this century, J.P. Hill and his associates investigated several aspects of the reproductive processes of the native cat, Dasyurus viverrinus, including the early development of the egg (Hill, 1910), events in the reproductive cycle (Hill and O'Donoghue, 1913), the development of the follicle and CL (Sandes, 1903; O'Donoghue, 1912) and the arrangement of foetal membranes, placentation and parturition (Hill, 1900). From these several studies it was concluded that the native
cat, which would breed between late May and August each year, was polyovular and monoestrous. At oestrus, copulation generally occurred once and often lasted for several hours, but in a few cases would extend intermittently over 2 to 3 days. Since unsegmented ova were recovered from the uteri between 4 and 7 days after the last copulation, Hill (1910) and Hill and O'Donoghue (1913) concluded that ovulation did not occur in most cases until 5 or 6 days after coitus. This variation in the interval between copulation and fertilisation could account for some of the differences in estimates of gestation period (8, 16, 20 days; Hill, 1900; Hill and O'Donoghue, 1913). In one other dasyurid species, Antechinus stuartii, the interval between copulation and ovulation is also variable (Selwood, 1980). Furthermore, in A. stuartii, early embryonic development is arrested at the 4 cell stage for up to 4 days and at the blastocyst stage for 2 to 3 days (Selwood, 1980).

After ovulation the cells of the membrana granulosa of the ruptured follicle hypertrophy and within 3 days the CL is formed irrespective of whether fertilisation occurs. Thus, the formation and development of the CL is the same in the pregnant and non-pregnant animal (Sandes, 1903; O'Donoghue, 1911, 1912). The CL persist throughout pregnancy and during the first few weeks of lactation (Sandes, 1903), but it is not clear how long they persist in the non-pregnant animal (O'Donoghue, 1912). Despite these studies little is known of either the temporal relationships of oestrus, ovulation and embryo development or the actual lengths of the oestrous cycle and the gestation period.

Three years ago a colony of native cats originating from a population near Hobart, Tasmania, was established at CSIRO, Division of Wildlife and Rangelands Research, Canberra, to study
reproduction, lactation and the energetics of growth of the young. In captivity, the females underwent oestrous cycles 3 to 4 times between May and August each year and so the species is polyoestrous (T. Fletcher, La Trobe University, Melbourne, pers. comm.; J.C. Merchant, pers. comm.), contrary to the observations made by Hill and his associates. Field observations by Dr B. Green (pers. comm.) have also shown them to be polyoestrous since in three instances females returned to oestrus after removal of their young and subsequently produced a second litter in the same breeding season.

In captivity, the oestrous or non-pregnant cycle ranges in length from 28 to 43 days (37.4 ± 0.65, Mean ± SEM, n = 27) while the mean gestation period, defined as the time from the first observed copulation to detection of neonates in the pouch, was 20.3 ± 0.63 days (Mean ± SEM, n = 4, range 19 to 22 days) (J.C. Merchant, pers. comm.). Removal of the pouch young in the breeding season results in a return to oestrus 10 to 14 days later (J.C. Merchant, pers. comm.). This suggests that in the oestrous cycle the follicular phase occupies about 15 days and therefore that pregnancy (20 days) occupies only the luteal phase.

The aim of this study was to describe the changes in plasma progesterone during a pregnancy and an oestrous cycle of the native cat to establish whether pregnancy does occupy only the luteal phase of the cycle and so determine whether pregnancy has an effect on the life-span of the CL. The profiles will then be compared with those of the possum and tammar.
SCOPE OF THIS THESIS

The past studies of reproduction in marsupials brought our understanding of function and mechanisms of regulation as far as possible without direct measurement of the putative hormones. The main aim of this study therefore, was to develop sensitive radioimmunoassays for progesterone and prolactin with which to deepen our understanding of luteal function and hypophysial-luteal interaction. In order to do this the major questions to be addressed were;

(a) does maternal recognition of early pregnancy occur in marsupials?

or (b) are the fertile and infertile cycles hormonally equivalent?

(c) is the marsupial CL autonomous?

and (d) what is the role of prolactin during lactation in marsupials?

The main species investigated is the tammar wallaby but comparative results for the possum and native cat are presented also.

The thesis is structured as follows. Chapter 2 describes general methods of animal maintenance, collection of blood samples, surgical procedures and histology. In Chapter 3 the validation of a radioimmunoassay for the measurement of plasma progesterone in the tammar, possum and native cat is described. As well, the development and validation of a radioimmunoassay for plasma prolactin in the tammar and possum is presented.

In Chapter 4, using the assays, the profiles of plasma progesterone and prolactin during oestrous cycles and pregnancies are described so that the hormonal equivalence hypothesis of Sharman
(1970) could be examined, and also the function of the CL of each species could be compared. The autonomy of the marsupial CL is examined in Chapter 5 by determining the effects of hypophysectomy on luteal function in lactating tammars and cycling possums.

In Chapter 6 the profile of plasma prolactin in the tammar and possum is described and compared with the eutherian pattern. The effect on prolactin concentrations of removing the tammar pouch young is examined.

The final chapter of the thesis presents a general discussion of the implications of the findings of this study.
CHAPTER 2.

GENERAL MATERIALS AND METHODS

2.1 ANIMALS - MAINTENANCE AND MANAGEMENT

Tammars

The wallabies were taken from the breeding colony established at the Division of Wildlife and Rangelands Research, CSIRO, in 1974 from Kangaroo Island stock. The animals were kept in open grassed paddocks with free access to water and a diet supplement of oats and lucerne.

Wallabies used in all experiments were greater than 2 years of age and weighed between 4.0 and 5.5 kg body weight. For certain experiments animals were removed to a CSIRO animal house and maintained in small pens (2 x 1 metre) which had sacking shelters. The pens were naturally lit and exposed to ambient temperature. Some animals were also held in individual wire cages in a room in the same animal house in which both light and temperature could be controlled. The floors of all pens were covered with straw or wood shavings. The diet for all animals maintained indoors comprised oats and lucerne chaff, fresh cabbage and water ad libitum.

Possums

The common brush tail possum, Trichosurus vulpecula, was obtained locally in the Canberra region, either from suburban homes, or nearby farming properties, with the permission of the Department
of the Capital Territory Conservation and Agriculture Branch. Possums were caught using traps baited with apple and/or aniseed solution, and housed in large pens (5 x 3 x 3 metres) which contained nest boxes and suspended sacks for shelter. Their diet comprised fresh fruit, particularly apples and oranges, carrots, rolled oats, sunflower seeds, bread and various types of eucalypt leaves (eg. Eucalyptus viminalis). Water was also freely available. Adult females ranged in body weight from 1.8 to 2.8 kg.

Native Cats

Native cats were taken from the captive colony established in 1980 at the Division of Wildlife and Rangelands Research, CSIRO from a population near Hobart, Tasmania. Groups comprising either 2 or 3 females and 2 intact adult males were held in grassed and sheltered enclosures (8 x 4 x 4 m). Three or four nest boxes were provided and the animals were fed dead mice and rats every other day. Water was freely available. Adult females ranged in body weight from 0.7 to 0.9 kg.

Donkey

An eighteen month old castrated donkey was purchased from a local landowner and held in an open grassed paddock. A diet supplement of thoroughbred stockfeed was provided three times per week.

2.2 COLLECTION OF BLOOD SAMPLES

Sodium heparin (mucous, Glaxo) was used as anticoagulant. It was diluted in sterile 0.9% saline and used at 500 IU/ml for single
samples, and at 250 IU/ml if repeated samples were collected over a few hours, or over several days or weeks.

Routine blood samples from wallabies and possums were taken from a lateral tail vein into heparinized syringes using 21 g or 23 g needles respectively. The samples were placed in glass centrifuge tubes and held on ice (less than 60 min) until centrifuged at 1000 g in a bench centrifuge for 10 minutes. Plasmas were decanted and stored at -15°C in volumes appropriate for the various hormone assays. If serum was required the blood was collected in the absence of a clotting agent, stored at 4°C for 24 hr, centrifuged and then separated into aliquots before freezing.

Two other methods of blood sampling were used. When an animal was to be used once only for a period of intensive sampling (eg. thyrotrophin releasing hormone injection) samples were collected from disposable winged infusion sets (Surflow, Terumo) inserted into the tail vein. The set consisted of a 19 g needle with a 30 cm extension tube (0.5 ml volume) attached. The latter was shortened to 5 cm and capped with a blunted 16 g needle and stopper. After each sample the tube was filled with heparinized 0.9% saline (250 IU/ml). For regular repeated sampling over several hours or days jugular catheters were inserted (see Section 2.3), and kept patent for up to 4 weeks by daily flushing with sterile 0.9% saline and refilling with dilute heparinised 0.9% saline (250 IU/ml).

In some experiments, long and/or short term, the total volume of blood collected during the experiment necessitated the return of red blood cells to maintain normal haematocrit levels and blood volume. Therefore samples were collected into sterile syringes, transferred to foil-capped, sterile, glass centrifuge tubes, and centrifuged at 500 g. The plasma was aspirated with sterile pasteur
pipettes and the plasma volume replaced with a buffered solution of sterile sodium citrate (2.63% sodium citrate, 0.327% citric acid, 0.222% sodium dihydrogen orthophosphate, 2.55% glucose). The red blood cells were then gently resuspended using syringes and 18 g needles with 5 cm extensions of polyethylene tubing attached. The resuspended red blood cells were stored at 4°C and were reinfused after mixing and warming to body temperature after the next blood sample was collected. With this method no haemolysis of red blood cells occurred and normal haematocrit values of 45-52% were maintained.

2.3 SURGICAL PROCEDURES

All surgery was performed under aseptic conditions. Instrument and operating sheets, cotton buds, gauze swabs and bottles of 0.9% saline were sterilized by autoclaving for 30 min under steam at 17 lb/sq in pressure. All instruments were sterilized by boiling.

Before surgery, the area around the site of incision was closely shaved and swabbed with an antiseptic solution (1% Cetavlon in 70% ethanol). Once placed on the operating table a sterile calico sheet was placed over the entire animal allowing access only to the swabbed area. Immediately after the operation all wounds were topically dressed with an antibacterial powder (Sulphanilamide or Tricin) and the animal was injected intramuscularly with 1.0 ml of long-acting penicillin, Vetspen Plus (Glaxovet) or Aquacaine (Commonwealth Serum Laboratories).
Anaesthesia

For tammars a 4% saline solution of Surital (sodium thiamylal, Parke-Davis), a barbiturate, was injected via the tail vein to induce anaesthesia. Depending on the body weight of the animal the initial dose required was 3-4 ml, and thereafter anaesthesia was maintained by small doses of 0.5 - 1.0 ml as required.

For possums anaesthesia was induced either by injection of 1.0 - 2.0 ml 2% Surital and then maintained with Halothane (Fluothane, ICI, Australia Limited) and oxygen, or induced directly with Halothane and oxygen using a face mask which was connected to a small animal anaesthetic machine (Stephens Anaesthetic Drawover Machine).

Jugular catheters

Catheters were placed in the external jugular vein of both tammars and possums using the method of Khin Aye Than and McDonald (1973) with some modifications. Catheters (silastic tubing, I.D. = 0.76 mm, O.D. = 1.65 mm, Medical Grade, Dow Corning, Australia) were sterilized in a solution of 1% Cetavlon in 70% alcohol for at least 24 h prior to operation, and were inserted at least four days before the commencement of an experiment. Depending on whether the animal had been catheterised previously, entry into the external jugular vein was made either directly or via a brachial branch vein leading from the arm. Use of the brachial vein meant that each animal could be catheterised at least three times.

A skin incision (2.0 cm) was made on the left or right side of the neck using the cutting setting of the electrocautery unit (Surgistat, Valleylab), and the external jugular vein and the
brachial vein freed of surrounding muscle and connective tissue by blind dissection. The vessel to be catheterised was ligated, opened and the catheter inserted so that the tip extended to the vena cava (≈ 3.5 cm for tammars; ≈ 2.5 cm for possums). The vessel was secured around the catheter with surgical thread before the free end of the tubing was passed beneath the skin to the midline at the back of the neck where a small incision had been made in the skin. A length of 7 to 9 cm was left exposed and plugged with a tapered piece of 18 g stainless steel wire. The wound was powdered with Tricin, closed with surgical clips (Autoclips, Clay Adams) and sprayed with plastic skin. The catheter was initially filled with heparinised saline (500 IU/ml) and then flushed daily with 0.9% saline and refilled with 0.2 ml heparinised saline (250 IU/ml).

Initially many catheters were pulled out by the animals. Therefore, so that the catheter could be firmly anchored a small amount of silastic glue was moulded around the tubing over a nylon thread. This mould of glue was positioned and the thread tied through the skin such that the catheter could not be pulled out from the back of the neck.

Catheters remained patent for varying lengths of time from less than two days to greater than four weeks. Failure was mostly due to fibrin build-up in the inner end of the catheter. This meant that samples could not be withdrawn but red blood cells could be returned.

Ovariectomy

This procedure was performed as described by Renfree and Tyndale-Biscoe (1978). Animals were starved overnight to allow easier access to the reproductive tract in the lower abdomen. After
induction of anaesthesia a 4 cm mid-ventral incision was made through the pouch skin, linea alba and peritoneum, anterior to the mammary glands, to reveal the lateral vaginae under which lay the paired uteri. The uteri were lifted to expose the ovaries which were removed from the bursa. A suture was tied around each hilus, the ovary cut free using fine scissors and the site cauterised.

Once the ovary had been removed the peritoneum and the muscle layer of the body wall were sutured together with Mersilk thread (No. 2 or 3). The site was topically dressed and the pouch skin closed with surgical clips.

**Hypophysectomy**

Hypophysectomy was performed on both female tammars and female possums by a parapharyngeal approach slightly modified from the method of Hearn (1975). Either the entire pituitary or the adenohypophysis alone was removed leaving the pars nervosa and pars intermedia intact. For sham operations the pituitary was exposed but not removed.

The animal (wallaby or possum) was anaesthetised and the lower jaw and throat shaved and swabbed before it was placed on its back on the operating table which was heated to a surface temperature of 35°C. Its head was held in a simple adjustable frame and the body supported by small sand bags. Initially, the trachea was opened by a small transverse incision between two tracheal rings and a glass cannula with a right angle bend at its midpoint inserted. To the other end was attached a piece of tubing leading to the anaesthetic machine. Thus anaesthesia, under Halothane and oxygen, and breathing rate could be closely monitored throughout the operation.
A 2.0 cm skin incision was made on the left side of the lower jaw immediately anterior to the submaxillary salivary gland. The buccinator and digastric muscles were separated by blunt dissection until the pterygoid bone was detected. The dissection was continued taking care to work posteriorly to the false palate (which was not punctured) until the nasopharynx was entered and the ventral surface of the sphenoid bone was exposed. Three retractors, one with arms (4 x 0.6 cm) were inserted to keep the incision open, and a light source positioned above to illuminate the area.

An area of 0.8 x 0.4 cm of the sphenoid bone ventral to the pituitary was removed with a dental drill using a size 10 burr. Haemorrhage of the bone was controlled with small plugs of bone wax (Ethicon), topical application of Vasolamin (S10%, Ilium, Troy Laboratories, Australia) and Vasolamin-soaked pieces of Gelfoam (Upjohn). Drilling continued until the dura mater was exposed. To expose the pituitary the dura was gently cauterised using the fine needle and low coagulant setting of the electrocautery unit. A blunt probe was then used to open the dura along the length of the pituitary, and to gently loosen the whole gland or just the anterior lobe. Suction was applied through a steel tube of sufficient diameter to effect removal of the gland which passed into a trap and so could be examined and weighed immediately. After removal, the sella turcica region was examined for fragments of pituitary before being plugged with Vasolamin-soaked Gelfoam.

After cleaning the nasal passages of clotted blood and saliva using suction, the tracheal cannula was removed, the passage cleared of accumulated debris and the tracheal rings stitched. Both incision sites were powdered with Tricin and then closed with autoclips. An intramuscular injection of 1.0 ml long-acting
penicillin and another of cortisol (10 mg/kg) (Sigma Chemicals) in saline were administered before the animal was returned to its cage in a warm air-conditioned room (20°C). Animals recovered from the operation within an hour and regained their appetite within two days. If at any time animals were obviously failing one or two daily injections of cortisol (10 mg/kg) in saline was generally effective in boosting appetite and condition.

The procedure for sham-hypophysectomy was exactly the same except that the pituitary was left intact after the dura mater had been opened. The choice of treatment was made randomly after the pituitary had been exposed. However, if at any time haemorrhage prevented either access to the pituitary or removal of the pituitary when this treatment was selected, the operation was classed as a failed hypophysectomy and not a sham-operation. The completeness of hypophysectomy in treated animals and the presence of an intact pituitary in sham-operated animals was confirmed by examination of histological sections of the sella turcica region by two independent observers.

Hypophysectomy of the possum was essentially the same as for the tammar but some adjustments were made to allow for the smaller size of the animal. In the possum the pituitary tissue was softer than in the tammar and often shattered as it was removed by aspiration. It was therefore difficult to determine from the weight of the recovered pieces and from re-examination of the sella turcica whether all of the pituitary gland had been removed. However this was finally confirmed, as in the tammars, by later examination of histological sections of the region.
**Bilateral adrenalectomy**

This operation was performed in two stages as described by Janssens and Tyndale-Biscoe (1982). In the first operation the right adrenal gland which lies very close to the vena cava was removed via a lateral incision made below the rib cage. The gland was freed from surrounding tissue and the artery and vein sealed using electrocautery. Approximately one week later the procedure was repeated for removal of the left adrenal gland. After the second operation a daily injection of 0.2 mg deoxycorticosterone (Sigma Chemicals) in oil successfully maintained the animals in good health.

**Vasectomy**

Male tammars and male possums were vasectomized 2 to 8 weeks before being used in experiments. The technique of vasectomy was similar for both species. Animals were anaesthetised and the neck of the scrotum shaved and swabbed. A small skin incision was made in the neck of the scrotum and the sheath to expose the vascular cord and vas deferens on the medial side. The vas deferens was dissected free of blood vessels, clamped and a 5 mm section removed. Both ends were cauterised before the sheath was stitched and the wound closed with surgical clips. The other vas deferens was treated similarly.

**2.4 AUTOPTSY PROEDURE**

Animals were weighed and then killed either by an overdose of Surital or by a blow to the back of the neck followed by cervical dislocation and cutting of the throat. If large volumes of blood
(150-200 ml) were required the animal was anaesthetised and blood taken through an 18 g needle from the heart.

The reproductive tract was removed and weighed. The uteri, and ovaries were grossly examined for evidence of a pregnancy and corpus luteum/follicles, before being dissected for weighing of individual components. Uteri were flushed via the cervix with 0.9% saline to recover eggs or blastocysts. For the later stages of pregnancy the uterus was carefully dissected and everted to recover vesicles or late stage embryos. All recovered material was weighed and/or measured with calipers or a micrometer eyepiece. Corpora lutea and other ovarian tissues were either frozen or fixed in Bouin's fixative.

Additional material was also collected depending on the experimental treatment the animal had received. After hypophysectomy adrenal glands and thyroid glands were weighed and preserved in 10% neutral buffered formalin. The sella turcica region was also fixed for later histological examination.

In all operated animals the sites of operation were closely examined for pieces of the removed tissue as well as for infection or adhesion.

2.5 HISTOLOGY

Vaginal smears

Oestrous in possums was diagnosed from vaginal smears taken by the method described by Poole and Catling (1974). Briefly the cloaca was everted and a glass tube carrying a cotton bud inserted into the urogenital sinus. The dampened cotton bud was used to extract material from the posterior vaginal sinus and this was
smeared on a microscope slide. The wet smear was fixed immediately in equal parts of ether and 95% alcohol for a minimum of 2 minutes before being stained in Shorr's stain (Shorr, 1941). Cornified cells stained bright pink/red while epithelial cells were blue/green. Other cell types such as leucocytes and prostatic bodies were also identified by their cellular characteristics and staining reactions. If no copulatory plug was observed the day of oestrus was taken as the day prior to the appearance of leucocytes in the smear as described by Pilton and Sharman (1962).

**Microtomy of skulls after hypophysectomy**

At autopsy skulls were crudely trimmed and fixed in 10% neutral buffered formalin. Specimens were partially decalcified in 5% disodium ethylenediaminetetra-acetate in 10% formalin and then trimmed to produce a block (about 1 x 1 x 1.5 cm) containing the sella turcica and lower edge of the hypothalamus. Decalcification was completed using Rapid Bone Decalcifier (RDO, Du Page Kinetic Laboratories Inc. Illinois). After dehydrating in alcohol, specimens were infiltrated with 0.5% celloidin, cleared in xylene and embedded in wax. Several specimens were mounted in each block and cut simultaneously. Serial sections were cut at 10 μ and every tenth section collected and mounted in sequence for examination.

Sections were stained with haematoxylin and eosin and scored for the presence or absence of pituitary tissue using a light microscope.
CHAPTER 3.

DEVELOPMENT OF RADIOIMMUNOASSAYS FOR PROGESTERONE AND PROLACTIN IN THE TAMMAR WALLABY

3.1 GENERAL PRINCIPLES OF BINDING ASSAYS

The basic principle of the hormone radioimmunoassay relies upon the reaction between an antihormone antibody and a hormone to produce an antibody-hormone complex such that at equilibrium there is a proportion of the complex present as well as free antibody and free hormone. In the presence of a fixed amount of antibody but varying amounts of free hormone, at equilibrium, the ratio of bound to free hormone will be quantitatively related to the total amount of hormone present. The incorporation of a tracer (either labelled hormone or labelled antibody) in the system provides a technical means of measuring the distribution of bound and free hormone. In most radioimmunosassays the tracer is added in the form of labelled hormone whose binding characteristics are assumed to be similar to those of the unlabelled hormone, thus allowing equal opportunity for binding of either to the antibody. When both the amount of tracer added and the antibody concentration are constant the addition of increasing amounts of unlabelled hormone displaces increasingly more tracer from the antibody. This then enables a standard curve to be constructed against which unknown samples can be quantitated.

The major difference between radioimmunoassay systems lies primarily in the method used to separate bound and free hormone.
portions. Generally, for the high molecular weight protein hormones a second antibody, specific to the gamma globulin of the species in which the first antibody was raised, is used to precipitate the antibody-hormone complex. In the radioimmunoassay system the primary antibody-hormone complex is too dilute to cause precipitation but if a second antibody and carrier non-immune serum of the same species as the first antibody are added a sizeable lattice is built, and the whole complex is precipitated. However the concentrations of both carrier and second antibody must be critically determined as too little or too much can interfere with the completeness of precipitation - this is known as the prozone effect (Court and Hurn, 1971). Precipitation is generally complete by 16 hours at 4°C. This system, known as a double antibody radioimmunoassay, has been used for measuring prolactin in the tammar wallaby and other marsupials.

For the smaller molecular weight substances such as steroids a simple physicochemical precipitation is often used in preference to a double antibody system. Adsorption methods utilise the non-specific surface adsorption of small molecules to particles, such as charcoal - the larger antibody-hormone complex cannot be adsorbed and so remains in the liquid phase while any free labelled hormone is firmly adsorbed. Dextran is used to coat the charcoal since it is thought to block larger pores which may trap the bound complex, but it does not interfere with the adsorption of the smaller molecular weight free hormone. Charcoal with a maximum pore size of 60 um is used and each new batch is tested - if in the system it reduces maximum binding or gives a high assay blank (non-specific binding) it is rejected as not suitable. Charcoal has been used to separate the bound and free fractions in the radioimmunoassay
developed for measuring plasma progesterone in tammars and other marsupials.

Whichever radioimmunoassay system is chosen, optimal assay conditions must be determined and rigidly controlled. Many factors can influence the binding of the antibody and the hormone. These include the concentration and pH of the buffer, and its carrier protein (bovine serum albumen, gelatin), the order in which reagents are added, incubation temperature and time, purity of solvents, cleanliness of glassware and presence of impurities in the drying manifold and/or compressed nitrogen or air used for drying down solvents constitute the blank in each assay.

To stipulate the conditions to be used the assay must be evaluated with respect to the following criteria; sensitivity, accuracy, specificity and precision. Sensitivity is defined as the 'minimal detection limit of an assay', and so is the least quantity which can be distinguished from a sample containing no hormone (the zero standard). Sensitivity can be increased or decreased by altering the concentrations of antiserum and/or tracer, and/or the incubation time. Generally the most sensitive standard curve is obtained by using the minimum amount of tracer while retaining adequate counting accuracy. Usually this is in the presence of the dilution of antiserum which is sufficient to bind approximately 50% of the tracer added.

Accuracy is defined as the extent to which a measurement of a hormone agrees with the exact amount of the hormone present and is assessed by recovery experiments. Different amounts of hormone are added to a plasma which contains low concentrations of endogenous hormone, and the amount of hormone measured is then compared with the amount added. Theoretically, a correlation coefficient of 1.0
should be obtained.

Precision is determined by examining the extent of agreement between replicate estimates of samples both within and between assays. Intra-assay variation is determined by replicate measurements of a plasma pool in a single assay, and inter-assay variation by measurements of plasma pools (quality control plasmas) in several different assays. The coefficients of variation (CV) of the mean of replicate determinations in each case are calculated as

\[
\%CV = \frac{\text{Standard deviation}}{\text{Mean}} \times 100
\]

Specificity can be defined as 'the degree to which the assay responds to substances other than that for which the assay was designed'. It is mainly dependent upon the specificity of the antiserum used, but there are often many factors in plasma or tissues which can show cross-reactivity in the system either in a specific or in a non-specific manner. To establish specificity, the amount of hormone measured should fall linearly with the dilution or volume of plasma or tissue extract assayed such that the dose response curve lies parallel with the standard curve. However, parallel dose response curves, though a necessary precondition for specificity, are not always sufficient evidence of complete identity. This is particularly true if the antiserum being used is not highly specific for the hormone being considered and shows significant cross-reaction with other closely related hormones.

During the development of the two radioimmunosassay systems for measuring prolactin and progesterone in marsupials full consideration was given to these various criteria.
3.2 PROGESTERONE RADIOIMMUNOASSAY

3.2.1 INTRODUCTION

The suitability of the radioimmunoassay technique for measuring plasma progesterone in the tammar and other marsupials has been rigorously tested. To achieve the desired level of sensitivity, accuracy and precision, extra precautions with, for example, the purity of solvents and cleanliness and suitability of glassware were essential to avoid step-by-step procedural losses and/or contamination. The aim of this section, therefore, was to develop and validate a radioimmunoassay under strictly controlled conditions so that it would be sensitive enough to measure changes in progesterone levels in female tammars during various reproductive states - delayed and non-delayed pregnancies and oestrous cycles, lactational and seasonal quiescence - as well as after experimental manipulations such as hypophysectomy, ovariectomy and/or adrenalectomy. In addition, the assay was validated for other marsupials (possum, native cat) to provide further comparative data.

The main progestin in the tammar is progesterone. Renfree and Heap (1977) and Renfree et al., (1983) found that minced luteal tissue, collected between 11 and 25 days after RPY, converted 40% of added labelled pregnenolone into progesterone in vitro. Other metabolites of pregnenolone represented less than 1% conversion of the substrate. Lemon (1970), however, who measured total progestins using a competitive protein binding technique, thought that 17α-OH progesterone may have been contributing to the levels in pregnant female tammars. Therefore, at the beginning of this study several plasmas, collected from tammars in late pregnancy, were extracted
with redistilled ether and chromatographed on Lipidex 500 columns, previously characterised for the separation of progesterone and 17α-OH progesterone. The two fractions collected for each plasma extract were assayed by radioimmunoassay using specific antisera (courtesy of G.E. Abraham, California) for each hormone. Significant cross-reaction was observed for progesterone only in the "progesterone" fraction but there was no detectable 17α-OH progesterone activity in any of the plasma extracts (results not presented). Therefore in the present study on the tammar the only progestin measured has been progesterone.

Curlewis et al. (1981) using chromatography and mass spectrophotometry showed that progesterone is also the main progestin in the possum. No chromatographic studies have been made to determine whether progesterone is the major progestin in the native cat. However the specificity of the progesterone antiserum, and the use of hexane for preferential extraction of progesterone from plasma means that only progesterone was being measured in the native cat.

### 3.2.2 MATERIALS AND METHODS

**Steroid assay buffer**

The buffer used in routine assay was 0.2 M sodium phosphate buffer, pH 7.0 containing 0.15 M sodium chloride, 0.1% gelatin (Labchem, Ajax Chemicals) and 0.1% sodium azide. The buffer was stored at 4°C for up to one month but could be used beyond this time as long as there was no evidence of bacterial growth. Gelatin was added to the buffer to decrease the adsorption of steroids to glassware.
Preparation of dextran-coated charcoal

A charcoal suspension was used to separate the unbound labelled steroid from the antibody-bound labelled steroid, and was prepared as follows: 6.25 g activated charcoal (Sigma charcoal, activated, untreated powder) and 0.625 g Dextran T70 (Pharmacia Fine Chemicals) were added to 250 ml glass distilled water, mixed and allowed to settle. The fines were decanted and the procedure repeated at least 3 times. After the final wash the slurry was made up to 100 ml with distilled water and stored at 4°C for up to 3 months. For use in the assay this stock solution (6.25%) was diluted 1:10 (v/v) in assay buffer, and 0.2 ml of the well-mixed suspension added to the appropriate tubes.

Radio-isotope of progesterone

(1, 2, 6, 7 - $^3$H) progesterone (Amersham) with a specific activity of between 84 and 110 Ci/mmol was used as tracer. The material was supplied as 250 uCi in 250 ul benzene:ethanol (9:1) and was made up to 5.0 ml with absolute ethanol and stored at -20°C. This solution was stable for 6 months.

For assay, 20 ul of the stock was dried and made up to 10 ml with assay buffer (1:500 dilution). This corresponded to 30 pg $^3$H-progesterone/0.1 ml ($= 10,000$ cpm) and was found to be the optimal concentration of tracer per assay tube (see Section 3.2.3).

Progesterone standard

Pure progesterone was obtained from Calbiochem and a stock solution of 1 mg/ml prepared in absolute ethanol. Serial 10 fold dilutions were made to obtain a final stock solution of 1 ug/ml in absolute ethanol. These solutions were stored at -15°C, and were
stable for at least a year.

For assay a solution of 20 ng/ml was made by drying 200 ul of the 1 ug/ml stock solution and adding 10 ml of assay buffer. A range of standards (10 pg/ml - 2000 pg/ml) was prepared by appropriate dilution of this 20 ng/ml solution, and could be stored at 4°C for up to 4 weeks.

**Progesterone antisera**

Both antisera (No. 230 and No. 334) were raised in sheep against the antigen progesterone-11-hemisuccinate-BSA (Steraloids) by Dr R.I. Cox, Hormone Assay Development Group, Division of Animal Production, CSIRO, Sydney, N.S.W. The specificity of both antisera are shown in Table 1. The gift of antiserum was received as a freeze-dried preparation. The material was weighed, reconstituted to the original serum volume in buffer and then diluted 1:10. Aliquots of 50 ul and 100 ul were stored frozen at -40°C.

For assay the aliquots were diluted appropriately to give maximum binding ranging from 45-55% of added counts. For antiserum No. 230 this was a working dilution of 1:1000 (final dilution 1:7000) and for No. 334 a working dilution of 1:1800 (final dilution 1:12,600) (see Section 3.2.3).

**Preparation of 'steroid-free plasma**

In the radioimmunoassay of steroid hormones, 'steroid-free' plasma can be used as a control to evaluate the non-specific effect of plasma in the assay. 'Steroid-free' plasma was prepared by adding 1.0 ml 6.25% charcoal to 9.0 ml of plasma. This solution was mixed constantly for approximately 4 hours at room temperature before the charcoal was removed by centrifugation. Aliquots of 'steroid-free' plasma were stored frozen at -20°C.
Table 1. Cross reaction of steroids with progesterone antisera (No. 230 and No. 334) raised in sheep against progesterone-11-hemisuccinate-BSA, performed by Dr R.I. Cox.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Cross reaction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. 230</td>
</tr>
<tr>
<td>Progesterone</td>
<td>100</td>
</tr>
<tr>
<td>11β-hydroxyprogesterone</td>
<td>6.7</td>
</tr>
<tr>
<td>17α-hydroxyprogesterone</td>
<td>0.3</td>
</tr>
<tr>
<td>20α-hydroxy-4-pregnene-3-one</td>
<td>0.3</td>
</tr>
<tr>
<td>20β-hydroxy-4-pregnene-3-one</td>
<td>0.3</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>1.6</td>
</tr>
<tr>
<td>3β, 17-dihydroxy-5-pregnene-2-one</td>
<td>&lt; 0.03</td>
</tr>
<tr>
<td>5β-pregnane-3α, 20β-diol</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>5β-pregnane-3α-2α-diol</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>5β-pregnane-3, 20-dione</td>
<td>3.5</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&lt; 0.03</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>0.3</td>
</tr>
<tr>
<td>11-deoxycorticosterone</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Solvents

n-Hexane (Analytical Reagent (A.R.) grade, May and Baker, Australia) was redistilled and then used for extraction of progesterone from plasma, serum and homogenised tissues. It was extremely important to use freshly redistilled solvent (only 1-2 days old) for each assay to ensure that solvent blanks remained below the sensitivity of the assay system (< 25 pg/ml, < 10 pg/assay tube) (see Section 3.2.3).
Analytical Reagent ethanol was used in the preparation of steroid tracer and standards.

**Scintillation fluid**

Two litres of toluene (A.R., sulphur free, Ajax chemicals), 300 ml of dioxan (Ajax chemicals) and 84 ml Spectrafluor (Amersham) were mixed, and 10 ml dispensed to each scintillation vial. Dioxan was added to this 2 phase system to facilitate the dissociation of labelled steroid from the antibody in the aqueous phase, and allowed the labelled steroid to move more rapidly into the non-aqueous scintillant phase to be counted. This reduced the equilibration time from 16-20 hours to 4-6 hours before a final count of 10 minutes per vial was made.

**Glassware**

For extractions, 5, 10 or 15 ml glass-stoppered Quickfit test tubes were used. Solvents were poured off into either 15 ml tapered, glass centrifuge tubes, or directly into the 75 mm x 11 mm disposable glass tubes in which the assay incubations were performed.

All glassware was soaked in Pyroneg detergent, washed, rinsed several times each in tap water and distilled water, and finally rinsed with 95% ethanol.

**Assay procedure**

The assay method is based on that of Abraham, Swerdloff, Tulchinsky and Odell (1971). To each volume of plasma was added 10 volumes of freshly redistilled hexane (i.e. 1.0 ml plasma plus 10 ml hexane, etc.). The stoppered tubes were manually shaken for 90
seconds and then allowed to stand for 5 minutes before the aqueous phase was frozen in a dry ice-ethanol bath. The organic phase was decanted into 15 ml glass centrifuge tubes and dried under air at 37°C. The plasma was thawed and the extraction procedure repeated. The second extract was added to the first, and after drying, 1.2 ml assay buffer was added. The samples were vortexed occasionally during an equilibration period of 4 hours at room temperature before duplicate 0.5 ml aliquots were pipetted into assay tubes.

Progesterone standards were prepared from a stock solution of 20 ng/ml to provide 10, 20, 50, 100, 200, 500, 1000 and 2000 pg/ml in assay buffer. Triplicate aliquots of 0.5 ml were taken for each dose to give a curve in the range of 5-1000 pg/assay tube.

To samples and standards was added 100 ul antiserum (No. 230, 1:1000 dilution; No. 334, 1:1800 dilution) and 100 ul labelled progesterone (10,000 cpm, 30 pg) to give an incubation volume of 700 ul. Control tubes for the estimation of maximum binding in the absence of unlabelled hormone (zero standard) contained 500 ul assay buffer, 100 ul antiserum and 100 ul tracer. Tubes for the measurement of non-specific binding (NSB) in the absence of antiserum contained 600 ul assay buffer and 100 ul tracer. Three tubes which received 800 ul buffer and 100 ul tracer served to measure total counts added. All tubes were thoroughly mixed and incubated overnight (14 to 18 h) in an ice-water bath at 4°C. To remove the free steroid, 0.2 ml 0.625% charcoal suspension was added to all tubes except the totals. After standing for 30 min in the ice-water bath, the charcoal was sedimented by centrifugation at 2,500 g for 15 min, at 4°C, using either an IEC-Centra-7R refrigerated bench centrifuge or a Servall automatic superspeed
refrigerated centrifuge, type RC-2. An aliquot of supernatant (0.5 ml) was then added to 10 ml scintillation fluid. After a 4 h extraction period the radioactivity in samples and standards was measured in an automatic liquid scintillation counter (Isocap 300, Searle) at an efficiency of 65%.

Calculations

The percentage of labelled steroid bound to the antiserum was calculated as follows:

\[
\% \text{ Bound (B)} = \frac{\text{mean cpm in standard or unknown} - \text{mean cpm NSB}}{\text{mean cpm of total counts (T)}} \times 100
\]

A standard curve was plotted either as %B/T vs log pg steroid/assay tube or as %B/B₀ vs log pg steroid/assay tube where B₀ is the amount of tracer bound at zero steroid (Figs. 1 & 2) and is calculated as:

\[
\% B \quad B_0 = \frac{\text{cpm of unknown or standard} - \text{mean cpm NSB}}{\text{cpm at zero standard} - \text{mean cpm NSB}} \times 100
\]

Unknown values could be read directly off the standard curve and multiplied by the appropriate dilution factor to give pg/ml plasma.

For routine assay measurements a computer program for the calculation of assay data compiled by Dr J.E.A. McIntosh (New Zealand) was modified for use with a Hewlett Packard 9825A calculator with graphics plotter by Dr G. Brown, Division of Wildlife Research, CSIRO. Using this program the standard curve is
calculated by a general iterative curve-fitting program which generates a log-logistic function without transformation. The fitting function is derived from Healy (1972). After the fitting procedure the data is transformed to T/B (ratio of total counts added to counts bound) so that a plot of T/B versus dose can be drawn. Fig. 3 presents typical plots for a progesterone standard curve, showing both the measured and fitted data points for antisera No. 334 and No. 230. Once the standard curve is fitted, the unknown samples are entered and a printout obtained of the calculated hormone concentration.

3.2.3 RESULTS Validation of the progesterone assay for the tammar Sensitivity. Both antisera (No. 334 and No. 230) were diluted to establish a standard curve appropriate for plasma progesterone levels in the tammar wallaby. Figs 4 and 5 illustrate the effect of antibody dilution on the standard curve for antiserum No. 334. Increasing the antibody dilution results in a displacement of the standard curve to the left and this is accompanied by an increase in the sensitivity of the curve. For example, at a final dilution of 1:3500 it takes 350 pg progesterone to induce displacement of 50% of the label from the antiserum while at a dilution of 1:12,600 100 pg is capable of comparable displacement (Fig. 5). A similar response is seen with antiserum No. 230 (Figs 6 and 7).

The initial slope of the dose-response curve is also influenced by the amount of chemical progesterone added as 3H-progesterone. This means that the percentage bound at zero-added
progesterone does not correspond to zero progesterone but corresponds to the amount of \( ^3\text{H}\)-progesterone added. The effect of changing the amount of labelled steroid added from 60 pg/tube to 15 pg/tube is illustrated for antiserum No. 334 in Figs 8 and 9. A reduction in \( ^3\text{H}\)-progesterone increases the initial slope of the curve and therefore increases the sensitivity of the assay. Nevertheless, there is a limiting concentration of tracer below which further reduction leads to no significant change in the position of the standard curve or the sensitivity of the assay: in this case 30 pg vs 15 pg. It is also necessary to balance the effect of antiserum dilution and added tracer with the increased sensitivity and the minimum number of counts required for accurate counting. Therefore the working conditions chosen for antiserum No. 334 were 1:12,600 final dilution, and 30 pg \( ^3\text{H}\)-progesterone. This gave maximum binding consistently around 50%, and assay sensitivity of 10 pg/tube or 25 pg/ml plasma (Figs 8 and 9). Similar manipulations were performed for antiserum No. 230 to give a final dilution of 1:7000 in the presence of 30 pg added label and a standard curve (Figs 10 and 11) which was also sensitive to 10 pg/tube.

**Specificity.** Table 1 shows that both antisera are highly specific for progesterone. Specificity for progesterone in plasma was examined by determining displacement relative to the standard curve in the presence of increasing volumes of plasma. Serial dilutions of plasma from a lactating female, ovariectomized female and two females in late pregnancy were parallel to the standard curve for progesterone (antiserum No. 230). Plasma from an ovariectomized/adrenalectomized female showed no displacement in the
assay (Fig. 12). This antiserum was used for routine determination of progesterone in tammar plasmas.

Similar dose response curves for plasmas were observed when antiserum No. 334 was used (Fig. 16). Parallel dose response curves were also obtained for plasmas from two female eastern grey kangaroos - one in lactation and the other in late pregnancy (Fig. 16).

Effect of plasma on the progesterone standard curve. Two experiments were performed to examine the effect of the addition of different types of plasma to the standard curve.

(a) Experiment 1. Standards prepared in ethanol were aliquoted in duplicate into extraction tubes, dried under air, 500 ul buffer added, and equilibrated overnight at 4°C. These standards were extracted and assayed in the usual way and compared with a set of standards which were not extracted before assay. Maximum binding was 52% for the non-extracted curve and 48% for the extracted curve - this difference is expected since the efficiency of the extraction procedure is 90%. In Fig. 13 the results have been expressed as %B/B₀ and the extracted standards corrected for efficiency of extraction. The two curves superimpose closely and the results confirm that the recovery of progesterone from buffer is consistent over the range of the standard curve. This result also meant that a non-extracted buffer curve could be used in routine assays.

(b) Experiment 2. In the second experiment several standard curves were prepared as before. After drying, 500 ul steroid-free plasma, 500 ul plasma from an ovariectomized tammar or 500 ul plasma from an ovariectomized and adrenalectomized tammar was added to each point of the curve. Plasmas were equilibrated at 4°C overnight, extracted and then assayed. The results are expressed as %B/B₀ and have been corrected for a recovery of 90%. The curves in the
presence of 'steroid-free' plasma and plasma from an adrenalectomized/ovariectomized tammar closely superimpose with the buffer standard curve (Fig. 14). Similarly maximum binding (%B/T) was unaffected by the presence of these two plasmas. However in the presence of plasma from an ovariectomized tammar maximum binding was depressed by 15%, and the curve, though parallel to the buffer curve, was displaced to the right. This displacement is due to endogenous progesterone of adrenal origin (Tyndale-Biscoe and Hinds, 1980).

Since all plasma curves were parallel to the buffer standard curve (with displacement occurring due to the presence of progesterone of adrenal origin in plasma from an ovariectomized tammar) it is concluded that non-specific factors in tammar plasma do not interfere with the binding of the antiserum and hormone.

**Accuracy.** (a) Evaluation of assay blanks. High blanks in the assay system are undesirable when low levels of hormone are being measured. One of the most common sources of blanks in the steroid system is the solvent used for extraction. When 20 ml n-hexane from an unopened A.R. Winchester was dried down and taken through the assay, readings of 50-100 pg/tube were obtained. This level was reduced to less than 10 pg/tube if the hexane was freshly redistilled and used within three days. It was also essential that hexane was stored in glass and dispensed through glass pipettes as the use of any plastics increased the blank readings 2 to 3 fold.

Other sources of blanks can be caused by unclean or unsuitable glassware. Initially screw-capped culture tubes were used for extraction. These proved unsatisfactory as water, buffer and hexane blanks from these tubes gave variable readings ranging
from 100-500 pg/tube. The source of the contamination appeared to be the plastic screw caps. When glass-stoppered Quickfit test tubes were used these blanks were reduced to less than the sensitivity of the assay.

The compressed air used for drying solvents was filtered through a trap containing cotton wool and did not contribute to the assay blanks. The drying apparatus was cleaned with ethanol before each assay to avoid contamination with steroids or other non-specific interfering materials.

Therefore to ensure that blanks remained consistently low hexane was freshly distilled and dispensed through glass only, all glassware was thoroughly washed and rinsed in distilled water and ethanol, and only glass Quickfit tubes were used for extraction.

(b) Extraction efficiency. Extraction efficiency was determined by the addition of 3H-progesterone to both buffer and plasma which contained 0-500 pg cold progesterone. A single or double extraction was performed in duplicate at each concentration of cold progesterone examined. Similar results were obtained independent of whether tracer was added in buffer, or added in solvent and dried down before the addition of plasma, provided there was an equilibration period of 2 hours before extraction.

A single extraction with hexane gave efficiencies varying from 50-75% (65.3 ± 9.3%) (Table 2) while a double extraction gave a consistently high efficiency of 90% (89.8 ± 2.3%) (Table 2) over the range of added cold progesterone examined. Very similar results were obtained for extraction from buffer. For this reason a double extraction has always been used and the results have been corrected accordingly.
Table 2. Efficiency of extraction (%) of $^3$H-progesterone from tammar plasma to which a range of concentrations of unlabelled progesterone was added.

<table>
<thead>
<tr>
<th>Concentration (pg) of cold progesterone added</th>
<th>Mean ± S.D.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>65.3 ± 9.3</td>
<td>10</td>
</tr>
<tr>
<td>50</td>
<td>55.71</td>
<td>10</td>
</tr>
<tr>
<td>100</td>
<td>75.67</td>
<td>10</td>
</tr>
<tr>
<td>250</td>
<td>75.55</td>
<td>10</td>
</tr>
<tr>
<td>500</td>
<td>50.62</td>
<td>10</td>
</tr>
</tbody>
</table>

Single extraction

Double extraction 89.91 93.92 87.88 88.91 92.87 89.8 ± 2.3 10

(c) Recovery of unlabelled progesterone from tammar plasma. The accuracy of the radioimmunoassay was tested by recovery experiments. To a plasma containing low levels of endogenous progesterone increasing amounts of standard progesterone (50, 100, 150, 200, 250 pg) were added. Twenty independent estimates of 500 ul plasma containing only endogenous progesterone were made, while 9 independent replicates of 500 ul plasma were made for each of the added doses. The amount of progesterone recovered was highly correlated with the actual amount added ($y = 1.01 x + 64.35$, $r = 0.991$; P < 0.001; n = 56), and was always within 13% of that expected (Fig. 15).

Precision. Intra- and inter-assay variation was evaluated by duplicate measurements of the same samples in the same assay and in several different assays. For ten duplicate determinations
performed in the same assay using plasmas with endogenous levels of progesterone ranging from 50 to 350 pg/ml the coefficient of variation was $7.5 \pm 1.0\%$ (M ± S.D., n = 30). There was no difference in intra-assay variation between extraction of 2 x 0.5 ml plasma (redissolved in 2 x 0.6 ml buffer and 2 x 0.5 ml taken for assay) and extraction of 1.0 ml (redissolved in 1.2 ml buffer and 2 x 0.5 ml taken for assay) ($7.5 \pm 1.0\%$ vs $8.0 \pm 0.8\%$). Therefore for routine assays 1.0 ml plasma samples were extracted and then 0.5 ml assayed in duplicate. This meant that twice as many samples could be determined in each assay. However quality control plasmas continued to be extracted by both methods to ensure precision was maintained.

In twelve different assays four replicate determinations of three pools of tammar plasma containing endogenous progesterone (52, 189 and 450 pg/ml) were made. The inter-assay coefficients of variation were 9.5%, 9.0% and 10.5% respectively.

Validation of the progesterone assay for the possum

The assay procedure used for the tammar was followed for the possum except that only 300 ul plasma (150 ul, in duplicate) was required for extraction.

Sensitivity. Using antiserum No. 334 (final dilution 1:12,600) the assay was sensitive to 10 pg/ml which when corrected for the volume of plasma extracted was 100 pg/ml plasma.

Accuracy and precision. The efficiency of the double extraction procedure determined from the recovery of $^3$H-progesterone equilibrated with endogenous plasma progesterone was $88.7 \pm 1.9\%$
(Mean ± S.D., n = 12). Recovery of cold progesterone added to 'steroid-free' possum plasma was always within 12% of that expected (n = 20) while intra- and inter-assay coefficients of variation were 10% (n = 20) and 13% (n = 5) respectively.

**Specificity.** Serial dilutions of plasma from an anoestrous female and a female possum in the late luteal phase of an oestrous cycle were parallel to the inhibition curves obtained with progesterone standards (Fig. 16).

**Effect of possum plasma on the standard curve.** In the absence of plasma from ovariectomized and ovariectomized/adrenalectomized possums only the effect of 'steroid-free' possum plasma on the standard curve could be determined. The curves were prepared as described for the tammar, except only 150 ul plasma was added. Maximum binding (%B/T) was unaffected by the addition of 'steroid-free' plasma (52% vs 51%). The results expressed as %B/B₀ and corrected for a recovery of 89% also confirm that the addition of 'steroid-free' possum plasma does not interfere with the binding of the antiserum (No. 334) to progesterone since the curves are superimposable (Fig. 16).

**Validation of the progesterone assay for the native cat**

The procedure followed was the same as described for the tammar but with only 300 ul plasma (150 ul in duplicate) being required for extraction.
Sensitivity. Using antiserum No. 334 (1:12,600, final dilution) the assay was sensitive to 10 pg/tube, or, when corrected, 100 pg/ml plasma.

Accuracy and precision. After a double extraction with hexane the recovery of \(^{3}H\)-progesterone from several different native cat plasmas was 87.9 ± 1.9% (M ± S.D., n = 14). Recovery of cold progesterone added to steroid-free native cat plasma was within 11% of that expected, while the intra- and inter-assay coefficients of variation were 7.0% (n = 8) and 9.0% (n = 2) respectively.

Specificity. Serial dilutions of plasma from a female native cat 15 days post-coitum were parallel to the standard curve for progesterone. Plasma dilutions from an adult male native cat showed no displacement in the assay (Fig. 17).

Effect of native cat plasma on the standard curve. For the native cat, only the effect of steroid-free plasma could be determined due to the unavailability of plasma from ovariectomized and/or ovariectomized/adrenalectomized animals. Standard curves with and without 150 ul ‘steroid-free’ plasma were prepared and assayed. The results obtained for the ‘steroid-free plasma were very similar to those described previously for the tammar and possum. There was no inhibition of maximum binding (47% in both cases), and when the results were expressed as \(\%B/B_0\) and corrected for a recovery of 88% the curve containing plasma superimposed with the buffer curve (Fig. 17). Therefore factors in plasma from native cats do not interfere in a specific manner with binding of the antiserum and hormone.
3.2.4. DISCUSSION

In this section a radioimmunoassay for progesterone based on the method developed by Abraham et al. (1971) has been validated for the measurement of plasma progesterone in three marsupials; the tammar, possum and native cat. Two highly specific antisera (No. 230 and No. 334) have been used and plasmas from all species tested (tammar, eastern grey kangaroo, possum and native cat) show parallel displacement with the standard curve. Steroid-free plasma from the tammar, possum and native cat do not interfere with the reaction of the hormone and antiserum. The sensitivity of the assay is 10 pg/tube, and the accuracy and precision of the assays for each marsupial are within the limits set by the literature (Concannon et al., 1975; Bonnin et al., 1978; Harder and Moorhead, 1980).

Since levels of progesterone in the tammar are very low the assay has been developed under strictly controlled conditions to reduce assay blanks and maintain assay accuracy and precision. Particularly important was the purity of the hexane used for extraction, and the cleanliness and suitability of glassware. Similar precautions were taken for the assay of progesterone in the possum and native cat. However plasma progesterone levels in these animals were several-fold higher than in the tammar so the introduction of solvent blanks or other non-specific factors would not have had as marked an effect on assay sensitivity.

It is concluded that the assay system for progesterone is suitable for measuring plasma levels of the hormone in these marsupials during various reproductive events.
3.3 PROLACTIN RADIOIMMUNOASSAY

3.3.1 INTRODUCTION

To establish the heterologous assay for plasma prolactin in the tammar, several antisera, raised against eutherian prolactins, were screened for cross-reaction with wallaby pituitary extracts. Antisera raised in turkeys and rabbits against ovine and bovine prolactin (Dr Alan Wallace, CSIRO, Sydney), showed no cross reaction, but two antisera raised in guinea pigs against human prolactin (Dr Alan McNeilly, Edinburgh, Scotland) showed excellent displacement with both pituitary extracts and plasmas from tammars. Using these antisera (33-9 and 33/1-8) a radioimmunoassay has been developed and validated for routine measurements of prolactin in the plasma of both tammars and possums.

3.3.2 MATERIALS

Buffer

For the assay a 0.01 M sodium phosphate buffer, pH 7.4, containing 0.15 M sodium chloride, 1.0% (w/v) bovine serum albumin (Sigma) and 0.1% sodium azide was used as diluent and hereafter is referred to as RIAD.

Antisera

First antibody. Guinea-pig anti-human prolactin was prepared and supplied by Dr Alan McNeilly (Edinburgh, Scotland). Guinea-pigs were immunized subcutaneously with 100 ug human prolactin in Freund's complete adjuvant and received 4 booster injections at 2 week intervals. Antiserum 33-9 was characterised and showed high
cross reactivity with human prolactin and prolactin from several eutherian species, but low cross reactivity (< 0.1%) with growth hormone, placental lactogen, LH and FSH from several eutherian species (McNeilly and Friesen, 1978b). A second antiserum, 33/1-8, was shown to have similar characteristics (Alan McNeilly, pers. comm.). Both antisera have been tested for use in the assay system. They were received as whole serum, diluted 1:10 with RIAD and stored in 50 ul aliquots at -40°C. Each aliquot was diluted appropriately with RIAD to give a final dilution in the assay of 1:36,000.

**Second antibody.** Several second antibody preparations were tested in the assay system before a satisfactory one was obtained. Sheep anti-guinea pig gamma globulin (Commonwealth Serum Laboratories, CSL), and 2 rabbit anti-guinea pig gamma globulins (from Growth Hormone Assay Kit, and Wellcome) gave less than 10% binding. Immunoelectrophoresis of the CSL sheep anti-guinea pig gamma globulin showed many lines of cross-reactivity with normal sheep serum suggesting that this antibody had not been raised against a specific immunoglobulin G but against a crude preparation of all immunoglobulins. However a commercially prepared donkey anti-guinea pig gamma globulin, tried on the advice of Dr Alan McNeilly, increased the level of maximum binding to greater than 30%, so it was decided to prepare a similar second antibody here.

Dr Mal Brandon (John Curtin School of Medical Research, Canberra) kindly prepared the antigen, highly purified guinea pig gamma globulin, from whole guinea pig serum by gradient elution (0.20 M - 0.40 M phosphate buffer, pH 8.0) on a 50 x 2.5 cm DEAE A50 Sephadex column. The first protein peak to elute was immunoglobulin
G2, and this was collected, concentrated and stored at -20°C until used for immunization. A castrated male donkey was immunized by intramuscular injection with 2.8 mg guinea-pig immunoglobulin G in Freund's complete adjuvant and by three booster injections of the same amount of antigen at 7, 14, and 25 days. On day 63 immunoelectrophoresis showed significant donkey anti-guinea pig gamma globulin activity and when tested in the assay the serum was useable at 1:20 dilution. After 69 days a large volume of blood (300 ml) was collected from the jugular vein, and the serum separated and stored frozen in 1 ml aliquots for use in the assay. A second volume of 220 ml was collected 8 days later and stored as before. Hereafter this donkey anti-guinea pig gamma globulin is referred to as donkey second antibody.

Normal guinea pig serum

Normal guinea pig serum (guinea pig complement [stabilized]) was obtained from Commonwealth Serum Laboratories as a lyophilized powder. Each vial when reconstituted by addition of 16 ml glass distilled water gave a solution equivalent to a 1 in 10 dilution of fresh guinea pig serum in saline. Aliquots of 500 ul were stored at -15°C, and diluted for assay to 30 ml with RIAD to give a working dilution of 1:600.

Normal guinea pig serum is included in the assay as a 'carrier protein' to increase the volume of the precipitate of the antigen-first antibody complex and the second antibody.

Standard prolactin preparation

Ovine prolactin (NIH-P-S12, 10 mg) was obtained from the National Institute of Arthritis, Metabolism and Digestive Diseases
In the assay it was used as the standard and also radio-iodinated for use as the tracer. The potency of the preparation as estimated by NIAMDD was 35 IU/mg. It contained less than 0.020 NIH-FSH-S1 units/mg, 0.0050 NIH-LH-S1 units/mg, 0.0050 Thyrotrophin stimulating hormone USP units/mg, and 0.010 Growth hormone IU/mg.

Each 10 mg of lyophilized powder was dissolved in 9.9 mls 0.05 M sodium phosphate buffer, pH7.4, plus 100 ul 1N NaOH, and the pH was adjusted to 8.5 by addition of 50 ul 1N HCl. Aliquots of 500 ul (500 ug) were frozen, and further dilutions made to give aliquots of 5 ug/25 ul for radio-iodination, and 200 ng/40 ul for use in the standard curve. All aliquots were frozen and stored at -40°C until used.

Columns and gels used for preparation of tracer

Sephadex G25 fine and Sephadex G100 gels were obtained from Pharmacia Fine Chemicals (Aust. Pty. Ltd.). Before use both gels were swollen in distilled water for more than 48 h at room temperature, and then stored at 4°C.

Sephadex G25 column. Sephadex G25 fine was degassed for 15 min, packed in a glass column (0.7 x 15 cm) and equilibrated as follows. After running through one volume (7 ml) of 0.05 M sodium phosphate buffer, pH7.4 the gel was conditioned with 0.5 ml 5% bovine serum albumin. (This conditioning presaturates any possible sites in the gel which might bind the labelled protein.) At least 2 more volumes of buffer were run through before the iodinated material was layered on the top of the column.
**Sephadex G100 column.** Sephadex G100 was degassed for 60 min before packing in a glass column, 1.5 cm x 40 cm. The column was equilibrated by running through a minimum of 100 ml of 0.05 M sodium phosphate buffer, pH7.4 plus 0.1% bovine serum albumin (w/v), (also degassed). Prior to loading the tracer on the column the flow rate of buffer through the gel was adjusted to 1 drop/12 secs. This rate consistently produced a sharp elution profile.

### 3.3.3 METHODS

**Radioiodination of prolactin and tracer purification**

Iodination of ovine prolactin (NIH-P-S12) was carried out by the lactoperoxidase method of Thorell and Johansson (1971) with some modifications. All procedures were performed at room temperature. Reactants were added rapidly to 5 ug (25 ul) of the hormone in the following order: 10 ul 0.5 M sodium phosphate buffer, pH7.4; 0.5 mCi Na125I (Amersham) (5 ul); 4 ug (10 ul) lactoperoxidase and 10 ul 0.088 mM H2O2. The reaction was stopped after 45 seconds by dilution with 500 ul 0.05 M sodium phosphate buffer, pH7.4. To remove unreacted iodide the solution was immediately subjected to gel filtration on an equilibrated and conditioned Sephadex G-25 column (0.7 x 15 cm) (Section 3.3.2). The reaction vial was rinsed with 100 ul 0.05 M sodium phosphate buffer, pH7.4. Fractions of 0.5 ml were collected into 0.1 ml 5% bovine serum albumin and 10 ul subsamples counted to determine the distribution of iodinated material and free iodine (Fig. 18). Higher molecular weight substances are eluted earlier, so the protein peak precedes the free iodine peak. Routinely the iodinated prolactin eluted in fractions 9-11 (4.5 - 6 ml ) and the free iodine in fractions 18 - 22 (9 - 11 ml ).
Using this procedure greater than 80% of the added Na\textsuperscript{125}I was incorporated into the protein giving a specific activity of between 40 and 80 uCi/ug.

The binding activity of this preparation was very short-lived. Within 3 days the maximum binding capacity of the tracer had dropped from an initial level of greater than 30% to less than 8% indicating that degradation of the iodinated material had occurred very rapidly. It was therefore necessary to introduce another step to further purify the labelled tracer. This was done using a Sephadex G100 column (1.5 x 40 cm) equilibrated with 0.05 M sodium phosphate buffer, pH7.4, containing 0.1% bovine serum albumin (w/v). After the initial purification step on the G25 column the 2 or 3 fractions of highest activity in the protein peak were pooled and layered on the G100 column. The flow rate of eluent was adjusted to 1 drop/12 secs and fractions of 1 ml (25 drops) collected using a Fractomette fraction collector. Subsamples of 20 ul from fractions 15 to 55 were counted in an automatic gamma counter (Searle Analytic 1197). A typical elution profile consisted of one major peak of protein the forward shoulder of which was not very sharp (fractions 20-37) and probably comprised aggregates of iodinated hormone (Fig. 19).

Immediately after preparation the binding capacity of fractions 37-42 was always greater than 45% while for the trailing fractions (43-47) binding was around 50%. Significantly lower binding (< 35%) was present in the forward shoulder of the eluted peak (20-35). Since peak activity was always present in fractions 40-45 these were pooled, diluted with 500 ul 5% bovine serum albumin and frozen in 500 ul aliquots at -15°C. This preparation gave maximum binding activity consistently greater than 30% for a minimum
of three weeks.

A summary of the steps involved in the iodination and purification of the tracer is presented in Fig. 20.

**Assay procedure**

Assays for marsupial prolactin were carried out by a double antibody procedure as described by McNeilly and Friesen (1978b). Unless otherwise specified all dilutions were made in RIAD, all standards were assayed in triplicate and all samples were assayed in duplicate. For routine assays plasma samples of 50 or 100 ul were used.

**Day 1.** Standards ranging from 0.019 - 10.0 ng NIH-P-S12/100 ul RIAD, or unknown (100 ul), were added to disposable plastic 5 ml serology tubes (Medical Plastics South Australia). Anti-human prolactin (As 33-9, or As 33/1-8) was suitably diluted in RIAD and 100 ul added to each tube to give a final dilution of 1:36,000. This dilution of antiserum routinely bound 30-50% of added counts.

Control tubes for the estimation of maximum binding in the absence of unlabelled hormone contained 400 ul RIAD and 100 ul antiserum, while tubes for the measurement of non-specific binding of tracer received 500 ul RIAD. All other tubes (standards and samples) received an additional 300 ul RIAD to bring the total incubation volume for Day 1 to 500 ul. All tubes were vortexed and incubated at 4°C for 24 h.
Day 2. The tracer was diluted in RIAD and 100 µl (=20,000 cpm) was added to each assay tube. Four tubes received 100 µl of tracer for estimation of total counts. All tubes were vortexed and incubated at 4°C for 48 h.

Day 4. Donkey second antibody (100 µl at 1:20 dilution) and normal guinea-pig serum (100 µl at 1:600 dilution) were added. After vortexing, incubation was continued at 4°C for another 16 h before the antibody-bound and the free hormone were separated by centrifugation at 2500 g for 30 mins in a Servall automatic superspeed refrigerated centrifuge type RC-2 or Centri-7R refrigerated bench centrifuge. The supernatants were aspirated off and the antibody-bound iodinated prolactin in the precipitate was measured in an automatic gamma counter (Searle Analytic Model 1197).

Titration of second antibody and normal guinea pig serum

Second antibody is used to precipitate the antigen-first antibody complex and normal guinea pig serum is added as a 'carrier protein' to increase the volume of the precipitating complex. It is important to optimize the conditions of precipitation since too much second antibody as well as too little can decrease the volume of the precipitate. Similarly too much normal non-immune serum can produce too large a precipitate which can then trap unbound hormone. The optimum conditions for precipitation have been determined in the following experiment by titration of the second antibody and normal guinea pig serum in the presence of a fixed concentration of first antibody (1:6000 working dilution).
To a series of tubes was added:

Day 1. 100 ul first antibody (1:36,000) in RIAD
100 ul tracer (20,000 cpm) in RIAD
400 ul RIAD
(Non-specific binding tubes were also prepared - 500 ul RIAD plus
100 ul tracer).

Day 3. 100 ul second antibody diluted 1:4 - 1:80 in RIAD
100 ul normal guinea pig serum diluted 1:300 -
1:600 in RIAD

Day 4. All tubes were centrifuged and the precipitates
counted. A titration curve was obtained by plotting the dilution of
the second antibody against the percentage of added counts
precipitated for a fixed concentration of normal guinea pig serum
(Fig. 21 a&b).

A marked prozone effect is seen. In the presence of excess
donkey second antibody (< 1:10) there is decreased precipitation,
while insufficient donkey second antibody (> 1:30) does not
precipitate all of the antigen-first antibody reaction and so the
amount of radioactivity decreased. Where the concentration of
donkey second antibody is optimal (1:10 to 1:25), maximum
precipitation occurs and a plateau is obtained (Fig. 21a). Maximum
precipitation was maintained in the presence of normal guinea pig
serum dilutions of 1:600, 1:500 and 1:400 (Fig. 21b). Therefore the
conditions chosen for use in the radioimmunoassay were donkey second
antibody at 1:20 and normal guinea pig serum at 1:600.
In a second experiment it was found that the same conditions applied when using antiserum 33/1-8 (1:36,000).

3.3.4 ASSAY VALIDATION PROCEDURES AND RESULTS

Sensitivity
The recommended final dilution for antisera 33-9 and 33/1-8 which would bind 30-50% of the tracer (depending on the age of the tracer) was 1:36,000 in both cases. To determine whether the same dilutions would give as sensitive a curve with adequate binding in this laboratory, each antiserum was titrated. Several standard curves were prepared and antiserum added to give final dilutions of 1:24,000, 1:30,000, 1:36,000 and 1:42,000. For antiserum 33-9 a dilution of 1:36,000 gave a curve sensitive to 1.56 ng/ml (Fig. 22) and maximum binding of > 30%. Although the curve produced at a dilution of 1:42,000 was more sensitive than the curve at 1:36,000, maximum binding was reduced to < 25% and so the apparent increase in sensitivity was negated by the corresponding decrease in precision.

Similarly, antiserum 33/1-8 was titrated over a range of dilutions (1:30,000, 1:36,000 and 1:42,000). The optimum dilution giving a curve sensitive to 1.56 ng/ml was 1:36,000 (Fig. 23). As for antiserum 33-9 a dilution of 1:42,000 of antiserum 33/1-8 appeared to produce a more sensitive curve but in doing so binding was reduced and counting accuracy reduced.

Specificity of the heterologous assay for marsupial prolactin
Cross reactivity. (a) Purified hormone fractions and pituitary homogenates. To assess the specificity of the assay for marsupial prolactin various pituitary homogenates and purified
pituitary fractions from different species of marsupial were incubated. The purified wallaby prolactin fraction (74.9(3) Fr 3) was prepared by Dr J. Hawkins using the ammonium sulphate fractionation method of Neill and Reichert (1971). The presence of prolactin-like activity in this preparation was confirmed using a pigeon crop gland bioassay. Other purified pituitary fractions were provided by Dr S.W. Farmer (Gallo, Licht, Walker Farmer, Papkoff and Hawkins, 1978; Farmer et al., 1981). These preparations are listed in Table 3. A range of concentrations (0.01 - 32 ng/ml) of each hormone fraction was prepared, and 100 µl aliquots incubated in the system as described above.

Adenohypophyses of tammars (Macropus eugenii), eastern grey kangaroos (M. giganteus), brush possum (Trichosurus vulpecula), native cats (Dasyurus viverrinus) and kowari (Dasyuroides byrnei) were also assessed. The glands had been collected within 15 min of

Table 3. Provenance of purified hormones used to validate the heterologous radioimmunoassay for marsupial prolactin.

<table>
<thead>
<tr>
<th>Species</th>
<th>Hormone</th>
<th>Code</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tammar wallaby (Macropus eugenii)</td>
<td>Prolactin</td>
<td>74.9(3)Fr 3</td>
<td>J. Hawkins</td>
</tr>
<tr>
<td></td>
<td>LH</td>
<td>Ex241</td>
<td>Gallo et al. (1978)</td>
</tr>
<tr>
<td></td>
<td>FSH</td>
<td>Ex240D</td>
<td>Gallo et al. (1978)</td>
</tr>
<tr>
<td>Eastern grey kangaroo (M. giganteus)</td>
<td>Prolactin</td>
<td>EG13B</td>
<td>Farmer et al. (1981)</td>
</tr>
<tr>
<td>Western grey kangaroo (M. fuliginosus)</td>
<td>Prolactin</td>
<td>WG13C</td>
<td>Farmer et al. (1981)</td>
</tr>
<tr>
<td>Sheep</td>
<td>Prolactin</td>
<td>NIH-P-S12</td>
<td>NIH</td>
</tr>
</tbody>
</table>
death, stored at -15°C, and crude aqueous homogenates prepared prior to assay. Each anterior pituitary was homogenised in 1 ml RIAD, serial dilutions (1:1000 to 1:512,000) prepared and 100 ul of each dilution tested in the assay.

The binding of the tracer to antiserum 33-9 was displaced in a parallel manner by crude pituitary homogenates of all species examined (tammar, eastern grey kangaroo and possum) (Fig. 24). A similar parallel displacement was also observed for purified prolactin fractions from the 3 macropodid species (tammar, eastern grey kangaroo and western grey kangaroo), but not by purified tammar LH or FSH (Fig. 24). The two latter preparations have been shown to have cross-reactivity in heterologous radioimmunoassays for LH (Sutherland et al., 1980) and FSH (Gallo et al., 1978). Purified marsupial growth hormone was not available to test but McNeilly and Friesen (1978b) have shown that there is no cross-reaction of this antiserum with growth hormone of several eutherian species.

The binding of the tracer to antiserum 33/1-8 was also displaced in a parallel manner by crude pituitary homogenates of several species (tammar, possum, native cat and kowari) and by purified tammar prolactin (74.9(3) Fr 3) (Fig. 25).

(b) Plasma from tammars and possums. (i) Antiserum 33-9.

To demonstrate parallelism of immunoreactivity between standard ovine prolactin and endogenous tammar prolactin in plasma samples, serial dilutions (10-200 ul) of four different tammar plasmas were tested. Dilution-response curves were obtained for plasma from a castrate male, ovariectomized female and a lactating female; with increasing volumes of plasma there was an increased displacement of tracer from the antiserum such that the curves obtained appeared to
be parallel to the standard curve prepared in RIAD (Fig. 26).
However increasing volumes of plasma from an hypophysectomized
tammar induced no significant displacement of the tracer (Fig. 26).

(ii) Antiserum 33/1-8). Serial dilutions of plasma from 4 possums and 2 tammars were tested. Parallel dose response curves were obtained for plasma from an adult male, lactating female and non-lactating female possum and a lactating tammar. No significant displacement of the tracer from the antiserum was induced by plasma from a hypophysectomized female of either species (Fig. 27).

Accuracy and precision

Tammar. Accuracy was assessed by the recovery of ovine prolactin standard (6-100 ng) added to a normal tammar plasma which had previously been assayed at 4.0 ng/ml. Each point was assayed in quadruplicate. The amount of prolactin recovered was closely correlated with the actual amount added \( y = 0.867 x + 4.22, r = 0.998, P < 0.001; \ n = 24 \) (Fig. 28a) and was always within 12% of the expected value.

The coefficients of variation determined from assays of replicate samples of three pools of tammar plasma containing 6.5, 38 and 82 ng/ml were 8.5%, 7.7% and 7.2% respectively \( (n = 15 \) for each pool) within assay, and 12.0%, 11.7% and 11.5% respectively \( (n = 6 \) for each pool) between assays.

Possum. Ovine prolactin standard (3.13 - 50 ng) was added to a normal possum plasma in which prolactin was not detectable. Each point was determined in quadruplicate. Recovery of prolactin was closely correlated with the amount added \( y = 0.969 x - 0.017, r = 0.996, P < 0.001; \ n = 24 \) (Fig. 28b) and was always within 14% of
the expected value.

Intra-assay coefficients of variation determined from replicate assays of two pools of possum plasma containing 8.5 ng/ml and 32.5 ng/ml were 10.0% and 6.5% respectively (n = 10 for each pool). Inter-assay coefficients of variation for the same two pools were 13.0% and 11.0% respectively (n = 6 for each pool).

Measurement of plasma prolactin levels in the tammar and possum

To determine whether the assay system would measure changes in prolactin levels in vivo in response to agents and procedures known to alter blood prolactin levels in other species, blood samples were collected before and after injection of thyrotrophin releasing hormone and before and after entire hypophysectomy from tammars and possums. In the tammar, samples were also collected before and after injection of bromocriptine (CB154, Sandoz Aust; 5 mg/kg i.m.). All tammar plasma levels reported in thesis were determined using antiserum 33-9. Possum plasmas were assayed using antiserum 33/1-8 only.

Response in plasma prolactin levels to i.v. injection of thyrotrophin releasing hormone. Two intact male tammars, two intact female tammars and one ovariectomised female were used. Each animal was injected intravenously (i.v.) at time 0 with 40 ug thyrotrophin releasing hormone (Roche) and blood samples collected from the tail vein from -30 min to 140 min. Four intact female possums and two intact male possums were treated similarly with 20 ug thyrotrophin releasing hormone i.v. and sampled from jugular catheters from -60 min to 180 min.
Both male and female tammars, and male and female possums showed an increase in plasma prolactin levels in response to an i.v. injection of thyrotrophin releasing hormone. In the tammars there was a 10 fold increase (20-200 ng/ml) to peak levels within 10 min and a return to near pre-injection levels by 140 min (Fig. 29). In the possums the peak values of 30-157 ng/ml were reached between 5 and 10 min after injection and returned to basal levels between 50 and 120 min. The size of the increase in the possums was more variable than in the tammars (Fig. 30).

Both species showed a similar response to that reported for many eutherian species.

Response in plasma prolactin levels to i.m. injection of bromocriptine. Bromocriptine (CB154) was prepared for injection by dissolving equal quantities of the drug and D,L-tartaric acid in one volume of 70% alcohol. An equal volume of warmed (40°C) sterile 0.9% saline was added to give the required concentration (25 mg/ml). Control groups received a solution of tartaric acid, alcohol and saline. All animals were injected intramuscularly (i.m.) in the rump with 0.2 ml solution per kilogram body weight to give a total dose of CB154 of approximately 25 mg per animal.

The effect of CB154 injection on plasma prolactin concentrations has been examined in three separate experiments.

In the first experiment (September) one group of five non-lactating animals received a single injection of CB154 (5 mg/kg), a second group received the same dose on three successive days, and the third group was injected with saline vehicle on three successive days. Blood samples were collected prior to injection, 5 h later, and then on days 1 to 7, 14, 21 and 28 post-injection.
In the second experiment (March) one group of five lactating females received a single injection of CB154 (5 mg/kg) and another group of five females was injected with saline vehicle. Blood samples were collected at shorter intervals at -2, -1, 0, 0.3, 0.6, 1, 1.5, 2, 2.5, 3, 4, 5, 7, 9 and 22 h post-injection.

In the third experiment a group of five females in late lactation (November) were injected with saline vehicle on day 0 and with CB154 (5 mg/kg) on the following day. Blood samples were collected on Day -4, and Day -1, and then on Day 0 and Day 1 at more frequent intervals (0, 0.5, 3 and 7 h post-injection). Three further samples were taken on Days 2, 3 and 6.

There was no significant decrease in plasma prolactin levels in the tammar in response to injection(s) of CB154 (Fig. 31 a,b&c). In March (Fig. 31a) levels in the treated and control animals were similar throughout the sampling period, while in September (Fig. 31b) although the levels in the animals given daily injections of CB154 for 3 days were reduced, the decrease was not significantly different from the control (analysis of variance). In November, in the lactating females, the drop in plasma prolactin levels after saline treatment was greater than the decrease observed after injection of CB154 (Fig. 31c). This result could be confounded by the presence of the sucking young since the sucking stimulus at this stage elicits a large release of prolactin (Chapter 6, Section 6.1.2).

This result for the tammar is in contrast to the response in eutherians in which CB154 is a potent inhibitor of prolactin secretion. In only one other species, the chicken, has CB154 been shown to have no effect on the release of prolactin (Chadwick and Hall, 1983).
Response in plasma prolactin to hypophysectomy.

(a) Tammars: Blood samples were collected on Days 0, 1, 7 and 14 after entire hypophysectomy \( n = 6 \) and after sham hypophysectomy \( n = 11 \) of lactating females.

Plasma prolactin concentrations on the day of operation (Day 0) were not different in the two groups, but by Day 14 levels were significantly lower \( 6.5 \text{ ng/ml} \) vs \( 38 \text{ ng/ml} \), \( P < 0.05 \) in the hypophysectomised females (Fig. 32a). In 4 of the 6 hypophysectomised tammars levels had declined to less than the sensitivity of the assay \( (< 2 \text{ ng/ml}) \), but in the remaining 2 animals concentrations of \( 15 \text{ ng/ml} \) were measured. This 'measurable prolactin' present in some plasmas after hypophysectomy is due to interference by the plasma with the second antibody precipitating reaction (see page 84) and is not due to incomplete hypophysectomy (see page 83).

(b) Possums: Blood samples were taken on alternate days after full hypophysectomy \( n = 6 \) and after sham hypophysectomy \( n = 3 \) performed on different days after oestrus.

Plasma prolactin levels in cycling possums were low to undetectable (Fig. 32b) so an effect of hypophysectomy could not be demonstrated. As with some plasmas from hypophysectomised tammars plasma from some hypophysectomised possums contained 'measurable prolactin' levels.
Interference due to plasma or serum from hypophysectomised animals

Tammars. All plasmas tested were from animals in which full hypophysectomy or anterior hypophysectomy had been confirmed by serial section of the sella turcica. When 100 ul of plasma or serum from a hypophysectomised tammar was added to each point of the standard curve there was a consistent inhibition of binding (Fig. 33). The degree of inhibition varied markedly between plasmas of different hypophysectomised animals ranging from less than 5% to greater than 20% (Fig. 33). However the inhibition produced by any one plasma was the same irrespective of which antiserum (33-9 or 33/1-8) was used. This inhibition appears to be non-specific as transformation of the data to percentage $\frac{B}{B_0}$ produces standard curves which superimpose quite closely (Fig. 34). A similar though not so marked effect is seen in the luteinising hormone radioimmunoassay (Sutherland et al., 1980) conducted by C.A. Horn in this laboratory.

Since the interference of plasmas from different animals in the standard curve was so variable it was not possible routinely to add this plasma to the curve. Therefore all plasma concentrations are derived from a standard curve prepared in buffer alone.

Possums. All plasmas tested were collected from animals in which full hypophysectomy or anterior hypophysectomy had been confirmed by serial section of the sella turcica. Plasma (100 ul) was added to each point of the standard curve and binding compared with the curve prepared in buffer alone. Two of the four plasmas tested had no effect on the binding of the antiserum (33/1-8) to the tracer, since the curves in the presence of these plasmas super-
impose quite closely with the standard curve in buffer (Fig. 35 and Fig. 36). However the remaining two plasmas caused significant inhibition of maximum binding (10% and 22% respectively) (Fig. 35), and when the data was transformed to percentage B/B₀, a significant, though parallel, displacement to the right of the buffer curve was observed (Fig. 36). Hence, as with some plasmas from hypophysectomised tammars, some plasmas from hypophysectomised possums also show some non-specific interference. Since this interference varies between plasmas it has not been possible to add plasma to the standard curve to correct for the effect. Therefore plasma concentrations are derived from a standard curve prepared in buffer only.

A number of experiments have been performed in an attempt to determine the nature of the interference due to plasma from hypophysectomised animals. This has been done using tammars and tammar plasma due to the limited availability of possum material. The questions posed were; (1) Is the effect due to residual prolactin of unknown origin, or (2) Is it due to non-specific interference with either the first antibody reaction and/or the second antibody reaction?

(1) Is there a source of residual prolactin? If functional fragments of the anterior pituitary gland were left in situ at the time of hypophysectomy injection of thyrotrophin releasing hormone should stimulate the release of prolactin. If, however, the effect is non-specific there should be no response to thyrotrophin releasing hormone. This test could not exclude sources of prolactin which may not be responsive to a thyrotrophin releasing hormone stimulus.
Six lactating females were hypophysectomised and a further six lactating females sham-operated in April 1982. Day 0 was defined as the day of operation, and blood samples were collected on days 0, 1, 7, and 14 for measurement of plasma prolactin. Between days 10 and 14 the nine surviving animals were injected intravenously with 20 ug thyrotrophin releasing hormone. Blood samples of 1.0 ml were collected via an indwelling catheter inserted in the lateral tail vein at the first sample time. The sampling schedule was as follows: -60, -30, 0, 5, 10, 20, 30, 45, 60, 90, 120 and 180 min. Thyrotrophin releasing hormone was injected at 0 min.

The five sham-operated females showed a 5 to 10 fold increase in plasma prolactin within 5 min of the injection of 20 ug thyrotrophin releasing hormone (Fig. 37a). This response was very similar to that observed in other intact tammars. In contrast, there was no significant change in the 'measurable' plasma prolactin levels of the 4 hypophysectomised tammars given the same dose of thyrotrophin releasing hormone (Fig. 37b).

(2) Is plasma from hypophysectomized animals interfering with the reaction of either the first or second antibody? To examine this possibility four standard curves were prepared using antiserum 33-9 (1:36,000). To two curves, plasma from an hypophysectomized tammar which was known to have an effect on binding was added. On Day 4 one second antibody (donkey anti-guinea pig gamma globulin) was used to precipitate one standard curve in the presence and one in the absence of plasma from a hypophysectomized female. A different second antibody, rabbit antiguinea pig gamma globulin (Wellcome), was added to the remaining two curves. (The rabbit second antibody had been titrated with normal
guineapig serum and the conditions for its use were the same as for the donkey second antibody, 1:20 with normal guinea pig serum 1:600).

Maximum binding for both curves prepared in buffer was similar (46% vs 51%). However the inhibitory effect of the plasma on binding was greater in the presence of the rabbit second antibody (30% inhibition vs 15%) (Fig. 38). When the data was transformed to percentage B/B₀ the curves in plasma both superimposed with their corresponding buffer curve, although the curves with the rabbit second antibody were not as sensitive (3 ng/ml vs 1.56 ng/ml) (Fig. 39).

These results show that the effect of plasma from hypophysectomized tammars may be related to the properties of the second antibody and not the first antibody. Therefore to determine if the reduction in binding was due to interference of the plasma with the second antibody reaction, three series of maximum binding and non-specific binding tubes were incubated. On Day 4 just prior to the addition of second antibody, plasma from the same hypophysectomized tammar was added to one series of tubes, normal plasma containing normal levels of prolactin (10 ng/ml) to another, while buffer was added to the third series. Theoretically, the addition of plasma at this stage means that it should not interfere with the first antibody - tracer reaction, only with the second antibody precipitating reaction. Various dilutions of donkey second antibody (1:10, 1:15, 1:20, 1:25, 1:30) in the presence of dilutions of normal guinea pig serum (1:600, 1:500, 1:400, 1:300) were then added.
For all of the conditions examined plasma from a hypophysectomised tammar suppressed maximum binding (Fig. 40). A minimum of interference (12%) occurs in the plateau region for the conditions of second antibody (1:20) and normal guinea pig serum (1:600) which are in routine use. This level of interference is the same as when the plasma is added on Day 1. No other plasmas showing less than 12% interference in binding were available to test. The normal plasma also affected binding except for one series of dilutions of donkey second antibody in the presence of normal guinea pig serum (1:300) (Fig. 40). However previous results have shown that these conditions of normal guinea pig serum are not optimal for precipitation of the first antibody-hormone complex. Interestingly the level of prolactin in this normal plasma, when assayed under routine conditions, gave a reading of 10 ng/ml - in this assay when added on Day 4 the level was 4.0 ng/ml. This means that either the plasma has interfered with the second antibody reaction equivalent to 4.0 ng/ml, or that it has affected the binding with the first antibody (theoretically not probable) but displacement was incomplete due to the ongoing reaction with the second antibody.

Although the results of these experiments do not exclude a source of prolactin other than the pituitary that is insensitive to thyrotrophin releasing hormone, they support the view that the effect of plasma from hypophysectomised tammars on binding of tracer is due to a non-specific factor, present in varying concentrations in plasmas from different hypophysectomised tammars, which interferes with the second antibody reaction. The highly variable effect of different plasmas may be due to changes in plasma characteristics in animals deprived of their pituitary as has been suggested for the rat (Neill and Reichert, 1971). A similar
situation may apply to plasma from hypophysectomised possums since binding is also affected to different degrees by plasmas from different animals.

3.3.5 DISCUSSION

In this section I have shown that the heterologous radioimmunoasay assay system developed by McNeilly and Friesen (1978b) for measuring rabbit prolactin will also measure prolactin in the plasma of the tammar and possum. The assay system in the presence of either antiserum 33-9 or 33/1-8 meets the stringent criteria required to demonstrate validity. Cross-reaction studies have demonstrated its specificity for prolactin of marsupials: dose response curves for purified marsupial prolactin fractions, pituitary homogenates of several marsupials and plasma from tammars and possums were parallel to the standard curve for ovine prolactin (NIH-P-S12), while no displacement was observed with purified tammar LH or FSH. Purified tammar growth hormone was not available for testing but McNeilly and Friesen (1978b) have shown no cross-reaction of the antisera with growth hormone from several different eutherian species.

Plasma from most hypophysectomised tammars and possums showed little or no cross-reactivity. The marked effect on binding caused by some plasmas was due to interference with the second antibody reaction. It was dependent on the second antibody used but independent of which first antibody was used. The nature of this cross-reacting material remains a mystery but could be due to changes in plasma properties which occur as a result of hypophysectomy. It seems unlikely to be due to incomplete hypophysectomy since injection of thyrotrophin releasing hormone
into hypophysectomized tammars does not induce a release of prolactin.

The assay is sensitive to 2 ng ovine prolactin/ml (0.2 ng/tube) while the limits of precision and accuracy are well within the limits reported in the literature (Neill and Reichert, 1971; McNeilly and Friesen, 1978b; Schulte, Seal, Plotka, Verme, Ozoga and Parsons, 1980).

Treatment of tammars and possums with thyrotrophin releasing hormone caused a rapid and significant rise in plasma prolactin, similar in time course to that seen in other species (Davis and Borger, 1972; Convey, Tucker, Smith and Zolman, 1972). However bromocriptine (CB154), a potent inhibitor of prolactin secretion in all eutherian species examined, does not affect prolactin secretion in the tammar; injection of CB154 at two times of the year and during late lactation had no significant effect on the plasma prolactin concentration. In only one other species, the chicken, has injection of bromocriptine had no effect on prolactin release (Chadwick and Hall, 1983).

After hypophysectomy of the tammar, plasma prolactin declined to undetectable levels in most animals, but this decline could not be demonstrated in all cases due to the interference by the plasma with the second antibody reaction. In the possum, basal levels in intact animals were low or undetectable so an effect of hypophysectomy on plasma prolactin concentrations could not be demonstrated.

It is concluded that this heterologous radioimmunoassay can be used to measure relative changes in prolactin levels in wallaby and possum plasma and so will be useful in the investigation of the role of prolactin in the two seasonally breeding marsupials.
3.4 SUMMARY

In this chapter methods for the radioimmunoassay of plasma progesterone and prolactin have been validated for the tammar and possum. The progesterone radioimmunoassay has also been validated for the native cat.

The assays for the three marsupials meet the criteria of specificity, sensitivity, accuracy and precision required for validation, and compare very favourably with assays published in the literature.

Both radioimmunoassays will be used to measure changes in the two hormones during various reproductive states as well as after different experimental manipulations in the tammar and possum. Additional comparative data will be obtained where possible for the native cat.
Fig. 1. Typical standard curve (semi-log plot) for progesterone using antiserum No. 334 (final dilution 1:12,600) plotted as percent B/T vs log dose (x-x) and percent B/B₀ vs log dose (o-o).

B - amount of $^3$H-Progesterone bound in the presence of unlabelled hormone.

B₀ - amount of $^3$H-Progesterone bound in the absence of unlabelled hormone.

T - total counts added.
Fig. 2. Typical standard curve (semi-log plot) for progesterone using antiserum No. 230 (final dilution 1:7,000) plotted as percent B/T vs log dose (x-x) and percent B/B₀ vs log dose (o-o).
FIG. 2.
Fig. 3. Typical calculated standard curve for progesterone using (a) antiserum No. 334 (final dilution 1:12,600), and (b) antiserum No. 230 (final dilution 1:7,000), plotted as T/B vs hormone dose.

* - actual T/B

+ - fitted T/B
FIG. 3.

(a) 21 17 13 9 5

(b) 21 17 13 9 5

HORMONE CONCENTRATION (pg/tube)
Fig. 4. Titration of antiserum No. 334 (percent B/T, semi-log plot). Added $^3$H-Progesterone was constant at 30 pg/assay tube.

Key to final dilutions of No. 334;

- $\times-\times$ 1:3,500
- $\circ-\circ$ 1:7,000
- $\circ-\circ$ 1:12,600
- $\triangle-\triangle$ 1:21,000
FIG. 4.
Fig. 5. Titration of antiserum No. 334 (percent B/B₀, semi-log plot). Added ³H-Progesterone was constant at 30 pg/tube.

Key to final dilutions of No. 334:

- X-X 1:3,500
- O-O 1:7,000
- o-o 1:12,500
- a-a 1:21,000
Fig. 6. Titration of antiserum No. 230 (percent B/T, semi-log plot). Added $^3$H-Progesterone was constant at 30 pg/assay tube.

Key to final dilutions of No. 230:

- - 1:3,500

x-x 1:5,250

o-o 1:7,000

△-△ 1:10,500

■-■ 1:14,000
FIG. 6.
Fig. 7. Titration of antiserum No. 230 (percent B/B₀, semi-log plot).

Added $^3$H-Progesterone was constant at 30 pg/assay tube.

Key to dilutions of No. 230;

- - 1:3,500

× × 1:5,250

o - o 1:7,000

△ △ 1:10,500

■ ■ 1:14,000
FIG. 7.

![Graph showing the relationship between PERCENT B/B₀ and HORMONE CONCENTRATION (pg/tube). The graph includes multiple curves representing different data sets, each distinguished by a different symbol.](image-url)
Fig. 8. Effect of concentration of $^3$H-Progesterone on binding in the progesterone standard curve (semi-log plot) presented as percent B/T. Final dilution of antiserum (No. 334) was constant at 1:12,600 per tube.

Key to concentration of $^3$H-Progesterone:

- X-X  15pg
- o-o  30pg
- △-△  60pg
FIG. 8.

![Graph showing hormone concentration (pg/tube) vs. percent B/T.](image-url)
Fig. 9. Effect of concentration of $^3$H-Progesterone on binding in the progesterone standard curve (semi-log plot) presented as percent $B/B_0$. Final dilution of antiserum (No. 334) was constant at 1:12,600.

Key to concentration of $^3$H-Progesterone:

- 15pg
- 30pg
- 60pg
FIG. 9.

![Graph showing hormone concentration vs. percent B/Bo](image-url)
Fig. 10. Effect of concentration of $^3$H-Progesterone on binding in the progesterone standard curve (semi-log plot) presented as percent B/T. Final dilution of antiserum (No. 230) was constant at 1:7,000 per tube.

Key to concentration of $^3$H-Progesterone;

- x-x 15pg
- o-o 30pg
- ▲▲ 60pg
FIG. 10.

PERCENT B/T

HORMONE CONCENTRATION (pg/tube)
Fig. 11. Effect of concentration of $^3$H-Progesterone on binding in the progesterone standard curve (semi-log plot) presented as percent $B/B_0$. Final dilution of antiserum (No. 230) was constant at 1:7,000 per tube.

Key to concentration of $^3$H-Progesterone:
- X-X 15pg
- O-O 30pg
- △-△ 60pg
FIG. 11.
Fig. 12. Dose response curves (percent B/B₀, semi-log plots) for extracts of dilutions of tammar plasma in the progesterone using radioimmunoassay (antiserum No. 230 (1:7,000)). Increasing volumes of plasma (100 - 500 μl, in duplicate) were tested and compared against the buffer standard curve (x-x).

Key to plasmas from tammars;

- Lactating female
- Ovariectomized female
- Ovariectomized/adrenalectomized female

Females in late pregnancy
FIG. 12.

Dilutions of plasma (ul/tube)

PERCENT B/B°

HORMONE CONCENTRATION (pg/tube)
Fig. 13. Effect of extraction on inhibition curves for progesterone standards prepared in buffer; non-extracted buffer standards (x-x); extracted buffer standards (o-o). Results presented as percent B/B₀; semi-log plot; antiserum No. 334 (1:12,600).
FIG. 13.

HORMONE CONCENTRATION (pg/tube) vs. PERCENT B/BO
Fig. 14. Inhibition curves for progesterone standards in the presence of buffer (x-x) and different tammar plasmas using antiserum No. 334 (1:12,600). Results are presented as percent B/B₀ (semi-log plot).

Key to tammar plasmas:

Δ-Δ Steroid-free plasma
●-● Plasma from an ovariectomized tammar
○-○ Plasma from an adrenalectomized/ovariectomized tammar
FIG. 14.

PERCENT B/B₀ vs HORMONE CONCENTRATION (pg/tube)
Fig. 15. Recovery of progesterone added to tammar plasma. Plot of progesterone measured versus progesterone added to plasma.
FIG. 15.

\[ y = 1.01x + 64.35 \]
\[ r = 0.991 \]
Fig. 16. Dose response curves (percent B/B₀, semi-log plots) for extracts of dilutions of possum, tammar and eastern grey kangaroo using plasma in the progesterone radioimmunoassay antiserum No. 334 (1:12,600). Increasing volumes of plasma (10 - 200 ul, in duplicate) from an anoestrous female possum (□-□) and a possum in the late luteal phase of an oestrous cycle (○-○) were compared with the buffer standard curve (x-x). For plasma from tammars, increasing volumes of 200 - 500 ul in duplicate from an adrenalectomized/ovariectomized female (△-△), an ovariectomized female (▼-▼) and a female in late pregnancy (■-■) were tested and compared with the buffer standard curve (x-x).

For the eastern grey kangaroo increasing volumes of plasma from a lactating female (★-★) and a female in late pregnancy (▲-▲) were tested and compared with the buffer curve (x-x).

Standard curves for progesterone were prepared in assay buffer (x-x) and in steroid-free possum plasma (○-○).
FIG. 16.

Dilutions of plasma (ul/tube)

HORMONE CONCENTRATION (pg/tube)

PERCENT B/Bo
Fig. 17. Dose response curves (percent B/B₀, semi-log plots) for dilutions of native cat plasma in the progesterone radioimmunoassay (antiserum No. 334, 1:12,600). Increasing volumes of plasma (25 - 200 ul) from a male native cat (Δ-Δ) and a female native cat 15 days post-coitum (Δ-Δ) were tested, and compared with the buffer standard curve (x-x). Standard curves for progesterone were prepared in assay buffer (x-x) and in steroid-free native cat plasma (o-o).
FIG. 17.

Dilutions of plasma (ul/tube)

PERCENT B/Bo

HORMONE CONCENTRATION (pg/tube)
Fig. 18. Separation of iodinated prolactin and free iodine on a 0.7 x 15 cm column of Sephadex G25.
FIG. 18.

Iodinated prolactin

Free iodine

COUNTS PER MINUTE x 1000/10UL FRACTION

VOLUME ELUTED (ml)
Fig. 19. Elution profile of purification of $^{125}$I-ovine prolactin on a 1.5 x 40 cm column of Sephadex G100.
FIG. 19.

COUNTS PER MINUTE x 1000/20uI FRACTION

VOLUME ELUTED (ml)
Fig. 20. Flow chart for iodination and tracer purification of prolactin.
FIG. 20.

Iodination Reaction

5 ug prolactin (NIH-P-S12)
+ 10 ul 0.5 M sodium phosphate buffer, pH7.4
+ 0.5 mCi Na\(^{125}\)Iodine (5 ul)
+ 4 ug (10 ul) lactoperoxidase
+ 10 ul 0.088 mM \(H_2O_2\)

React 45 seconds

+ 500 ul 0.05 M sodium phosphate pH7.4

Rinse with 100 ul buffer

Sephadex G25 column

Collect 0.5 ml fractions into 0.1 ml 5% BSA

Count 10 ul subsamples

Free iodine

Pool \(125\)I-prolactin

Sephadex G100

Elute with 0.05 M sodium phosphate buffer pH7.4, plus 0.1% BSA

Flow rate = 1 drop/12 secs

Collect 1 ml fractions

Count 20 ul subsamples

Pool peak fractions

Add 5% BSA

Store frozen
Fig. 21. Titration of donkey second antibody and normal guinea pig serum (NGPS) for the prolactin radioimmunoassay.

(a) Titration of second antibody in the presence of constant dilutions of antiserum 33-9 (1:6,000, working dilution) and NGPS (1:600, working dilution).

(b) Double titration of donkey second antibody and NGPS (•-•, 1:600; x-x, 1:500; o-o, 1:400; △-△, 1:300, working dilutions) in the presence of antiserum 33-9 (1:6,000, working dilution).
FIG. 21.

% RADIOACTIVITY PRECIPITATED

RECIPROCAL OF DILUTION OF SECOND ANTIBODY
Fig. 22. Effect of antiserum dilution (33-9) on the standard curve for ovine prolactin. Final dilutions were 1:42,000 (△-△), 1:36,000 (x-x), 1:30,000 (o-o), and 1:24,000 (o-o). Added $^{125}$I-ovine prolactin was constant at 20,000 cpm per tube. Semi-log plot of percent $B/B_0$ vs Hormone concentration. $B$, amount of $^{125}$I-ovine prolactin bound in the presence of hormone; $B_0$, amount of $^{125}$I-ovine prolactin bound in the absence of unlabelled hormone.
FIG. 22.

HORMONE CONCENTRATION (ng/ml)

PERCENT B/B₀
Fig. 23. Effect of antiserum dilution (33/1-8) on the standard curve for ovine prolactin. Final dilutions were 1:42,000 (Δ-Δ), 1:36,000 (x-x) and 1:30,000 (o-o). Added 125I-ovine prolactin was constant at 20,000 cpm per tube. Semi-log plot of percent B/B₀ vs Hormone concentration.
FIG. 23.

PERCENT B/Bo

HORMONE CONCENTRATION (ng/ml)
Fig. 24. Inhibition curves for prolactin (semi-log plots, percent B/B₀) of dilutions of aqueous homogenates of anterior pituitaries of tammar (▼▼), brush possum (▼▼) and eastern grey kangaroo (■■); and of purified prolactin fractions of western grey kangaroo (WG 13C) (□□), eastern grey kangaroo (EG 13B) (○○) and tammar (74.9(3) Fr 3) (●●); and of purified LH (Ex 241) (▲▲) and FSH (Ex 240D) (▲▲) of tammar; and ovine prolactin (NIH-P-S12) (××) with antiserum 33-9 (1:36,000). Added ¹²⁵I-ovine prolactin was constant at 20,000 cpm per tube.
Dilutions of pituitary homogenates $\times 10^3$

HORMONE CONCENTRATION (ng/ml)

PERCENT B/Bo
Fig. 25. Inhibition curves for prolactin (semi-log plots, percent B/B₀) of dilutions of aqueous homogenates of anterior pituitaries of tammar (▼▼), brush possum (▼▼), native cat (■■) and Dasyuroides byrnei (□□); and of purified wallaby prolactin (74.9(3) Fr 3) (●●); and ovine prolactin (NIH-P-S12) (××) with antiserum 33/1-8 (1:36,000).

Added ¹²⁵Ι-ovine prolactin was constant at 20,000 cpm per tube.
FIG. 25.

Dilutions of pituitary homogenates ($x10^3$)

PERCENT B/B0

HORMONE CONCENTRATION (ng/ml)
Fig. 26. Dose response curves (percent B/B₀, semi-log plots) for dilutions of tammar plasma samples in the prolactin radioimmunoassay, using antiserum 33-9 (1:36,000). Increasing volumes (10 - 200μl) of plasma from a castrate male (○-○), ovariectomized female (▲-▲), lactating female (●-●) and a hypophysectomized female (▼-▼) were tested and compared with the standard curve for ovine prolactin (NIH-P-S12) (x-x).
FIG. 26.

Dilutions of plasma (ul/tube)

10  25  50  100  200

HORMONE CONCENTRATION (ng/ml)
Fig. 27. Dose response curves (percent $B/B_0$, semi-log plots) for dilutions of possum plasma in the prolactin radioimmunoassay, using antiserum 33/1-8 (1:36,000). Increasing volumes (25 - 200 ul) of plasma from an adult male (■-■), a lactating female (○-○), a non-lactating female (▲-▲) and a hypophysectomized female (○-○) were tested and compared with the standard curve for ovine prolactin (NIH-P-S12) (x-x). Dilutions of plasma from a lactating tammar (▼-▼) and a hypophysectomized tammar (□-□) were also tested.
FIG. 27.

Dilutions of plasma (ul/tube)

25  50  200

HORMONE CONCENTRATION (ng/ml)
Fig. 28. Recovery of ovine prolactin added to (a) tammar plasma using antiserum 33-9 and (b) possum plasma using antiserum 33/1-8. Plot of prolactin measured versus prolactin added to plasma.
FIG. 28.

(a) 
\[ y = 0.967x + 4.22 \]
\[ r = 0.998 \]

(b) 
\[ y = 0.969x - 0.017 \]
\[ r = 0.996 \]
Fig. 29. Effect of intravenous injection of thyrotrophin releasing hormone (40 ug) on plasma prolactin levels in (a) 2 adult male tammars (X-X, o--o) and (b) 2 adult female (●-●, □-□) and one adult ovariectomized female tammar (▲-▲). Solid arrow indicates time of injection.
FIG. 29.
Fig. 30. Effect of intravenous injection of thyrotrophin releasing hormone (20 ug) on plasma prolactin levels in (a) 2 adult male possums (o--o, ■■■) and (b) 4 adult female possums (x-x, ◆◆◆, □□□, ▲▲▲). Solid arrow indicates time of injection. Dashed horizontal line = sensitivity of assay.
FIG. 30.

(a)  

(b)  

PLASMA PROLACTIN (ng/ml)

TIME AFTER INJECTION (minutes)
Fig. 31. Plasma prolactin (ng/ml, Mean ± SEM) in female tammars treated with bromocriptine and/or saline vehicle in
(a) Lactational quiescence (March); controls (n = 3),
○-○, CB154, 5 mg/kg body weight (n = 5), △-△.
(b) Seasonal quiescence (September); non-lactating females (n = 5 per group). Controls, ●-●; CB154,
5 mg/kg body weight, single injection, △-△; CB154,
5 mg/kg body weight, daily injection for three
days, ○-○.
(c) Seasonal quiescence (November); lactating females
(> 30 weeks) (n = 5) (●-●), and a post-lactating
female (X-X). Saline vehicle was injected on Day 0
and CB154 (5 mg/kg body weight) on Day 1.
FIG. 31.

(a) 

(b) 

(c)
Fig. 32. Plasma prolactin levels (Mean ± SEM) in (a) Lactating tammars after sham hypophysectomy (n = 11) (●●) and after hypophysectomy (n = 6) (x-x) and (b) cycling possums after sham hypophysectomy (n = 3) (●●) and after hypophysectomy (n = 6) (x-x). Solid horizontal line indicates sensitivity of assay.
FIG. 32.

(a) PLASMA PROLACTIN (ng/mL)

(b) DAYS AFTER OPERATION
Fig. 33. Inhibition curves for prolactin (percent B/T, semi-log plots) in the presence of buffer (x-x) and of plasmas from three hypophysectomized female tammars (□-□, ▲-▲, ▼-▼). The final dilution of antiserum 33-9 was 1:36,000.
Fig. 34. Inhibition curves for prolactin (percent B/B₀, semi-log plots) in the presence of buffer (x-x) and of plasmas from three hypophysectomized female tammars (□-□, ▲-▲, ▼-▼). The final dilution of antiserum 33-9 was 1:36,000.
FIG. 34.

HORMONE CONCENTRATION (ng/ml)

PERCENT B/B0
Fig. 35. Inhibition curves for prolactin (percent B/T, semi-log plots) in the presence of buffer (x-x) and of plasmas from 4 hypophysectomized female possums (o-o, △-△, ■-■, •-•). The final dilution of antiserum 33/1-8 was 1:36,000.
FIG. 35.

HORMONE CONCENTRATION (ng/ml)

PERCENT B/T
Fig. 36. Inhibition curves for prolactin (percent $B/B_0$, semi-log plots) in the presence of buffer (x-x) and of plasmas from 4 hypophysectomized female possums (o-o, △-△, ■-■, ●-●). The final dilution of antiserum 33/1-8 was 1:36,000.
FIG. 36.

PERCENT B/B₀

HORMONE CONCENTRATION (ng/ml)
Fig. 37. Effect of intravenous injection of thyrotrophin releasing hormone on plasma prolactin levels in tammars after (a) sham hypophysectomy (n = 5) •-•, ■-■, o-o, x-x, △-△, and (b) hypophysectomy (n = 4) ○-○, x-x, □-□, △-△. Day of treatment was between Days 10 and 14 post-operation.
FIG. 37.

(a) Plasma prolactin (ng/ml) as a function of time after injection.

(b) A different aspect of the same data, showing prolactin levels over time.

TIME AFTER INJECTION (minutes)
Fig. 38. Effect of different second antibodies on standard curves (percent B/T, semi-log plots) for ovine prolactin prepared in the presence and absence of plasma from a hypophysectomized tammar (antiserum 33-9, 1:36,000).

Key to standard curves:

- **x-x** Buffer curve, donkey second antibody
- **o-o** Curve plus plasma, donkey second antibody
- **□-□** Buffer curve, rabbit second antibody
- **△-△** Curve plus plasma, rabbit second antibody
FIG. 38.

![Graph showing percent B/T against hormone concentration (ng/ml).]
Fig. 39. Effect of different second antibodies on standard curves (percent $B/B_0$, semi-log plots) for ovine prolactin prepared in the presence and absence of plasma from a hypophysectomized tammar (antiserum 33-9, 1:36,000).

Key to standard curves;

- $\times-\times$ Buffer curve, donkey second antibody
- $\circ-\circ$ Curve plus plasma, donkey second antibody
- $\square-\square$ Buffer curve, rabbit second antibody
- $\triangle-\triangle$ Curve plus plasma, rabbit second antibody
FIG. 39.

PERCENT B/B₀

HORMONE CONCENTRATION (ng/ml)
Fig. 40. Effect of presence of plasma from a hypophysectomized tammar (○-○) and plasma from an intact tammar (●-●) on the titration of donkey second antibody and NGPS in buffer (×-×) (antiserum 33-9, 1:36,000).
(a) NGPS 1:300; (b) NGPS 1:400; (c) NGPS 1:500; (d) NGPS 1:600.
FIG. 40.

(a) NGPS 1:300

(b) NGPS 1:400

(c) NGPS 1:500

(d) NGPS 1:600

PERCENT B/T

RECIPROCAL OF DILUTION OF SECOND ANTIBODY

In this figure, changes in the reciprocal of dilution of second antibody were measured throughout the estrus cycle in the native cat. Changes in plasma luteal cytochrome content were established for the three species, each of which show a different pattern of production, may reflect differences in the production of their respective estrous cycles.
CHAPTER 4.

ASPECTS OF LUTEAL FUNCTION IN THE TAMMAR, POSSUM AND NATIVE CAT

4.1 INTRODUCTION

In this chapter the function of the CL during the breeding season of the tammar, possum and native cat has been examined. Using the radioimmunoassays for progesterone and prolactin, changes in the plasma concentrations of both hormones during pregnancy and the oestrous cycle, and subsequently their precise relationships at parturition, post-partum oestrus and oestrus were established for the tammar. In the possum, changes in both hormones were measured throughout both cycles, while in the native cat, plasma progesterone profiles during the breeding season are presented.

A comparison of the profiles from the three species, each of which shows a different pattern of reproduction, may reflect differences in the function of their CL. Within species comparison will enable examination of the hypothesis that the oestrous cycle and pregnancy are hormonally equivalent in marsupials (Sharman, 1970).
4.2 THE TAMMAR

4.2.1 THE PROFILES OF PLASMA PROGESTERONE AND PROLACTIN IN THE PREGNANT AND NON-PREGNANT TAMMAR

In the first experiment the profiles of plasma progesterone during delayed and non-delayed pregnancies and oestrous cycles have been determined so that the following hypotheses could be examined; (1) that pregnancy influences either the secretory activity of the CL, as suggested by Lemon (1972), or the life span of the CL, or both, and (2) that a peak of plasma progesterone occurs in the first week of pregnancy but not at this time in the oestrous cycle, as suggested for the quokka (Cake et al., 1980).

In a second experiment the profiles of plasma prolactin were determined during a delayed oestrous cycle which was followed by a pregnancy.

(1) Plasma progesterone profiles

Experimental design. Adult animals were taken from the breeding colony at the beginning of the breeding season in February and 2 groups set up according to the design used by Merchant (1979). The females of the first group (Group A) were each to be followed through a pregnancy and an oestrous cycle that had been delayed by lactation, while the animals in the second group (Group B) were to be followed through an oestrous cycle and a subsequent pregnancy, both to be uninterrupted by lactation (Fig. 41).

Detection of oestrus depended on the observation of a copulatory plug which was visible in the urogenital sinus of most females after mating. Vasectomised males were substituted for intact males at appropriate times (Fig. 41, see below) to aid in the
diagnosis of the day of oestrus but more particularly to establish that copulation *per se* did not influence the hormonal patterns of the female.

**Group A:** At the start these 9 animals were already carrying small pouch young born 26 to 37 days before and all had been associated with intact males at post-partum oestrus and so were presumed to be pregnant with a dormant blastocyst. Their pouch young were removed (Day 0) to initiate resumption of the pregnancy, and before the subsequent post-partum oestrus, vasectomised males were substituted for intact males. Ten days after birth, the pouch young were once again removed to reactivate the non-pregnant cycle and the females were checked for oestrus at the end of this cycle.

**Group B:** These 9 females were also carrying small pouch young at the start but they had been isolated from males at post-partum oestrus and were therefore not pregnant. Their young, approximately 18 days old, were removed to initiate the cycle and they were run with vasectomized males until after their next oestrus, which was the start of a normal oestrous cycle. At the end of this cycle, they were mated to intact males and so began a pregnancy uninterrupted by lactation. The animals that mated at this time were killed between Days 10 and 14 to establish that they were pregnant and to obtain timed embryos for another study.

**Collection of blood samples.** Catheters were inserted in the jugular veins of the females in Group A 3 days before removal of pouch young. Most of these catheters had ceased to flow or were lost by Day 10, and thereafter blood samples were taken from a
lateral tail vein. Because patent catheters could not be maintained in Group A animals, catheters were not inserted in the females in Group B. The days on which blood samples (3 to 5 ml) were taken from each group are shown on Fig. 41. Since 1.2 ml of plasma was required for each assay, steps were taken to prevent progressive anaemia developing; red blood cells were resuspended in sterile sodium citrate buffer and returned to the donor and the number of samples collected in the middle of the cycles was reduced.

Progesterone was measured as described in Chapter 3 and all the samples from each animal were analysed in the same assay to allow internal comparison between a pregnancy and an oestrous cycle.

**Results.**

**Group A: Pregnancy and oestrous cycle delayed by lactation.** Five females gave birth and mated post-partum 26 to 28 days after the pouch young were removed (Table 4). One animal (4780) did not give birth but returned to oestrus on Day 28 and then underwent a second uninterrupted cycle. Birth and/or oestrus were not detected for 2 other animals (4782, 4730) while birth in the ninth animal was delayed and occurred between Days 35 and 38 (Table 4).

The 5 animals that gave birth underwent a second non-pregnant cycle after their pouch young were removed on Day 10 post-partum, and returned to oestrus on Day 30 to 31 (Table 4). The progesterone results for these animals have been analysed together and are considered to represent the normal pattern as all either gave birth or entered oestrus within the expected time as described by Merchant (1979). The results for the remaining 4 animals were not included because for unknown reasons they did not go through a normal
Table 4. Results from Group A females (pregnancy and oestrous cycle delayed by lactation). Intervals (days), from removal of pouch young (RPY) to birth or oestrus, RPY to the progesterone peak, progesterone peak to birth or oestrus, and the maximum level of progesterone (pg/ml) at the peak.

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>PREGNANT CYCLE</th>
<th>NON-PREGNANT CYCLE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days</td>
<td>Progest-</td>
</tr>
<tr>
<td></td>
<td>from</td>
<td>erone</td>
</tr>
<tr>
<td></td>
<td>RPY to</td>
<td>at Peak</td>
</tr>
<tr>
<td>days</td>
<td>(pg/ml)</td>
<td>Birth</td>
</tr>
<tr>
<td>4426</td>
<td>5</td>
<td>413</td>
</tr>
<tr>
<td>4657</td>
<td>6</td>
<td>360</td>
</tr>
<tr>
<td>4665</td>
<td>6</td>
<td>1270</td>
</tr>
<tr>
<td>4680</td>
<td>6</td>
<td>656</td>
</tr>
<tr>
<td>4786</td>
<td>5</td>
<td>341</td>
</tr>
<tr>
<td>Mean</td>
<td>5.6</td>
<td>608</td>
</tr>
<tr>
<td>± SEM</td>
<td>0.3</td>
<td>174.8</td>
</tr>
</tbody>
</table>

4780 | 6 | 468 | 22 | 28a | 8b | 284 | 23 | 31 |
4782 | 5 | 275 | - | - | - | 281 | - | - |
4926 | 6 | 355 | 29 | 35 | - | - | - | - |
4730c | 7 | 367 | - | - | - | - | - | - |

a Oestrus, no birth.
b Interval (days from oestrus to peak).
c Died Day 16 RPY.
pregnant cycle. The progesterone results for these animals are presented in Appendix 1.

The progesterone profiles for the first 5 animals are shown in Fig. 42 and the means are presented in Fig. 43. In all the animals the concentration of progesterone for the first 4 days after removing the pouch young was under 200 pg/ml. There was then a transient peak on either Day 5 or 6 of 608 ± 174.8 pg/ml (Table 4) and a subsequent return to the previous level until Day 10. In the second part of pregnancy plasma progesterone was consistently elevated (>300 pg/ml) until parturition when it returned to the basal level and remained low (<200 pg/ml) during the 10 days of lactation. In the non-pregnant cycle there was a distinct peak of progesterone on Day 6 or Day 7 after removal of the pouch young (i.e. Day 16 or 17 post-partum) which was lower and less variable than that of the pregnant cycle (Table 4). By analysis of variance the differences were statistically significant (t = 3.88) on a one-tailed test P = 0.03, and on a sign test P = 0.03. Not only did the peak occur at a precise time after RPY but the interval to birth or oestrus was equally consistent; 22 and 24 days respectively (Table 4).

Group B: Oestrous cycle and pregnancy, uninterrupted by lactation. At the beginning of the non-delayed oestrous cycle, copulatory plugs from mating with vasectomized males were observed for 7 of the 9 females. Five of these females subsequently mated with intact males at the end of this non-pregnant cycle and pregnancy was confirmed for each animal at autopsy 10 to 14 days later (Table 5).
Table 5. Results from Group B females (oestrous cycle and pregnancy, uninterrupted by lactation). Intervals (days) from initial RPY to oestrus, oestrus to oestrus, oestrus to progesterone peak, peak to next oestrus, and the maximum level of progesterone (pg/ml) at the peak.

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Days from RPY</th>
<th>Days from Oestrus</th>
<th>Progest-erone (pg/ml)</th>
<th>Days from Oestrus Peak to Oestrus</th>
<th>Days from Oestrus to Progesterone Peak</th>
<th>Autopsy at Day 10-14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days</td>
<td>Days</td>
<td></td>
<td>Days</td>
<td>Days</td>
<td>post-coitum</td>
</tr>
<tr>
<td></td>
<td>from Oestrus</td>
<td>to Peak</td>
<td></td>
<td>from Oestrus Peak to Oestrus</td>
<td>from Oestrus to Progesterone Peak</td>
<td></td>
</tr>
<tr>
<td>4419</td>
<td>29</td>
<td>8</td>
<td>517</td>
<td>24</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td>4646</td>
<td>31</td>
<td>8</td>
<td>311</td>
<td>25</td>
<td>33</td>
<td>7</td>
</tr>
<tr>
<td>4710</td>
<td>31</td>
<td>6</td>
<td>263</td>
<td>24</td>
<td>30</td>
<td>6</td>
</tr>
<tr>
<td>4756</td>
<td>33</td>
<td>6</td>
<td>475</td>
<td>25</td>
<td>31</td>
<td>6</td>
</tr>
<tr>
<td>4795</td>
<td>31</td>
<td>7</td>
<td>372</td>
<td>23</td>
<td>30</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>31.0</td>
<td>7.0</td>
<td>399.6</td>
<td>24.2</td>
<td>31.2</td>
<td>7.0</td>
</tr>
<tr>
<td>± SDI</td>
<td>0.63</td>
<td>0.45</td>
<td>48.9</td>
<td>0.4</td>
<td>0.58</td>
<td>0.45</td>
</tr>
</tbody>
</table>

*Delayed non-pregnant cycle
The patterns of plasma progesterone in these 5 animals were very consistent (Figs. 44 and 45), beginning with high levels (>400 pg/ml) at the end of the preceding cycle. Oestrus occurred one or two days after plasma progesterone had fallen to less than 200 pg/ml and remained at this level until Day 6, 7 or 8 (Table 5) when there was a marked elevation for 1 or 2 days, before a return to basal concentrations. The transient nature of this peak is clearly shown in the progesterone profiles for the individual animals (Fig. 44) and, as in the delayed oestrous cycle, the interval from the peak to oestrus (24.2 ± 0.4 days) was consistent (Table 5). Levels increased after Day 10 and remained elevated until the end of the cycle 1 or 2 days before the next oestrus. The pattern of plasma progesterone during the first 10 days of the succeeding pregnancy was very similar to the preceding cycle; the peak occurred on the same day and the maximum progesterone concentration (399 ± 48.7 pg/ml) was the same as in the oestrous cycle (389.6 ± 48.9 pg/ml) (Table 5). Analysis of variance showed no significant difference between the two peaks nor between either peak and the peak of the non-pregnant cycle of Group A females.

For the other 4 females in the Group, even though oestrus was detected in only 2 of these females at the beginning of the oestrous cycle all 4 showed transient peaks in plasma progesterone (Fig. 45, Table 5). Similarly, at the end of this cycle only 1 of the 4 females was observed to enter oestrus, but 3 of the 4 showed another peak of progesterone 31, 30 and 30 days after the previous peak, indicating that they were continuing to cycle (Fig. 46, Table 5).
Experimental design. Early in the breeding season 4 females without new young were separated from males to prevent a post-partum mating. After births occurred the young were removed to initiate a non-pregnant cycle. At the end of this cycle the females entered oestrus, mated with intact males and underwent a pregnancy. Blood samples were collected at 2 to 3 day intervals throughout both cycles by Dr J. Hawkins during 1975. Plasma was stored at -20°C until assayed in February, 1981.

Results. Plasma prolactin levels remained relatively constant between 15 to 35 ng/ml during the oestrous cycle initiated by RPY. No change was observed either when the young were removed, or at the end of the cycle when the females entered oestrus (Fig. 47a). Copulatory plugs were detected for 3 of 4 animals on Day 28 to 30 after RPY. The male was observed chasing the fourth female on Day 27 but no mating was detected. This female neither gave birth nor entered oestrus at any subsequent time. Levels of plasma prolactin in the 3 mated females throughout their succeeding pregnancy were similar to the preceding oestrous cycle. However for the 2 females which successfully gave birth there was a 2 to 3 fold increase either on the day before or on the day on which the young was detected in the pouch (Fig. 47b). After birth prolactin declined to pre-partum concentrations. In the third animal, which died on Day 21, pregnancy was confirmed at autopsy.

Discussion

The results of the first experiment confirm the findings of Merchant (1979) that the interval between removal of pouch young and
post-partum oestrus in pregnancy is shorter than the interval between removal of pouch young and oestrus in non-pregnant females. This difference in cycle length is reflected in the patterns of both plasma progesterone and prolactin; the fall in progesterone preceding oestrus occurred earlier in pregnancy, and the surge in prolactin occurred around parturition or post-partum oestrus but not at oestrus. The differences in hormone patterns between the 2 cycles indicate that some aspect of pregnancy, possibly the conceptus, affects ovarian events either directly, or indirectly via the pituitary, such that the life span of the CL is shortened and the onset of oestrus is hastened. However since blood samples were collected only once a day for progesterone and every 2 to 3 days for prolactin, neither the precise temporal relationships of the hormone changes relative to each other and to birth and oestrus, nor whether there is any evidence that one affects the other, could be resolved.

Accordingly, the aim of the next experiment was to determine, at shorter intervals, the concentrations of progesterone and prolactin in females undergoing successively a pregnancy and an oestrous cycle.

4.2.2. PRECISE TEMPORAL RELATIONSHIPS OF PROGESTERONE AND PROLACTIN AT OESTRUS, PARTURITION AND POST-PARTUM OESTRUS IN THE TAMMAR.

Experimental design

Eight females carrying small pouch young in February and presumed to be carrying a dormant blastocyst in one uterus were selected from the breeding colony. They were housed indoors under artificial lighting conditions of 12 h light : 12 h darkness with
intact or vasectomised males where appropriate.

To initiate the pregnancy, pouch young were removed. Over the period of parturition and post-partum oestrus between Days 24 and 30 after RPY, blood samples of 4 ml were taken from a lateral tail vein at 0800, 1600 and 2400 h. At each sampling time the females were examined for the presence of a new born young in the pouch and for signs of recent copulation. Oestrus was assumed to have occurred during the 8 h preceding the first observation of the copulatory plug.

Seven of the 8 females gave birth. At the post-partum oestrus these 7 females were mated with vasectomised males and so were non-pregnant. The oestrous cycle was initiated by removal of the pouch young 10-14 days after birth. As in the previous cycle, blood samples were collected at 8 hourly intervals over the expected time of oestrus between Days 25 and 33 after RPY.

After collection of the blood and separation of the plasma, separate volumes were taken for each hormone and frozen at -20°C until assayed. All the samples for both cycles of each animal were run in the same assay in order to allow a direct comparison between them.

Results

In the pregnant cycle, 7 of the 8 females gave birth between Days 25 and 28 after RPY (26.1 ± 0.2 days, Mean ± SEM). A post-partum mating occurred between 0 and 16 h after the detection of the neonate in the pouch (26.5 ± 0.2 days after RPY, or 8 ± 2.2 h after birth) (Table 6). The eighth animal did not give birth but mated on Day 30 and so was assumed to have been non-pregnant. In the second, non-pregnant cycle, 5 of the 7 females mated between Days 27 and 32 after RPY. The mean interval from RPY to oestrus for these 5
Table 6. Intervals in days from removal of pouch young (RPY) to birth, post-partum oestrus and the peak of prolactin at the end of the pregnant cycle; and from RPY to oestrus in the non-pregnant cycle for 8 tamarins; and the intervals (hours) between birth and post-partum oestrus and the peak of prolactin in 7 pregnant females.

<table>
<thead>
<tr>
<th>Animal</th>
<th>PREGNANT CYCLE</th>
<th>NON-PREGNANT CYCLE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RPY to Birth</td>
<td>RPY to Post-partum</td>
</tr>
<tr>
<td></td>
<td>(days)</td>
<td>(days)</td>
</tr>
<tr>
<td>4239</td>
<td>25.67</td>
<td>26.0</td>
</tr>
<tr>
<td>4260</td>
<td>26.0</td>
<td>N.D.</td>
</tr>
<tr>
<td>4268</td>
<td>27.33</td>
<td>27.3</td>
</tr>
<tr>
<td>4510</td>
<td>25.67</td>
<td>26.0</td>
</tr>
<tr>
<td>4521</td>
<td>26.0</td>
<td>26.67</td>
</tr>
<tr>
<td>4557</td>
<td>26.3</td>
<td>26.67</td>
</tr>
<tr>
<td>4768</td>
<td>25.67</td>
<td>26.0</td>
</tr>
<tr>
<td>3194</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Mean</td>
<td>26.1</td>
<td>26.5</td>
</tr>
<tr>
<td>± SDM</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

N.D. = event not detected.

* Value not included in mean as uncertain whether cycle was a failed pregnancy.
non-pregnant cycles \((29.6 \pm 0.8 \text{ days, Mean} \pm \text{SEM})\) (Table 6) was significantly longer than in the previous pregnant cycle (paired t-test; \(t_3 = 6.66, P < 0.01\)).

**Progesterone**

**Pregnancy.** Plasma progesterone concentrations were maximal \((\approx 500 \text{ pg/ml})\) prior to parturition. Within 8 h of the young being detected in the pouch these had declined to basal levels of 200 pg/ml or less in 5 females (Fig. 48). In only 2 females was plasma progesterone still elevated when the young was first detected; in one (4239) the neonate had not reached the pouch but was on the fur near the urogenital sinus. The concentration of progesterone was 360 pg/ml at this time and had declined to 120 pg/ml by the next sampling period (Fig. 48). In the other case (4768), although the young was already attached to the teat, progesterone levels were still high (542 pg/ml) and did not decline to less than 200 pg/ml until 16 h later. Post-partum oestrus occurred in all animals immediately progesterone declined to 200 pg/ml, i.e. between 0 and 16 h after birth (mean 8.0 h) (Table 6).

**Oestrous cycle.** Maximum levels of progesterone at the end of the oestrous cycle were not different from the pregnant cycle (Fig. 49). In the absence of parturition, however, plasma progesterone declined gradually to basal concentrations over a period of 24 h or more after Day 28 RPY (Fig. 48). This contrasts sharply with the pregnant cycle (Figs 48 and 49). In addition, oestrus did not occur until 16 h or more after levels had fallen to 200 pg/ml.
Prolactin

Pregnancy. A transient but very high peak of plasma prolactin (50 ng/ml - 200 ng/ml) occurred at the end of pregnancy, 25.9 ± 0.2 days after the previous young had been removed (Table 6). The peak occurred suddenly either 8 h before birth or at the time birth was first detected, and levels had returned to basal (20 ng/ml) by 16 h after parturition (Fig. 48). The close coincidence of the fall in progesterone and the pulse of prolactin, preceding birth and post partum oestrus is shown in Fig. 50. The female (3194) which failed to give birth but mated on Day 30 did not show a peak of plasma prolactin (Fig. 48).

Oestrous cycle. No change from basal concentrations of prolactin (10 - 20 ng/ml) was detected at the end of the oestrous cycle for any animal (Fig. 48).

The difference in plasma prolactin between the 2 cycles is illustrated in Fig. 51 in which the levels have been synchronised with the time of post-partum oestrus or oestrus.

Conclusion

The differences in the length of the delayed cycles in pregnant and non-pregnant females reported by Merchant (1979) were again confirmed here and reflect real differences in the profiles of progesterone and prolactin. These differences suggest that the conceptus is involved in the onset of parturition.

4.2.3. DISCUSSION

The results confirm the very low levels for plasma progesterone in the tammar reported by Lemon (1972) using a
competitive protein binding technique, and are lower than preliminary values by radioimmunoassay reported by Renfree & Heap (1977) for this species. The basal and peak concentrations are the lowest so far reported for any marsupial although recently Walker & Gemmell (1983) reported similar levels in Bennett's wallaby *Macropus rufogriseus rufogriseus* during pregnancy. The two other monovular marsupial species so far studied have peak levels of progesterone of 4 ng/ml (*Trichosurus vulpecula* - Thorburn *et al.*, 1971; Shorey and Hughes, 1973a) and 2.5 ng/ml (*Setonix brachyurus* - Cake *et al.*, 1980), whereas the peak values in two polyovular species are considerably higher, being 16 ng/ml in *Didelphis virginiana* (Harder and Fleming, 1981) and 20 ng/ml in *Isoodon macrourus* (Gemmell, 1979, 1981). However, in none of these other species has the peak level of progesterone in pregnant animals been found to differ significantly from that in non-pregnant animals, and the results presented here do not support Lemon's (1972) conclusion that there is a difference in the maximum level of progestins at the end of the cycles in the tammar. Indeed, in this study, comparison of the levels on Day 25 after RPY of pregnancy (471.8 ± 26.2, Mean ± SEM, n = 12) with levels on Day 25 after RPY of the oestrous cycle (445.4 ± 42.0, n = 7) showed that they were not significantly different (unpaired t-test, P < 0.15). Lemon (1970) thought that 17α-OH progesterone might be contributing to the values she obtained in pregnant females whereas my antisera, having minimal cross-reaction with this steroid (Table 1), would not have detected this. However, as mentioned earlier, assay of 17α-OH progesterone in chromatographic fractions of plasma extracts using a radioimmunoassay specific for 17α-OH progesterone did not detect this steroid in plasmas collected in late pregnancy from the tammar. Furthermore, Renfree and Heap (1977) identified
progesterone as the major progestin in the tammar and in vitro studies of luteal steroid metabolism indicate that there is less than 1% conversion of available substrate to steroids other than progesterone (Renfree et al., 1983). Since ovariectomy or ablation of the CL in pregnancy reduces plasma progesterone to less than 200 pg/ml, Lemon (1972) concluded that the CL, and not an extra-ovarian source, produces the progesterone in late pregnancy. Subsequent studies have supported her conclusion since, although the placenta and endometrium have the capacity to synthesise progesterone, the rate of conversion of precursors is less than 2% (tammar - Renfree and Heap, 1977; Heap, Renfree and Burton, 1980; quokka - Bradshaw, McDonald, Hähnel and Heller, 1975).

The pattern of plasma progesterone in the cycles in the tammar is similar to that reported by Lemon (1972), except for the presence of the early transient peak. Lemon (1972) did not detect the peak for two reasons; the infrequency of sampling (every third day) and the pooling of some samples for chromatography before assay (Lemon, 1970). Since, in any one animal, the peak occurs on either Day 5 or 6 it is not surprising that it was missed, although progesterone levels were variable in the first half of the cycle; some of the highest values occurred on Day 5 and 7 after RPY. The results of the present study have amply confirmed the phenomenon of a progesterone surge in the first week of the cycle, as reported during early pregnancy in the quokka by Cake et al. (1980), and have further shown that it is inhibited during lactation. However, the results do not support their conclusion that it is exclusively associated with pregnancy. In the tammar the surge cannot be due to the presence of the embryo, and it is not specifically associated with the resumption of CL activity after lactation-controlled delay
since it occurred in females undergoing an oestrous cycle and pregnancy uninterrupted by lactation. Notwithstanding that the peak occurred in both cycles of all the females, the level in the delayed pregnancy (Group A) was just significantly higher than in the delayed oestrous cycle, which appears to support the thesis of Cake et al. (1980). However one factor that was not controlled, either in Group A or in their experiment, may have caused this difference. The pouch young which were removed to initiate pregnancy were already about one month old and so therefore were the CL, whereas in the second non-pregnant cycle the pouch young were removed at Day 10, so that the CL at reactivation were younger. Nevertheless, since the peak of progesterone occurred in all cycles examined, I conclude that there is no basis for the hypothesis that maternal recognition of early pregnancy occurs in the tammar. Since the reproductive cycle of the quokka is very similar it seems unlikely to be true for that species either.

In the study by Cake et al. (1980), only three animals were sampled and they were not the same animals for each cycle so the evidence for the thesis even in the quokka is not strong. Wallace, Green and Renfree (1983) have subsequently reported that a significantly higher progesterone concentration occurs in both pregnant and non-pregnant quokkas killed on Day 3 after RPY compared to those killed on either Days 0 or 6 after RPY. However their study, designed and completed before the work of Cake et al. (1980) was published, cannot resolve the other question - is the magnitude of the peak significantly different in the two cycles? Recently, Walker and Gemmell (1983) reported a peak of progesterone in the early stages of pregnancy initiated by RPY in Bennett's wallaby, but they did not determine whether it also occurs in the oestrous
cycle. The rise in progesterone early in the cycle has not been reported for any non-macropodid species (possum, opossum, bandicoot, native cat), but is present in all macropodid species examined to date. Thus it is probably a feature of the macropodid pattern of reproduction, particularly in those species which show embryonic diapause.

Clearly, in the tammar, the pulse is a normal concomitant of CL development and it occurs just before the onset of luteal cell hypertrophy on Day 8 post-oestrus or Day 7 after RPY (Tyndale-Biscoe, 1979). It appears that the pulse does not reflect a change in stored progesterone in the CL since Renfree et al. (1979) and Hinds, Evans and Tyndale-Biscoe (1983) observed no differences in either concentration or total content of progesterone in quiescent CL and those obtained from tammars on Day 5 of delayed pregnancy. This suggests that the pulse may be caused by a transient change in the rate of secretion of progesterone on Day 5 or 6, which is in agreement with in vitro incubation studies (Hinds et al., 1983).

It is unclear what the function of this transient peak may be but since it occurs in both the quokka and the tammar during the brief period when lutectomy or ovariectomy causes embryo development to fail (Tyndale-Biscoe, 1963a; Sharman and Berger, 1969; Tyndale-Biscoe, 1970; Young and Renfree, 1979), it would seem to be important. In the quokka, development of the blastocyst and CL resumes simultaneously 2 days after RPY although the luteal phase in the uterus does not develop until Day 7 (Tyndale-Biscoe, 1963a). In addition, normal embryonic development can be induced in ovariectomised females and in females with quiescent CL by injections of progesterone (10 mg/kg) (quokka - Tyndale-Biscoe, 1963a; red kangaroo - Clark, 1968), although lower doses lead to
embryonic death (quokka - Tyndale-Biscoe, 1963a; tammar - Berger and Sharman, 1969). These findings led to the proposal that the embryo is directly stimulated by the secretions of the CL (Tyndale-Biscoe, 1963c; Clark, 1968) but it appeared to be refuted when Tyndale-Biscoe (1970) found that quiescent blastocysts reactivated after transfer to the uteri of primed recipients ovariectomised on Day 8. Only 1 of 5 transferred blastocysts reached an advanced stage of development so, either the conditions of the luteal uterus were not adequate to maintain growth, or, it may be that for development to continue successfully past the unilaminar blastocyst stage the embryo must briefly experience a high concentration of progesterone.

Since the interval from the progesterone peak to birth and/or oestrus (22 and 24 days respectively) is relatively constant the peak may synchronise the remaining events of the cycle. In tammars, artificial reduction of the daylength by 3 h during seasonal quiescence induced reactivation of the CL and blastocyst, and births occurred 29-36 days later (Sadleir and Tyndale-Biscoe, 1977). A peak of plasma progesterone occurred 8-14 days after the change in daylength but the interval from the peak to birth/oestrus was still 22/24 days (Hinds and den Ottolander, 1983). Thus the longer interval to birth after photoperiod change was not due to a lengthening of the events of embryogenesis but to an initial delay before development resumed; presumably this is the time taken to transform the photoperiod signal into an endocrine response. Therefore the interval from the peak to birth/oestrus is fixed and it appears that the blastocyst must await the brief increase in progesterone secretion from the CL before it can resume development.
These experiments have also amply confirmed the observation of Merchant (1979) that delayed pregnancy is shorter than the delayed oestrous cycle in the tammar. Moreover, the difference reflects real dissimilarities in the profiles of progesterone and prolactin, as well as LH and prostaglandin F₂α (Tyndale-Biscoe, Hinds, Horn and Jenkin, 1983), and so the hypothesis (Sharman, 1970) that the two cycles are hormonally equivalent is not supported for this macropod; thus, pregnancy shortens the duration of progesterone secretion from the CL, although it does not influence the maximum concentration, there is a peak of prolactin and prostaglandin before and at parturition respectively which do not occur at oestrus, while post-partum oestrus and the LH surge occur earlier (Tyndale-Biscoe et al., 1983). These differences indicate that the conceptus is not only involved in the onset of parturition but also in the timing of post-partum oestrus and ovulation.

A role for the foetus in parturition has not been shown for any marsupial although foetal corticosteroids are involved in the initiation of parturition in the domestic eutherians (Thorburn et al., 1977; Thorburn and Challis, 1979). Nevertheless, by Day 24 3β-steroid dehydrogenase activity is present in tammar foetal adrenal tissue and significant concentrations of corticosteroids are present in foetal plasma (Call, Catling and Janssens, 1980; Catling and Vinson, 1976). Thus there may be a causal relationship between the onset of foetal adrenal activity and the marked changes in plasma prolactin and progesterone. However, any suggestion that either foetal corticosteroids are involved in the events leading to birth in marsupials, or that the mechanisms of parturition are similar to the domestic eutherians is speculative.
The close coincidence of the fall in plasma progesterone and the pulse of prolactin before birth implicates prolactin as a luteolytic agent as it is in the rat (Wuttke and Meites, 1971) and mouse (Grandison and Meites, 1972) at the end of the cycle. However since the peak is absent at the end of the oestrous cycle such a role may be unlikely in the tammar. The fact that it occurs only at parturition strongly suggests that the conceptus is involved in its initiation. However, irrespective of the cause or effect of the pulse of prolactin the pituitary must be important for parturition since tammars hypophysectomised in mid-pregnancy fail to deliver term foetuses (Hearn, 1974).

The precipitate decline in progesterone at parturition appears to have the secondary effect of inducing oestrus, as this behaviour occurred at about the same time after the decline of progesterone in both pregnant and non-pregnant animals. In other mammals a decline in the ratio of progesterone to oestrogen is an effective stimulant for oestrous behaviour. In the tammar a peak of oestrogen occurs after birth (J.D. Harder, pers. comm.), and this change in the ratio may ensure that oestrus and the LH surge occur at a precise time post-partum. Therefore, because the endocrine changes associated with parturition may provide the preconditions for oestrus and ovulation, the foetus may exert an influence on these as well by a cascade effect. Previous experiments with hybrids between eastern and western grey kangaroos (Kirsch and Poole, 1972; Poole, 1975) had shown that foetal genotype is important in the determination of gestation length but, because oestrus is suppressed by lactation in these species (Clarke and Poole, 1967; Poole and Catling, 1974), the foetus was not implicated in the control of oestrus and ovulation. However, there
is no evidence at present in non-macropodid marsupials that the foetus determines the time of parturition or influences the time of oestrus or ovulation.

In several eutherian species, high concentrations of progesterone inhibit lactogenesis but do not affect established lactation, and there is increasing evidence that the withdrawal of progesterone at parturition triggers the onset of lactogenesis (Kuhn, 1977). In the tammar, lactose concentrations increase abruptly within 1 day of parturition. Similarly, a significant increase in lactose concentrations occurs within 24 h of lutectomy on Day 18 of pregnancy (Findlay, Ward and Renfree, 1983) suggesting that progesterone withdrawal may trigger the onset of lactogenesis in this species also. However, in tammars in which elevated progesterone levels were maintained artificially at the end of pregnancy, births occurred during treatment and lactose concentrations in the mammary glands were the same as in the controls. Only in those animals which retained the foetus in the uterus did lactose concentrations remain low (Findlay et al., 1983). Thus, other changes occurring at or before parturition, which may be initiated either by the foetus or the mother, can trigger lactose synthesis even when plasma progesterone levels are maintained at artificially high concentrations - one of these changes may be the surge in plasma prolactin. In the bandicoot, other factors must be involved also as withdrawal of progesterone is not necessary for either parturition or the establishment of lactation (Gemmell, 1979, 1981). Nevertheless in many marsupials pregnancy itself is not necessary for the establishment of lactation. Normal growth and development of neonates transferred to a gland of a non-pregnant female at a corresponding post-oestrus
stage of development has been documented for several species of marsupial (possum - Sharman, 1962; red kangaroo - Sharman and Calaby, 1964; Merchant and Sharman, 1966; tammar - Tyndale-Biscoe et al., 1984). Since lactose concentrations increase in the non-pregnant female at oestrus (Findlay et al., 1983), at the end of pregnancy the conceptus, by initiating the prolactin pulse may ensure the abrupt demise of the CL and thereby synchronise the early onset of lactose synthesis with its birth.

4.3 THE POSSUM

4.3.1 PLASMA PROGESTERONE AND PROLACTIN DURING THE OESTROUS CYCLE AND PREGNANCY IN THE FEMALE POSSUM, TRICHOSURUS VULPECULA

Monitoring of cycles and collection of samples

Cycles were monitored in 3 breeding seasons; April to June, 1981, November, 1981 and April to June, 1982. At the beginning of each major breeding season adult females, ranging in body weight from 1.7 to 2.8 kg, were trapped and the first born young removed to initiate a return to oestrus. Pilton and Sharman (1962) found that the interval from RPY to oestrus was 8.02 ± 0.18 days (n = 61). Oestrus was determined in the first instance by the presence of a flush of leucocytes in the vaginal smear (Day 1) (Pilton and Sharman, 1962), and later was confirmed by the presence of a preovulatory surge of LH (Day 0) (Horn, 1981). Copulatory plugs were recorded also but proved less reliable as an indicator of oestrus since they were difficult to distinguish from the increase in vaginal secretions at this time (Hughes and Rodger, 1971). However, mating was confirmed if either prostatic bodies and/or
sperm were present in the vaginal smear.

Irrespective of the detection of oestrus, blood samples (2.5 ml) were collected either daily or on alternate days from a lateral tail vein in May of the first year, and thereafter from jugular catheters or tail veins until birth or oestrus occurred.

In the first breeding season five females were housed with 3 intact males. At the end of the first cycle one female gave birth on Day 17, while 3 of the remaining 4 returned to oestrus on Days 26, 27 and 30. Samples were not taken during the next cycle as body weights and the haematocrit readings in some animals were declining. By late October, 4 of the 5 females had a pouch young, and these were removed to initiate another cycle. On the day of removal of pouch young, jugular catheters were inserted and daily blood samples collected for approximately 30 days from all animals. Oestrus was detected in only one of these females and she subsequently gave birth on Day 18.

In April, 1982, 4 males were trapped and 2 of these were vasectomised. In May, cycling was initiated in 6 females by removal of pouch young, and they were held with vasectomised males. Therefore the 4 females which returned to oestrus were known to be non-pregnant. Intact males were then substituted. At the end of this non-pregnant cycle, 2 of the 4 females returned to oestrus, and one subsequently went through a pregnancy; birth occurred on Day 18. The second female died 6 days after this oestrus and was found to be non-pregnant at autopsy. Blood samples (2.5 ml) were collected from jugular catheters inserted 2 days after removal of pouch young. The catheters remained patent for 1 to 4 weeks and during this time sterile resuspended red blood cells were returned to the vascular system each day to prevent anaemia developing.
Results

A total of 8 oestrous cycles and 3 pregnancies were followed during this study. Of these, 9 cycles were timed from the pre-ovulatory LH surge (Day 0) and 2 from the flush of leucocytes in the vaginal smears (Day 1). In 2 possums (6058, 6039) an oestrous cycle and a pregnancy were obtained, but in only one case (6058) were these successive cycles.

Plasma progesterone concentrations

Oestrous cycles. In the first breeding season, four females were sampled after oestrus but none gave birth at the expected time between Days 16 to 26, although 3 returned to oestrus on Days 26, 27 and 30 (Table 7). Thus it was assumed that they had undergone infertile oestrous cycles (Fig. 52a and b). In the second year plasma progesterone profiles for 4 females known to be non-pregnant were obtained (Fig. 53a and b). The variance in the concentration of progesterone increased as the levels increased so the data was transformed to natural logarithms to make it approximate a log normal distribution before a Student's t-test was applied. Comparison of the mean profiles for the two seasons showed no significant differences between levels on Days 6, 8, 10, 18 and 20. All profiles were unimodal. Levels remained below 1 ng/ml until 4 days after oestrus, reached 2 ng/ml by Day 9 and then increased markedly between Days 9 to 12 to reach maximum concentrations (5.5 to 15 ng/ml) between Days 12 and 16 (Table 7, Figs. 52 and 53). Concentrations remained high for 1 to 3 days and then declined to less than 2 ng/ml between Days 16 and 22.

The first increase in progesterone above 2 ng/ml occurred consistently after Day 8, and was more predictable than (i) the
Table 7. Temporal changes in plasma progesterone during the pregnant and non-pregnant cycle in the possum.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Days from RPY to oestrus</th>
<th>Days from oestrus to progestogen &gt; 2 ng/ml</th>
<th>Day of max progestogen concentration (ng/ml)</th>
<th>Maximum progestogen concentration (ng/ml)</th>
<th>Days from oestrus to progestogen &lt; 2 ng/ml</th>
<th>Days from oestrus to progestogen &gt; 2 ng/ml</th>
<th>Length of luteal phase</th>
<th>Length of follicular phase</th>
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<tbody>
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<td>Non-pregnant cycle</td>
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<td></td>
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<td></td>
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<tr>
<td>6039</td>
<td>6</td>
<td>9</td>
<td>14</td>
<td>8.0</td>
<td>18</td>
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<td>9</td>
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<td>9.0</td>
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<td>27</td>
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<tr>
<td>6046</td>
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<td>18</td>
<td>-</td>
<td>9</td>
<td>-</td>
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<td>11</td>
<td>14</td>
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<td>25</td>
<td>6</td>
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<td>6</td>
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<td>6070</td>
<td>8</td>
<td>11</td>
<td>16</td>
<td>11.5</td>
<td>21</td>
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<tr>
<td>Pregnant cycle</td>
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<tr>
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<td>4.96</td>
<td>18</td>
<td>-</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
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<td>7</td>
<td>9</td>
<td>14</td>
<td>11.5</td>
<td>20</td>
<td>-</td>
<td>11</td>
<td>-</td>
</tr>
<tr>
<td>6058</td>
<td>-</td>
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<td>12</td>
<td>7.8</td>
<td>18</td>
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<td>Mean</td>
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<tr>
<td>± SEM</td>
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</table>
maximum concentration reached, (ii) the day (12 - 16) this level was reached, (iii) the day (13 - 19) when levels began to decline and (iv) the day (16 - 22) when levels were again less than 2 ng/ml (Table 7). Therefore the period of active progesterone secretion by the CL, defined as the difference in days from the increase to greater than 2 ng/ml and the return to less than 2 ng/ml, is variable, ranging from 6 to 12 days (9.1 ± 0.8, n = 8) (Table 7). The length of the follicular phase, however, may be less variable as 4 of 5 females returned to oestrus 8 days after progesterone had declined to less than 2 ng/ml (Table 7). Conversely, the interval from removal of pouch young to oestrus varied from 6 to 10 days (7.6 ± 0.5 days) (Table 7).

**Pregnancies.** The progesterone profile in pregnancy was also unimodal, and very similar to the oestrous cycle (Fig. 54a and b); concentrations were equally as variable in their magnitude and duration, as well as in the time of increase from, and return to basal levels (Table 7). They remained below 2 ng/ml until at least Day 8, increased to maximum concentrations between Days 12 to 14, then declined to less than 1 ng/ml by Day 20 (Fig 54). For 2 possums the progesterone concentrations were below 1.5 ng/ml on Day 18 when the young was first recorded in the pouch, while in the third animal plasma progesterone was 4 ng/ml on the day of birth (Day 17) and did not decline to less than 1 ng/ml until Day 20.

No significant differences (student's t-test, P>0.2 in all cases) were detected between the 3 pregnant and 8 non-pregnant cycles when mean levels were compared on Days 4, 8, 11, 14, 16, 18, 20 or 26 post-oestrus.
Other cycles. Samples for progesterone were collected from 5 other animals in which a return to oestrus was not detected after removal of pouch young. For 4 females very low to undetectable levels were measured (Appendix II.). Since, in the possum, the major source of progesterone is the CL (Shorey and Hughes, 1975) the low progesterone levels would suggest that follicular growth resulting in ovulation and formation of an active CL had not occurred. In the fifth possum, progesterone levels typical of the luteal phase in other animals were observed, but the first increase above 1 ng/ml occurred more than 26 days after removal of pouch young. Ten days later levels declined to less than 1 ng/ml and there was another delay of about 30 days before levels again increased implying that the length of the oestrous cycle was approximately 40 days in this female (Appendix II.).

Plasma prolactin concentrations. Levels were determined for the 4 oestrous cycles and 2 pregnancies monitored in 1981. Most of the samples collected in 1982 were lost during the assay when the incubation volume coagulated after the addition of the second antibody. This meant that the bound and free phases could not be separated. The same effect occurred when duplicate samples were re-assayed using new buffers and tracer. The clotting was greater during the period that the jugular catheters were patent and the red blood cells were being returned to the circulation than at any other time during the sampling period. Therefore either the return of red blood cells or the catheter itself induced an immune response which interfered with the second antibody reaction in the assay. Whatever the cause of the interference the results obtained were unreliable and so have been excluded.
Oestrous cycles. During 2 of the 4 infertile oestrous cycles monitored in 1981 prolactin levels remained stable at low to undetectable levels, and between 8 to 12 ng/ml respectively. In the third possum a surge of prolactin occurred on the day prior to oestrus (40 ng/ml) and on Day 17 post-oestrus (42 ng/ml), while in the fourth female an increase in prolactin occurred on Day 17 to 18 (26 ng/ml). In this possum levels were also high on Days 20 and 22 post-oestrus (Fig. 55a and b). It must be noted, however, that these females may have undergone either a non-pregnant cycle or a pregnancy that failed as they were associated with intact males at oestrus.

Pregnancy. Two pulses of prolactin occurred during pregnancy in the 2 possums (Fig. 56). In the first possum there was a peak on the day of oestrus (30 ng/ml) and on the day of birth (Day 18, 31 ng/ml), while in the second possum a pulse occurred 2 days before oestrus (22 ng/ml) and one day before birth (16 ng/ml).

Discussion

Except for the greater range in maximum progesterone concentrations in the mid-luteal phase of the cycle, the levels and pattern of plasma progesterone measured in this study are comparable with those reported previously for the possum (Thorburn et al, 1971; Shorey and Hughes, 1973a). There is no transient peak of plasma progesterone in the first week of the cycle as in the tammar (Section 4.2.1.), only a major increase after Day 8 post-oestrus which Shorey and Hughes (1975) showed to be of luteal origin. The unimodal pattern is similar in both the pregnant and non-pregnant states; peak levels of 9.1 ± 0.9 ng/ml (Mean ± SEM, n = 11) which
occurred between Days 12 and 14, and the decline to basal levels between Days 15 to 20 were not significantly different between the two cycles. This implies that pregnancy does not affect the life span of the CL which is in accordance with the previous findings for this species (Pilton and Sharman, 1962; Shorey and Hughes, 1973a, b) and another, the American opossum, Didelphis virginiana, (Harder and Fleming, 1981), but is in sharp contrast to the findings for the tammar wallaby (Section 4.2.2; Tyndale-Biscoe et al, 1983).

Nevertheless there is marked variation both within and between cycles with respect to maximum progesterone concentrations and their duration (i.e. the life-span of the CL), whereas the length of the follicular phase at the end of the cycle is relatively constant. The variations in oestrous cycle length observed in this study (25 to 30 days, n = 5), and by Pilton and Sharman (1962) (22 to 32 days, n = 49), therefore, may be attributed to differences in the active life-span of the CL. However, in the possum with an oestrous cycle length of about 40 days, progesterone levels were increased above basal concentrations for only 10 days implying that in this case either the follicular phase was prolonged, or the animal was entering anoestrous.

The results for plasma prolactin, though limited, indicate that a significant release of prolactin occurs around oestrus and also at the end of the luteal phase in both the pregnant and the non-pregnant (or failed pregnant) cycle. The peak was not detected in all animals sampled on a daily basis and so is probably of relatively short duration (<12 h). Nevertheless the results indicate a similarity between the pregnant and non-pregnant cycles which contrasts with the pattern in the tammar (Section 4.2.2).
The significance of either pulse of prolactin is unknown; at the end of the luteal phase it may be luteolytic as it coincides with the decline in progesterone, but since the samples were infrequent it is not possible to define the temporal relationships of these hormones more closely for the possum.

4.4 THE NATIVE CAT

4.4.1 PLASMA PROGESTERONE IN THE NATIVE CAT, DASYURUS VIVERRINUS

Experimental design

Seven females between 2 and 3 years old, weighing 729 to 969 g, and known to have produced young in the previous breeding season were selected at the beginning of April. Another female, a one year old which was entering her first breeding season, was also included.

Near the end of May the females were checked on alternate days for overt signs of oestrus (oedema of the urogenital sinus, moistening of the pouch and slight enlargement of the pouch edges) (O'Donoghue, 1911; Hill and O'Donoghue, 1913) and observed twice daily for mating. After the first animal entered oestrus and copulated, vaginal smears and blood samples were taken from all of the females 3 times each week. Blood samples of 0.8 to 1.0 ml were collected by heart puncture using a heparinised 1.0 ml syringe and a 12 mm 26 g needle. This method proved efficient, ensured that blood samples were free of contamination and was less distressing for the animal than collection from the femoral or ear vein. Vaginal smears were taken using a hypodermic syringe and a small glass tube by the method of Pilton and Sharman (1962), and were fixed and stained as
described earlier (Chapter 2.5). Smears were assessed for the presence of sperm and prostatic bodies and changes in cell types if mating was not detected. Throughout the cycle the development of the pouch was recorded, and was similar to the description of O'Donoghue (1911).

Daily checks for births were made from Day 15 to 25 p.c. but only one litter was produced. Therefore the initial aim of comparing an oestrous cycle and a pregnancy in several animals could not be achieved.

Results

Matings were recorded for 7 of the 8 females between 26 May and 4 June. The pouch of each female was highly vascularised and glandular between Days 18 and 24 p.c. but neonates were found in only the one year old female (5 young on Day 18) during this time. The remaining animals returned to oestrus 35 to 41 days (37.5 ± 0.96 days, Mean ± SEM, n = 6) after the first mating (Table 8).

The failure to produce young may have been due to the frequency of handling and blood sampling but this was unlikely since only one of several other 2 and 3 year old females in the colony produced a litter in their first cycle of the season. Alternatively it may have been due to a failure to either ovulate and/or conceive, or of embryos to develop to term or of parturition to occur. To investigate this further, 5 of the 7 females were killed at different times after observed mating (Days 7, 10, 15, 17 and 18 p.c.). Another 5 animals which had not been sampled for blood or frequently handled were also killed at various times after mating. Two of the original group of females (B26 and T6) were followed through their second cycle but, as before, no births were
detected and they returned to oestrus on Days 35 and 43 post-oestrus (Table 8). Therefore in their next cycle both females were unilaterally hysterectomised on Day 7 post mating and then autopsied, one on Day 13 (B26) and the second on Day 17 (T6) after mating.

At autopsy the reproductive tract was weighed and examined. The number of CL visible on the ovaries was counted before the left uterus and left ovary were dissected and each weighed. The remainder of the tract was then fixed in formal saline as part of another study. The left uterus was either flushed or everted to recover developing embryos which were measured and the stage of development estimated (Hill, 1910). The pouch area was removed and three mammary glands dissected and weighed. The remaining three glands and pouch skin were fixed in formal saline for another study.

Table 8. Oestrous cycle length in the native cat, Dasyurus viverrinus, defined as the interval (days) from mating to mating.

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>1st cycle</th>
<th>2nd cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>L15</td>
<td>35</td>
<td>-</td>
</tr>
<tr>
<td>B15</td>
<td>35</td>
<td>-</td>
</tr>
<tr>
<td>L19</td>
<td>38</td>
<td>-</td>
</tr>
<tr>
<td>B17</td>
<td>41</td>
<td>-</td>
</tr>
<tr>
<td>B26</td>
<td>39</td>
<td>35</td>
</tr>
<tr>
<td>T 6</td>
<td>37</td>
<td>43</td>
</tr>
<tr>
<td>Mean</td>
<td>37.5</td>
<td>37.9</td>
</tr>
<tr>
<td>± SEM</td>
<td>0.96 (n = 6)</td>
<td>1.1 (n = 8)</td>
</tr>
</tbody>
</table>
**Autopsy results.** Table 9 shows the increase in the weights of the mammary glands, reproductive tract, uteri and ovaries during the first 18 days after oestrus, the number of CL visible on each ovary and the number and stage of development of eggs or embryos recovered from the left uterus. There was an increase in the weight of the mammary glands from Day 7 to 13 p.c., and a four-fold increase in the weight of the reproductive tract between Days 7 and 17 p.c.. This increase was mostly due to an increase in uterine weight. Ovarian tissue weight also increased. On Days 7 and 10 the CL were distinct, pink, vascularised bodies on the surface of the ovaries but were difficult to dissect from the ovary as the tissue was very soft. Between Days 13 and 18 p.c. they were prominent white or yellow bodies and could be dissected easily from surrounding ovarian tissue. The number of CL on each ovary was often dissimilar and the total number ranged from 12 to 30 between animals (Table 9). The number of CL present on any one ovary was never equal to the number of embryos recovered from the corresponding uterus. When the CL were dissected from the ovary to confirm the number actually present very little other ovarian tissue remained; there were no corpora albicantia from the previous cycle, and no growing follicles. Individual CL dissected from the same ovary weighed between 1.5 and 4.0 mg.

Embryos at various stages of development were recovered on Days 7, 10 and 13 p.c., but thereafter, on Days 15, 17 and 18 nothing was recovered. On these days the uterine flushings contained little if any debris. Unfertilised ova were recovered on Day 13 from only one animal (L16). The stage of development of the eggs or embryos recovered from different animals on the same day
Table 9. Results of autopsy of native cats at various times after observed mating.

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Day of Autopsy</th>
<th>Weights of:</th>
<th>Number of Visible Corpora Lutea</th>
<th>Number and Stage of Development of Embryos Recovered from Left Uterus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Three Mammary Glands (mg)</td>
<td>Reproductive Tract (g)</td>
<td>Left Uterus (g)</td>
</tr>
<tr>
<td>L15</td>
<td>7</td>
<td>173.5</td>
<td>7.56</td>
<td>-</td>
</tr>
<tr>
<td>B26a</td>
<td>7</td>
<td>-</td>
<td>-</td>
<td>2.87</td>
</tr>
<tr>
<td>T6b</td>
<td>7</td>
<td>-</td>
<td>-</td>
<td>1.52</td>
</tr>
<tr>
<td>L18</td>
<td>10</td>
<td>185.6</td>
<td>9.96</td>
<td>3.4</td>
</tr>
<tr>
<td>L31*</td>
<td>10</td>
<td>180.0</td>
<td>12.77</td>
<td>3.6</td>
</tr>
<tr>
<td>B26a</td>
<td>13</td>
<td>281.6</td>
<td>-</td>
<td>11.3</td>
</tr>
<tr>
<td>L16*</td>
<td>13</td>
<td>251.0</td>
<td>19.8</td>
<td>10.1</td>
</tr>
<tr>
<td>L19</td>
<td>15</td>
<td>198</td>
<td>28.24</td>
<td>11.3</td>
</tr>
<tr>
<td>B15</td>
<td>15</td>
<td>275</td>
<td>26.6</td>
<td>-</td>
</tr>
<tr>
<td>L22*</td>
<td>15</td>
<td>153</td>
<td>25.0</td>
<td>10.2</td>
</tr>
<tr>
<td>B17</td>
<td>17</td>
<td>167</td>
<td>30.03</td>
<td>11.7</td>
</tr>
<tr>
<td>T6b</td>
<td>17</td>
<td>314</td>
<td>-</td>
<td>8.2</td>
</tr>
<tr>
<td>B14*</td>
<td>18</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L30*</td>
<td>18</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a,b: Animals unilaterally hysterectomised on Day 7 after mating.

* Animals not previously handled, no blood samples taken.

c + = CL present.
after mating were variable, but within animals the stage of each embryo was similar. In two animals degenerate eggs as well as expanding blastocysts were recovered (Table 9).

**Plasma progesterone profiles in the oestrous cycle (or failed pregnancy).** A total of 8 complete cycles (whether non-pregnant or failed pregnant) and 7 incomplete cycles were monitored for 7 animals. Since blood samples were collected only three times each week, irrespective of the time after observed mating, the results for these cycles have been combined and presented as a composite profile synchronised from the day of first mating in each cycle (Fig. 57a). For 2 animals two complete and consecutive cycles were followed and these have been presented as individual profiles to illustrate the similarity in patterns and concentrations from cycle to cycle (Fig. 58).

Plasma progesterone concentrations declined from 1.29 ± 0.2 ng/ml (Mean ± SEM, n = 10) on the day of copulation to less than 0.5 ng/ml 4 to 6 days later, before slowly increasing to 1.5 ng/ml by Day 11. Maximum concentrations (4.5 to 15 ng/ml), reached between Days 11 to 13, were maintained until Day 17. By Day 19 levels had declined to 2.5 ng/ml and were basal (< 1 ng/ml) on Day 21 (Figs 57a and 58). Levels remained low until about 7 days before the next mating, after which there was a small but significant increase (P < 0.01) which was maintained from 5 days before mating until 1 day after mating (Fig. 57b).
Plasma progesterone profile in pregnancy

During the one observed pregnancy the concentrations and changes in plasma progesterone were very similar to those occurring in the oestrous cycles (or failed pregnancies). Levels were low initially but increased markedly after Day 10 p.c. Maximum concentrations of 6 to 7.3 ng/ml were maintained from Day 12 to Day 17 p.c., after which there was a rapid fall to basal levels by Day 19, one day after 5 young were observed in the pouch. Thereafter levels remained low (< 0.5 ng/ml) until sampling ceased on Day 33 after mating (Fig. 59).

Discussion

In the native cat 2 peaks of plasma progesterone were observed during the cycle. A major peak with maximum concentrations ranging from 4.5 to 15 ng/ml occurred between Days 12 and 17 after the first observed mating, and a second small but significant increase occurred before mating. There is no early peak of progesterone as described for the tammar. Except for the pro-oestrous rise, the concentrations and pattern are similar to that described for the possum (Shorey and Hughes, 1973b; Section 4.3) and the opossum (Harder and Fleming, 1981). The source of the pro-oestrous increase in the native cat may be the luteinized follicles (corpus luteum atreticum) described by Sandes (1903). Since ovulation probably occurs about 5 to 6 days after mating (Hill and O'Donoghue, 1913) and the CL takes 3 to 4 days to form and reach its maximum size (Sandes, 1903), it is not surprising that the major increase in plasma progesterone did not occur until after Day 10 when the CL were most prominent on the ovaries. Although only one pregnancy was monitored the close similarity of the progesterone
profile indicates that pregnancy does not affect the life span of the CL as had been suggested by the observations of Sandes (1903). Also since births occur between Days 18 and 22 (J.C. Merchant, pers. comm.) pregnancy occupies only the luteal phase of the cycle and so parturition coincides with the end of the secretory life-span of the CL. Such a pattern is very similar to that described for the possum (Shorey and Hughes, 1973a, b; Section 4.3) and opossum (Harder and Fleming, 1981).

Sandes (1903) and O'Donoghue (1911) showed that after ovulation, the formation of the CL is similar whether or not pregnancy intervenes, but that the CL of pregnancy may persist during the first 6 weeks of lactation (Sandes, 1903). Although O'Donoghue (1911) also thought that the CL of the non-pregnant cycle persisted, in the present study no luteal tissue from the previous cycle (non-pregnant or failed pregnant) was present at autopsy between Days 7 and 18 of the next cycle. In the American opossum, Hartman (1923) also observed that the CL of one cycle were no longer present during the next cycle. Since the workers in the early 1900's believed that the native cat was monoestrus they may have confused the CL of the second cycle with persisting CL. However the persistence of the CL of pregnancy cannot be resolved without further investigation. This study indicates that after parturition the CL no longer actively secrete progesterone, but whether they remain visible on the ovaries or involute is not known. In the bandicoot for example, the CL of pregnancy continue to secrete progesterone for the first nineteen days after parturition (Gemmell, 1979, 1981) but do not involute until after 40 days of lactation (Hughes, 1962; Lyne and Hollis, 1979).
The recovery of different stages of embryos from animals autopsied on the same day after mating supports the conclusion of Hill (1910) that ovulation may occur at varying intervals after the last copulation, but it does not exclude the possibility that development may be arrested during the early stages as has been described for another dasyurid, *A. stuartii* (Selwood, 1980). Nevertheless, if there is no delay in development, when ovulation does occur it must be highly synchronised as all developing embryos recovered from one uterus were always at a very similar developmental stage. The recovery of expanding blastocysts and degenerate eggs from the same uteri in the native cat indicates that embryo wastage occurs during pregnancy as well as at parturition (only 6 teats available for lactation). Embryo wastage also occurs in the Tasmanian devil, *Sarcophilus harrisii*, with more than a third of the cleaving eggs failing to reach the 8-cell stage (Hughes, 1982). Thus in both species the variation in development of the early cleaving eggs may reflect the wastage of embryos rather than either variation in time of ovulation or a delay in development.

The discrepancy between the number of CL and the number of embryos recovered can be attributed to any number of factors. For example, the occurrence of biovular follicles (O'Donoghue, 1911), the presence of follicles with no ovum (O'Donoghue, 1911), the failure of some eggs to be fertilised or a failure to continue development at any stage after fertilisation. In this study development of the embryos did not proceed successfully beyond the vesicle stage in any animal, irrespective of the frequency of handling or blood sampling. The failure of implantation may be a function of the age of the females as only captive one-year old cats successfully produced and raised young. If so, in the older animals
(2 to 3 years) the conditions in the uterus must be inadequate for complete nourishment of the embryo, despite the 4 to 5-fold increase in the weight of the uteri by Days 13 to 15 and the high plasma progesterone concentrations until Day 17.

Interpretation of the findings in this study has been complicated by the failure of most of the females to produce young. Nevertheless it is concluded, tentatively, that the life-span of the native cat CL is not affected by an intervening pregnancy, as in the possum, opossum and bandicoot.

4.5 SUMMARY AND CONCLUSIONS

In this chapter the function of the CL of the tammar, possum and native cat has been examined by following the changes in peripheral progesterone concentrations during pregnancies and oestrous cycles. Changes in plasma prolactin were also monitored in the tammar and possum.

In the tammar, during the oestrous cycle and pregnancy the pattern of plasma progesterone was characterised by an early peak between Days 5 and 8 and was followed by another increase in mid-cycle which was maintained until just before oestrus or parturition. At the end of a delayed pregnancy the decline in progesterone was more rapid and occurred earlier than in the delayed oestrous cycle. Plasma prolactin levels were low during both cycles except for a sharp surge just before parturition. The changes in the two hormones show that the hormonal status is not identical in the two cycles, and indicate that the conceptus and/or the state of pregnancy influences the life-span of the tammar CL.
In the possum the patterns of plasma progesterone and prolactin were the same during pregnancy as in the oestrous cycle. Progesterone levels were low until after Day 8, reached maximum levels by Day 12 and declined to basal after Day 16. There was a surge of plasma prolactin before oestrus and at the end of the luteal phase.

In the native cat, except for a small but significant pro-oestrus rise in plasma progesterone, the pattern and concentrations were similar to that in the possum, and were uninfluenced by an intervening pregnancy. The transient, early peak of plasma progesterone present in the tammar was not observed in either the possum or the native cat.

These studies have shown that the profiles of plasma progesterone in the possum and native cat are similar in both reproductive states. As in the opossum (Harder and Fleming, 1981), pregnancy does not affect the life-span of the CL in the possum and probably the native cat, and so the results support the hormonal equivalence hypothesis of Sharman (1970). Conversely, the results for the tammar do not support the hypothesis. During the delayed pregnancy the life-span of the CL is shortened and there are specific differences in the release of several hormones around parturition. The results provide no evidence for early maternal recognition of pregnancy in the tammar, possum and native cat.
Fig. 41. An outline of the experimental design for Group A and B.

RPY = removal of pouch young to initiate the delayed cycle; ♂ = intact male present at oestrus; ♂ = vasectomised male present at oestrus; ♂ under ordinate = blood sample taken this day.
FIG. 41.

GROUP A

- Lactation
- Delayed pregnant cycle
- Lactation
- Delayed non-pregnant cycle

Seasonal quiescence

Birth and post-partum oestrus

Birth and post-partum oestrus

0 10 20 30 40
Days after removal of pouch young

GROUP B

- Oestrous cycle
- Pregnancy

Seasonal quiescence

Birth and post-partum oestrus

Oestrus

Oestrus

0 10 20 30 10
Days after oestrus

Autopsy
Fig. 42. Plasma progesterone in five individual tammars through a delayed pregnancy, ten days of lactation and the first ten days of a delayed non-pregnant cycle (Group A). RPY = removal of pouch young; B = birth; 0 = oestrus; Closed area = lactation.
FIG. 42.

- Delayed pregnancy
- Lactation
- Delayed oestrous cycle

Tammar No.

4786

4683

4665

4657

4426

Days after RPY

Days after birth

Plasma progesterone (pg/ml)

Days after RPY
Fig. 43. Mean ± SEM plasma progesterone for five tammars which underwent a delayed pregnant cycle after removal of pouch young (RPY), ten days of lactation and the first ten days of a delayed non-pregnant cycle (Group A). For the period of lactation and the second cycle the results were synchronized to the day of birth. Open columns = day of birth and post-partum oestrus; Hatched columns = day of oestrus; Closed area = period of lactation.
FIG. 43.

- Delayed pregnancy
- Lactation
- Delayed oestrous cycle

Plasma progesterone (pg/ml)

Days after RPY

Days after birth

Days after RPY

No. births/oestrous
Fig. 44. Plasma progesterone in five individual tammars which underwent an oestrous cycle and the first ten days of a pregnancy. 0 = oestrus.
FIG. 44.

- Oestrous cycle
- Pregnancy

Tammar No.

Plasma progesterone (pg/ml)

Days after oestrus

<table>
<thead>
<tr>
<th>Tammar No.</th>
<th>Days after oestrus</th>
</tr>
</thead>
<tbody>
<tr>
<td>4795</td>
<td>0</td>
</tr>
<tr>
<td>4756</td>
<td>1</td>
</tr>
<tr>
<td>4710</td>
<td>2</td>
</tr>
<tr>
<td>4646</td>
<td>3</td>
</tr>
<tr>
<td>4419</td>
<td>4</td>
</tr>
</tbody>
</table>
Fig. 45. Mean ± SEM plasma progesterone for five tammars which underwent an oestrous cycle and the first ten days of a pregnancy. The values for the pregnancy were synchronized to the day of oestrus. Hatched columns = day of oestrus.
FIG. 45.

Oestrous cycle

Pregnancy

Days after oestrus

Plasma progesterone (pg/ml)
Fig. 46. Plasma progesterone in four individual tammars in which oestrus was not detected both at the beginning and the end of the oestrous cycle. 0 = oestrus; * = peak of progesterone.
FIG. 46.

![Graph showing oestrous cycle and pregnancy](image)

- Oestrous cycle
- Pregnancy

Days after oestrus

Plasma progesterone (pg/ml)
Fig. 47. Concentrations of plasma prolactin (Mean ± SEM) in four females during (a) an oestrous cycle (n = 4), and (b) a subsequent pregnancy (n = 3) until Day 21, then individual values for 2 females (x--x, x-x), RPY = removal of pouch young; hatched columns = oestrus; open columns = birth.
FIG. 47.

(a) Plasma prolactin (ng/ml)

(b) Days after RPY

No. oestrus

No. births

Days after oestrus
Fig. 48. Profiles of plasma progesterone (●-●) and prolactin (X-X) for individual females which underwent successively a delayed pregnancy and a delayed oestrous cycle. O = oestrus; B = birth.
FIG. 48.

PREGNANCY

OESTROUS CYCLE

Plasma progesterone (pg/ml)

Days after RPY

Plasma prolactin (ng/ml)
FIG. 48. (cont.)

PREGNANCY

OESTROUS CYCLE

Plasma progestrone (pg/ml)

Plasma prolactin (ng/ml)

Days after RPY
FIG. 48. (cont.)

PREGNANCY  OESTROUS CYCLE

Plasma progesterone (pg/ml)  Plasmas prolactin (ng/ml)

Tammar No. 4239

Tammar No. 3194

Days after RPY
Fig. 49. Changes in plasma progesterone (Mean ± SEM) at post-partum oestrus (n = 6) (●-●) and oestrus (n = 6) (○-○). Open columns = births.
FIG. 49.

Post-partum oestrus

Pregnancy

Oestrous cycle

No. births

Plasma progesterone (pg/ml)

Hours after oestrus

Days after RPY
Fig. 50. Changes in Mean ± SEM plasma progesterone (○-○) and prolactin (x-x) for 7 tammars around the time of parturition. Values have been synchronised with the first observation of the young in the pouch. Hatched columns = post-partum oestrus; py = pouch young.
FIG. 50.

Plasma progesterone (pg/ml) vs. Hours after young observed in pouch.

Days after RPY:
-24, 25, 26, 27, 28.

Plasma prolactin (ng/ml) vs. Post-partum oestrus:
-0, 2, 4.

Note: The graph shows the changes in plasma progesterone and prolactin levels after the young are observed in the pouch, with specific days after RPY indicated.
Fig. 51. Changes in plasma prolactin (Mean ± SEM) at post-partum oestrus (n = 6) (•-•) and oestrus (n = 6) (o-o).
FIG. 51.

Plasma prolactin (ng/ml)

Post-partum oestrus

Oestrus

Pregnancy

Oestrous cycle

Days after RPY

Hours after oestrus

24 25 26 27 28 29 30 31 32

0 16 32 48
Fig. 52. Plasma progesterone (ng/ml) in female possums (n = 4) in 1981. At oestrus (Day 0) although the animals mated with intact males none gave birth around Day 17 but 3 returned to oestrus between Days 26 and 30.

(a) Individual profiles (x-x, o-o, o-o, △-△). Arrows indicate oestrus.

(b) Mean ± SEM profile.
FIG. 52.

(a) Plasma progesterone (ng/ml)

(b) Days after oestrus
Fig. 53. Plasma progesterone (ng/ml) in female possums (n = 4) throughout the oestrous cycle in 1982. At oestrus (Day 0) the animals mated with vasectomised males.

(a) Individual profiles (x-x, o-o, o-o, ▲-▲). Arrows indicate oestrus.

(b) Mean ± SEM profile.
FIG. 53.

Plasma progesterone (ng/ml)

Days after oestrus
Fig. 54. Plasma progesterone (ng/ml) in female possums \( (n = 3) \) during pregnancy.

(a) Individual profiles (●-●, ○-○, ×-×). Arrows indicate births.

(b) Mean ± SEM profile. Open columns = births.
FIG. 54.

Plasma progesterone (ng/ml)

Days after oestrus

No. births
Fig. 55. Plasma prolactin (ng/ml) in the same female possums (n = 4) described in Fig. 52. At the first oestrus the females mated with intact males but failed to give birth around Day 17 and 3 returned to oestrus between Days 26 and 30.

(a) Individual profiles (x-x, o-o, o-o, △-△). Arrows indicate day of return to oestrus.

(b) Mean ± SEM profile.
FIG. 55.

(a) Plasma prolactin (ng/ml)

(b) Plasma prolactin (ng/ml)

Days after oestrus
Fig. 56. Plasma prolactin (ng/ml) in 2 possums (X-X, O-O) during pregnancy. Arrows indicate birth.
FIG. 56.

Days after oestrus

Plasma prolactin (ng/ml)
Fig. 57. Plasma progesterone profiles (Mean ± SEM) in native cats.

(a) Composite profile presenting results obtained from 8 complete cycles and 7 incomplete cycles, which have been synchronised from the day of first mating in each cycle. Solid arrows = observed mating and/or sperm in vaginal smear. Dashed arrow = oestrous smear. The number of samples on each day is indicated.

(b) Plasma progesterone levels before and after the day of first mating for all cycles. From 5 days before mating until 1 day after mating levels were significantly higher (P < 0.01) than 7 days before mating. The number of samples on each day is indicated.
FIG. 57.

(a) Plasma progesterone (ng/ml)

Days after mating

(b)
Fig. 58. Plasma progesterone profiles during 2 consecutive cycles for 2 native cats (a) animal T6 and (b) animal B26.

Solid arrow indicates observed mating and/or sperm in vaginal smear. Dashed arrow indicates oestrous smear.
FIG. 58.
Fig. 59. Plasma progesterone profile during pregnancy and early lactation for 1 native cat. O = oestrus, B = birth of 5 young.
CHAPTER 5

THE EFFECT OF HYPOPHYSECTOMY ON LUTEAL FUNCTION
IN THE LACTATING TAMMAR AND CYCLING POSSUM

5.1 INTRODUCTION

The role of the pituitary in the maintenance of pregnancy has been examined in only one marsupial, the tammar (Hearn, 1973, 1974). In those studies, however, no assessment of luteal function was made. Therefore in this chapter the effects of hypophysectomy on luteal function in the tammar were determined by measuring changes in plasma progesterone concentrations before and after treatment.

In the second part of the chapter the effects of hypophysectomy on luteal function and maintenance of pregnancy in the possum are described.

5.2 THE RESPONSE IN PLASMA PROGESTERONE TO HYPOPHYSECTOMY OF THE LACTATING TAMMAR

INTRODUCTION

Hearn (1974) demonstrated that hypophysectomy of lactating females caused loss of pouch young and induced immediate reactivation of the quiescent CL and blastocyst, and at autopsy on Day 20 after operation he found CL and embryos of equivalent size and development to those of intact females on Day 20 after RPY. These results suggested that the CL became autonomous once released
from the pituitary-induced inhibition.

The following experiments were designed to determine (a) whether the occurrence of the transient peak of progesterone on Day 5 or 6 after RPY is dependent upon the presence of the pituitary, and (b) whether plasma progesterone levels in hypophysectomised females differ from levels in intact females undergoing cycles.

In the first experiment survival of the hypophysectomised females was poor and the results obtained, though clear, needed supplementing. In both experiments the sham operation comprised exposure of the pituitary but not removal of the gland by aspiration as was done for the experimental group (Chapter 2.3). During each operation the pouch young was removed from the teat, and reattached after the operation.

EXPERIMENT I

Design

In early lactational quiescence (March) 12 lactating females were taken from the breeding colony and housed indoors in individual cages (0.9 x 0.5 x 0.5 m) under artificial lighting conditions (12 h lightness : 12 h darkness) and at a constant temperature of 20°C. Half of the females were to be hypophysectomised and half sham-operated. The choice of treatment was made randomly after the pituitary had been exposed.

Blood samples were collected on Days 0, 1, 3 to 7, 14 and 21 for later measurement of progesterone by radioimmunoassay. Aliquots were also taken on Days 0, 1, 7, 14 and 21 for determination of prolactin. The effects of hypophysectomy on plasma prolactin (Experiments I and II) are presented in Chapter 3. Each time an animal was handled, body weight and the presence or absence of a
pouch young was recorded. The body weight of the young was recorded on the day of operation and at the time of death/autopsy. Autopsies of the adults were performed at the time of death or on Day 21 post-operation, and the reproductive tract examined. Adrenal and thyroid glands were weighed and the region of the sella turcica fixed for later histological examination.

Results

(a) Histology. Plate 1 shows a diagrammatic transverse section through the head of a tammar wallaby. Histological examination of the region of the sella turcica confirmed that the entire pituitary was removed from 5 of the 6 hypophysectomised animals. In the sixth female only the posterior pituitary had been removed, leaving the anterior pituitary intact. The entire pituitary was intact in the 6 sham-operated females (Table 10, Plates 2, 3).

(b) Effect of hypophysectomy of the females on the growth of their pouch young. The pouch young of the sham-operated females all remained constantly attached to the teat after the operation and their body weight had almost doubled by Day 21 post-operation (Table 11). However out of five young of the fully hypophysectomised females four lost weight, did not remain attached to the teat and died on Days 7 (n = 2), 11 and 12 after operation. The fifth young, the second smallest in the experiment, showed a 45% increase in weight by Day 21. This rate of increase was very much lower than was observed for the smallest young of the sham-operated group (4802) which showed a 135% increase in body weight (Table 11). In the female (4789) in which only the posterior lobe of the pituitary
Table 10. Results of histological examination of the sella turcica region of the tammar after hypophysectomy (hypox) or sham hypophysectomy (sham hypox): Experiment I.

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Treatment at operation</th>
<th>Presence (+) or absence (-) of PLP</th>
<th>Presence (+) or absence (-) of ALP</th>
<th>Actual treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>4358</td>
<td>Sham hypox</td>
<td>+</td>
<td>+</td>
<td>Sham hypox</td>
</tr>
<tr>
<td>5149</td>
<td>&quot;</td>
<td>+</td>
<td>+</td>
<td>&quot;</td>
</tr>
<tr>
<td>4932</td>
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<td>+</td>
<td>+</td>
<td>&quot;</td>
</tr>
<tr>
<td>4975</td>
<td>&quot;</td>
<td>+</td>
<td>+</td>
<td>&quot;</td>
</tr>
<tr>
<td>5002</td>
<td>&quot;</td>
<td>+</td>
<td>+</td>
<td>&quot;</td>
</tr>
<tr>
<td>4802</td>
<td>&quot;</td>
<td>+</td>
<td>+</td>
<td>&quot;</td>
</tr>
<tr>
<td>4689</td>
<td>Hypox</td>
<td>-</td>
<td>-</td>
<td>Hypox</td>
</tr>
<tr>
<td>4969</td>
<td>&quot;</td>
<td>-</td>
<td>-</td>
<td>&quot;</td>
</tr>
<tr>
<td>4979</td>
<td>&quot;</td>
<td>-</td>
<td>-</td>
<td>&quot;</td>
</tr>
<tr>
<td>4946</td>
<td>&quot;</td>
<td>-</td>
<td>-</td>
<td>&quot;</td>
</tr>
<tr>
<td>4564</td>
<td>&quot;</td>
<td>-</td>
<td>+</td>
<td>&quot;</td>
</tr>
<tr>
<td>4789</td>
<td>Attempted Full Hypox</td>
<td>-</td>
<td>+</td>
<td>Posterior Hypox</td>
</tr>
</tbody>
</table>

*a Haemorrhage at anterior end.

* Gross examination at death on Day 7; sella turcica region empty.

PLP = posterior lobe of pituitary.

ALP = anterior lobe of pituitary.
Table 11. Effect of hypophysectomy (hypox) or sham hypophysectomy of lactating tammars on the growth of their pouch young: Experiment I.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Treatment</th>
<th>Body Weight of Pouch Young (g) Day 0</th>
<th>Day 21 or at death</th>
<th>Percent Increase in Body Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4358</td>
<td>Sham Hypox</td>
<td>21.4</td>
<td>38.8</td>
<td>81.3</td>
</tr>
<tr>
<td>5149</td>
<td>&quot;</td>
<td>21.1</td>
<td>36.0</td>
<td>70.6</td>
</tr>
<tr>
<td>4932</td>
<td>&quot;</td>
<td>-</td>
<td>39.2</td>
<td>-</td>
</tr>
<tr>
<td>4975</td>
<td>&quot;</td>
<td>21.8</td>
<td>37.3</td>
<td>71.1</td>
</tr>
<tr>
<td>5002</td>
<td>&quot;</td>
<td>28.9</td>
<td>48.7</td>
<td>68.5</td>
</tr>
<tr>
<td>4802</td>
<td>&quot;</td>
<td>6.58</td>
<td>15.3</td>
<td>132.5</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>20.0</td>
<td>35.9</td>
<td></td>
</tr>
<tr>
<td>± SEM</td>
<td></td>
<td>3.6</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>4689</td>
<td>Hypox</td>
<td>8.0</td>
<td>11.6</td>
<td>45</td>
</tr>
<tr>
<td>4564</td>
<td>&quot;</td>
<td>23.2</td>
<td>20.5 (7)*</td>
<td>-11.6</td>
</tr>
<tr>
<td>4969</td>
<td>&quot;</td>
<td>21.5</td>
<td>13.4 (11)</td>
<td>-37.7</td>
</tr>
<tr>
<td>4979</td>
<td>&quot;</td>
<td>27.5</td>
<td>24.1 (12)</td>
<td>-12.4</td>
</tr>
<tr>
<td>4946</td>
<td>&quot;</td>
<td>23.4</td>
<td>20.3 (7)</td>
<td>-13.2</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>20.7</td>
<td>17.98</td>
<td></td>
</tr>
<tr>
<td>± SEM</td>
<td></td>
<td>3.3</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>4789</td>
<td>Posterior hypox</td>
<td>14.3</td>
<td>27.5</td>
<td>92.3</td>
</tr>
</tbody>
</table>

* ( ) Day pouch young died.
was removed, the growth of its young was similar to that of the young of the sham-operated females (Table 11).

(c) Effect of hypophysectomy of females in lactational quiescence. All sham-operated females survived to Day 21, and generally maintained body weight and appetite (Table 12). At autopsy the weights of the adrenals and thyroids were comparable with those of intact females (Table 12; Hearn, 1975). The reproductive tracts of the sham-operated females were small; there was a quiescent CL present on one ovary, and a dormant blastocyst was recovered from the ipsilateral uterus (Table 12).

Survival of the hypophysectomised females was poor. Two females (4946, 4564) died on Day 7, another (4979) on Day 12, while the two other females (4689, 4969) survived until Day 21 post-operation (Table 12). In this group the weights of the adrenals and thyroids were mostly reduced when compared with the sham-operated group, particularly in the 2 females which survived to Day 21. The exception was the unusually large adrenal weight of female 4564 which died on Day 7. Autopsy of the 3 longest surviving hypophysectomised females showed that their CL had been reactivated as the weight had increased significantly ($P < 0.005$) and they were carrying developing embryos (Table 12). The CL in one female (4564) which died on Day 7 after operation was heavier than in the sham-operated females but no embryo was recovered. In the other female which died on this day there was a small CL on one ovary and a blastocyst was recovered from the uterus (Table 12).

In the animal (4789) in which only the posterior pituitary gland was removed there was a decline in adrenal but not thyroid
Table 12. Weights of the adrenal and thyroid glands, ovaries and corpora lutea (CL), and the stage of embryo development in lactating tamarins autopsied on Day 21 after sham hypophysectomy or hypophysectomy.

<table>
<thead>
<tr>
<th>Animal Body Weight (Kg):</th>
<th>Weights (mg) of Tissues at Autopsy:</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>At Operation</td>
<td>At Autopsy</td>
<td>Adrenals</td>
<td>Thyroids</td>
<td>Ovary</td>
</tr>
<tr>
<td></td>
<td>mg</td>
<td>mg/kg</td>
<td>mg</td>
<td>mg/kg</td>
<td>mg</td>
</tr>
<tr>
<td>Sham Hypophysectomy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4358</td>
<td>4.25</td>
<td>4.45</td>
<td>502.8</td>
<td>113.0</td>
<td>291.0</td>
</tr>
<tr>
<td>5119</td>
<td>4.10</td>
<td>4.05</td>
<td>332.2</td>
<td>94.4</td>
<td>349.2</td>
</tr>
<tr>
<td>4932</td>
<td>4.00</td>
<td>3.85</td>
<td>323.8</td>
<td>84.1</td>
<td>220.6</td>
</tr>
<tr>
<td>4975</td>
<td>4.10</td>
<td>3.25</td>
<td>418.6</td>
<td>128.8</td>
<td>216.6</td>
</tr>
<tr>
<td>5002</td>
<td>3.60</td>
<td>3.55</td>
<td>362.0</td>
<td>99.4</td>
<td>247.0</td>
</tr>
<tr>
<td>4902</td>
<td>3.95</td>
<td>4.25</td>
<td>528.0</td>
<td>124.2</td>
<td>304.9</td>
</tr>
<tr>
<td>Mean</td>
<td>4.00</td>
<td>3.90</td>
<td>418.1</td>
<td>107.3</td>
<td>272.3</td>
</tr>
<tr>
<td>± SEM</td>
<td>0.09</td>
<td>0.18</td>
<td>33.5</td>
<td>7.2</td>
<td>21.6</td>
</tr>
</tbody>
</table>

Intact Females (n = 10)

| Mean                       | 495.8 | 106.2 | 63.0* |
| ± SEM                      | 29.8  | 5.9   | 3.1   |

Hypophysectomy

| Mean                       | 292.0 | 73.0 | 293.2 | 73.3 | 234.0 | 13.0 |

CRL = crown rump length.  CL = gross length.  * Hearn (1975).  ** Weight of ovary and CL.
a Student's t-test was used to compare weights of CL in sham-operated and hypophysectomised females;  P < 0.003.
b,c,d Animals autopsied at time of death on Days 7, 12 and 7 post-operation respectively.
weight, and a quiescent CL and dormant blastocyst were found on Day 21.

(d) **Plasma progesterone profiles.** After sham-hypophysectomy plasma progesterone levels remained at basal concentrations of about 200 pg/ml (Figs. 60 and 62). The low levels support the conclusion drawn at autopsy that there was no resumption of development of the CL in this group. Similarly, in the animal in which only the posterior lobe was removed plasma progesterone levels remained low (Fig. 60).

After hypophysectomy plasma progesterone levels increased in 4 of the 5 animals (Fig. 61); one animal (4946) showed an increase in plasma progesterone from 170 pg/ml on Day 4 to 400 pg/ml by Day 6 and two animals (4689, 4979) showed a rise in plasma progesterone from less than 200 pg/ml on Day 5 to 682 and 625 pg/ml respectively on Day 7 after operation. Similarly, in the fourth female (4969) progesterone levels were beginning to increase by Day 7. In the two females (4969, 4689) which survived until Day 21 the levels of progesterone increased from 200 pg/ml on Day 14 to more than 600 pg/ml on Day 21 (Figs. 61 and 62). The fifth hypophysectomised female died on Day 7 before any changes in plasma progesterone had occurred (Fig. 61).

The results of this experiment indicated that the patterns and concentrations of plasma progesterone after hypophysectomy were similar to females undergoing a pregnancy after RPY. However since the peak of progesterone was expected on Day 5 but did not occur until at least Day 7 the sampling regime was inadequate and did not allow full characterisation of the early peak. Therefore a second experiment was performed.
EXPERIMENT II

Design

In this experiment additional blood samples were taken to characterise the early peak and the animals were killed on Day 14 post-operation. Twelve lactating females were used; six were hypophysectomised and six were sham-operated in April. The schedule for collection of blood samples for progesterone was as follows; Days -1, 0, 1, 3 to 10 and 14. Full autopsies were performed either at the time of death or on Day 14. Another aspect of this experiment, the response to injection of thyrotrophin releasing hormone given between Days 10 and 14 post-operation, has been reported earlier (Chapter 3, Section 3.3.4).

Results

(a) **Histology.** Histological examination of the sella turcica region confirmed full hypophysectomy in 3 animals and the removal of only the anterior pituitary in the fourth animal. The remaining animals in this group died 2 days after operation and no material was kept for analysis. The pituitary was intact in five sham-operated animals (Table 13) (Plates 2, 3 & 4). The sixth animal in this group was put down at the time of operation when severe haemorrhage occurred.

(b) **Effect of hypophysectomy of the females on the growth of their pouch young.** As in Experiment I, a rapid weight loss and death of pouch young between Days 7 to 13 occurred in the females which were hypophysectomised while the young of sham-operated females gained weight, except in one case (5302) in which the mother had severe scours (Table 14).
Table 13. Results of histological examination of the sella turcica region of the tammar after hypophysectomy (hypox) or sham hypophysectomy: Experiment II.

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Treatment</th>
<th>Presence (+) or Absence (-) of PLP</th>
<th>Actual treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>5302</td>
<td>Sham hypox</td>
<td>+</td>
<td>Sham hypox</td>
</tr>
<tr>
<td>4513</td>
<td>&quot; &quot;</td>
<td>+</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>5016</td>
<td>&quot; &quot;</td>
<td>+</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>5634</td>
<td>&quot; &quot;</td>
<td>+</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>5379</td>
<td>&quot; &quot;</td>
<td>+</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>5136</td>
<td>Full hypox</td>
<td>-</td>
<td>Full hypox</td>
</tr>
<tr>
<td>5321</td>
<td>Attempted full hypox</td>
<td>+</td>
<td>Anterior hypox</td>
</tr>
<tr>
<td>5230</td>
<td>Full hypox</td>
<td>-</td>
<td>Full hypox</td>
</tr>
<tr>
<td>4896</td>
<td>&quot; &quot;</td>
<td>-</td>
<td>&quot; &quot;</td>
</tr>
</tbody>
</table>

PLP = posterior lobe of pituitary.

ALP = anterior lobe of pituitary.
Table 14. Effect of hypophysectomy (hypox) of lactating tammars on the body weight of their pouch young: Experiment II.

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Treatment</th>
<th>Body weight of Pouch Young (g): % Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 14 in Body Wt.</td>
</tr>
<tr>
<td>5302(^a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4513</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5016</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5634</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5379</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>35.0</td>
<td></td>
</tr>
<tr>
<td>± SEM</td>
<td>2.3</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Treatment</th>
<th>Body weight of Pouch Young (g): % Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>5136</td>
<td>Full hypox</td>
<td>27.5 (9)**</td>
</tr>
<tr>
<td>5321</td>
<td>Anterior hypox</td>
<td>35.5</td>
</tr>
<tr>
<td>5230</td>
<td>Full hypox</td>
<td>34.0</td>
</tr>
<tr>
<td>4896</td>
<td>Full hypox</td>
<td>34.7</td>
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<tr>
<td>Mean</td>
<td>34.7</td>
<td>24.6</td>
</tr>
<tr>
<td>± SEM</td>
<td>0.4</td>
<td>1.0</td>
</tr>
</tbody>
</table>

\(^a\) Animal 5302 had scours and lost 15.5% body weight.

\(*\) Day young first weighed.

\(**\) Day young died.
(c) **Effect of hypophysectomy of females in lactational quiescence.** As in Experiment I all sham-operated females were carrying quiescent blastocysts and had small CL at autopsy (Table 15). Their adrenal and thyroid weights were not significantly different from intact animals (Table 15).

In the 3 hypophysectomised animals and one adeno-hypophysectomised animal there was a significant decrease in the weights of the adrenals ($P < 0.02$), thyroids ($P < 0.05$) and ovaries (CL removed) ($P < 0.03$) when compared with the respective weights of the control group. At autopsy the weight of the CL had significantly increased ($P < 0.005$) and an expanding vesicle, equivalent to the stage reached 11 to 12 days after RPY (Renfree and Tyndale-Biscoe, 1973), was recovered from each female (Table 15).

(d) **Plasma progesterone profiles.** In sham-operated animals plasma progesterone levels remained at about 200 pg/ml after operation, except for one sample (628 pg/ml) collected on the day of operation in one animal (Fig. 63).

In the hypophysectomised females, including the animal in which only the anterior pituitary was removed, there was a peak in plasma progesterone ($497 \pm 23$ pg/ml, Mean $\pm$ SEM, $n = 4$) on either Day 7 ($n = 2$) or Day 8 ($n = 2$), and levels were increasing again by Day 14 (Fig. 64). Day by day t-tests of mean progesterone concentrations on equivalent days after operation for sham-operated and adeno- or fully hypophysectomised animals revealed that the levels were significantly higher ($P < 0.005$) in the hypophysectomised group on Days 6, 7, 8, 9 and 14 after operation (Fig. 65a and b).
Table 15. Weights of the adrenal and thyroid glands, ovaries and corpora lutea (CL), and stage of embryo development in lactating tannars autopsied on Day 14 after sham hypophysectomy or hypophysectomy.

<table>
<thead>
<tr>
<th>Anim No.</th>
<th>Body Weight (kg)</th>
<th>Weights (mg) at Autopsy:</th>
<th>Embryo Development</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Adrenals</td>
<td>Thyroids</td>
</tr>
<tr>
<td></td>
<td>Operation</td>
<td>At Audit</td>
<td>mg</td>
</tr>
<tr>
<td>Sham hypophysectomy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5302</td>
<td>5.05</td>
<td>3.80</td>
<td>567.0</td>
</tr>
<tr>
<td>4513</td>
<td>4.50</td>
<td>4.50</td>
<td>398.0</td>
</tr>
<tr>
<td>5016</td>
<td>5.20</td>
<td>4.75</td>
<td>547.0</td>
</tr>
<tr>
<td>5634</td>
<td>4.55</td>
<td>3.95</td>
<td>300.0</td>
</tr>
<tr>
<td>5379</td>
<td>4.40</td>
<td>4.30</td>
<td>409.0</td>
</tr>
<tr>
<td>Mean</td>
<td>4.74</td>
<td>4.26</td>
<td>444.2</td>
</tr>
<tr>
<td>SEM</td>
<td>0.16</td>
<td>0.17</td>
<td>49.9</td>
</tr>
<tr>
<td>Intact females (n = 10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>495.8</td>
<td>106.2</td>
<td>63.0</td>
</tr>
<tr>
<td>±SEM</td>
<td>29.8</td>
<td>5.9</td>
<td>3.1</td>
</tr>
<tr>
<td>Hypophysectomy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5136</td>
<td>4.80</td>
<td>4.65</td>
<td>213.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5321*</td>
<td>4.65</td>
<td>3.95</td>
<td>318.0</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5230</td>
<td>4.30</td>
<td>3.90</td>
<td>233.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4896**</td>
<td>4.60</td>
<td>3.85</td>
<td>250.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Mean</td>
<td>4.59</td>
<td>4.08</td>
<td>253.5</td>
</tr>
<tr>
<td>±SEM</td>
<td>0.10</td>
<td>0.19</td>
<td>22.8</td>
</tr>
</tbody>
</table>

*Anterior hypophysectomy.  ** Animal autopsied at time of death on Day 13 post-operation.  Students t-test was used to compare weights of organs in sham-operated and hypophysectomised females 14 days post-operation.  ▶ Hearn(1975).  

a, P < 0.0005;  b, P < 0.013;  c, P < 0.003;  d, P < 0.0005.
DISCUSSION

This study has shown that hypophysectomy induces reactivation of the CL and blastocyst, confirming the earlier findings of Hearn (1973, 1974) and Tyndale-Biscoe and Hawkins (1977). The source of the inhibition is the anterior pituitary since reactivation occurred only in animals which had been fully hypophysectomised, or adeno-hypophysectomised. Reactivation did not occur in the one female from which the posterior lobe was removed which supports the view that oxytocin is not involved in the inhibition (Tyndale-Biscoe and Hawkins, 1977). However, maintenance of lactation, and hence growth of the pouch young, is dependent on the anterior pituitary. The young of the adeno- or fully hypophysectomised females died within 7-13 days with one exception where growth was severely retarded. These findings are similar to those reported by Hearn (1974) who suggested the young died of starvation after lactation ceased 2 days post-operation. Since the young of the posterior hypophysectomised female continued to grow, more than doubling in weight in 21 days, this suggests that oxytocin may not be essential for the maintenance of this stage of lactation. However, the suckled gland in early lactation is very sensitive to low concentrations of oxytocin (Lincoln and Renfree, 1981a,b) and so oxytocin leaking from the median eminence may be sufficient to maintain lactation.

After hypophysectomy the pattern and concentration of plasma progesterone is comparable with that observed in intact females undergoing pregnancy after RPY. The magnitude of the early peak after hypophysectomy (527.1 ± 38.0 pg/ml, Mean ± SEM, n = 7) is not significantly different (Student's t-test, P < 0.4) from the peak (608 ± 174.8 pg/ml, n = 5) occurring after RPY. Although only 2
animals were sampled on Day 21 post-hypophysectomy the increase in progesterone by this time was also similar to levels in intact pregnant animals 21 days after RPY. The results therefore support the view that the CL, once released from the tonic inhibition exerted by the pituitary, becomes autonomous and requires no pituitary luteotrophic support to maintain progesterone production during pregnancy.

At autopsy the weight of CL from hypophysectomised animals was 2 to 3 fold greater than in the sham-operated animals and dormant blastocysts had also resumed development. Precise ageing of these embryos was difficult since wide variation occurs at all stages even in timed pregnancies (Renfree and Tyndale-Biscoe, 1973). However in both experiments the stages reached appeared to be retarded by 1 or 2 days with respect to stages after RPY. This was also reflected in the timing of the early peak of plasma progesterone. In intact females the peak occurs 5 to 6 days after RPY; in this study the peak occurred on Days 7 to 8, 1 to 2 days later than would be expected if the removal of the pituitary gland immediately removed the inhibition. There are two possible reasons for this discrepancy. The first is that the delay may be due to the trauma of the operation which affects many other metabolic functions for the first few days until a new homeostasis is achieved (Hearn, 1975). The second possibility is that reactivation occurs in response to cessation of lactation 2 days post-operation. It is more likely to be operational stress since Tyndale-Biscoe and Hawkins (1977) found that hypophysectomy of non-lactating females in seasonal quiescence also induced reactivation of the CL and blastocyst.
The studies of Tyndale-Biscoe and Hawkins (1977) demonstrated that prolactin is probably the main inhibitory agent produced by the pituitary. However, McDonald and Waring (1979) strongly criticised this hypothesis since in the American opossum Cook and Nalbandov (1968) had found that LH stimulates steroidogenesis in vitro, and in the bandicoot large CL persist during lactation (Hughes, 1962; Gemmell, 1979, 1981). Tyndale-Biscoe and Hawkins (1977) suggested that the sensitivity of the CL to pituitary inhibition may be confined to the macropodid species and the differences mentioned above may be related to the nature of luteal receptors in the different species. Certainly recent studies have supported this view; abundant receptors for prolactin but not LH are present in the luteal tissue of the tammar and the red kangaroo, but in the possum there are abundant LH receptors (Stewart and Tyndale-Biscoe, 1982). Furthermore, in vitro studies have shown that, unlike the opossum, steroidogenesis in the tammar CL is unaffected by the addition of LH or prolactin (Sernia, Hinds and Tyndale-Biscoe, 1980; Hinds et al., 1983). The current study showing that the plasma progesterone concentrations and profiles are the same after reactivation induced by either hypophysectomy or by removal of pouch young also reinforces the view that the quiescent CL is normally under a tonic pituitary inhibition but, after reactivation, it is autonomous.
5.3 THE EFFECT OF HYPOPHYSECTOMY ON LUTEAL FUNCTION IN THE CYCLIC POSSUM

INTRODUCTION

In the tammar the CL is devoid of LH receptors (Stewart and Tyndale-Biscoe, 1982) and it can grow and support pregnancy to term after hypophysectomy (Hearn, 1974; Section 5.2). Since peripheral progesterone levels in hypophysectomised females are similar to those in intact cycling females the tammar CL does not require any luteotrophic support from the pituitary for normal progesterone production (Section 5.1). In contrast in the possum, circulating progesterone levels are 10 to 20 fold higher (Thornburn et al., 1971; Shorey and Hughes, 1973a; Chapter 4) than in the tammar, and so the CL, which has abundant receptors for LH (Stewart and Tyndale-Biscoe, 1982) may be dependent on luteotrophic support to maintain progesterone production.

In the possum ovulation occurs by Day 2 after oestrus. By Day 4 the CL is well differentiated and by Days 7 to 10 it has reached its maximum size (Pilton and Sharman, 1962; Shorey and Hughes, 1975). The six-fold increase in the mass of the CL is accompanied by a three-fold increase in the rate of secretion of progesterone and marked proliferation of the uterine endometrium (Shorey and Hughes, 1973a,b). Removal of the CL before Day 8 post-oestrus inhibits the impending uterine secretory phase (Shorey and Hughes, 1975) which extends from Day 8 to about Day 17 of the cycle (Pilton and Sharman, 1962). However, for embryonic development to continue to term and for parturition to occur the CL cannot be dispensed with before Day 11 (Sharman, 1965). Therefore at least in the first part of the cycle there is considerable dependence on the
secretions of the CL, but it is not known whether the CL itself is autonomous, like the tammar CL, or whether it requires luteotrophic support from the pituitary for growth and progesterone production. The purpose of this study, therefore, was to determine whether the pituitary is necessary for ovulation, formation and growth of the CL, maintenance of progesterone production and/or parturition in the possum.

**EXPERIMENTAL DESIGN**

To examine the effect of hypophysectomy on ovulation, CL formation and growth, and progesterone secretion, the pituitary was removed on different days after oestrus. These were Days 1, 4 and 8 respectively. Removal of the CL after Day 11 does not prevent parturition (Sharman, 1965), and so if hypophysectomy is performed after this time (Day 12) the importance of the pituitary for parturition can be determined.

At the beginning of the experiment full hypophysectomies were to be performed on Days 1, 4, 8 and 12 post-oestrus, and sham operations on Day 1 post-oestrus, using 5 animals per group. Sham operations were not performed on each treatment day as the number of possums available was limited. After the first few operations it was found that post-operative recovery was poor, possibly due to the loss of the posterior lobe, and so in the later operations only the anterior lobe was deliberately removed. Thereafter the rate of survival improved although some animals developed an infection within 2 to 3 days of operation and were killed. These losses meant that the numbers of animals in the Day 8 and 12 groups were reduced in favour of Day 1 and 4 treatments.
Blood samples were collected every second day, beginning on the day of oestrus, and autopsies were performed at the time of death or on Day 18, the day after expected parturition.

RESULTS

(a) Histology. Hypophysectomy or sham hypophysectomy was performed on 20 females, of which 13 survived to autopsy at the end of pregnancy. In 6 of these surviving females full \((n = 4)\) or anterior \((n = 2)\) hypophysectomy was confirmed, while in 4, pieces of adeno-hypophysial tissue were found after histological examination of the sella turcica region. The entire pituitary was intact in the 3 sham-operated females (Table 16; Plates 5 & 6). In 3 of the 4 animals in which hypophysectomy was incomplete the remaining fragments of the anterior pituitary were closely associated with the posterior lobe, which was intact though small (Plate 7). In the fourth animal most of the anterior pituitary was present but it was damaged.

General

(b) Effects of hypophysectomy on the female possum

After hypophysectomy or sham hypophysectomy possums generally maintained their body weight (Table 17). Due to failed operations and poor survival the number of animals in each treatment group is small. Therefore to allow comparisons of gland weights and hormone profiles results for the animals operated on Days 1 and 4 have been combined.

There was no significant decrease in the weights of either the adrenal or thyroid glands of hypophysectomised possums 12 to 15 days after operation (Table 17). However the weights of the thyroid glands, but not the adrenal glands, were significantly lower
Table 16. Result of histological examination of the sella turcica region of the possum after sham hypophysectomy or hypophysectomy (hypox). The animals are listed as four groups. The upper three groups are referred to in Tables 17 and 18, the remaining group is referred to in Table 19.

<table>
<thead>
<tr>
<th>Anim No.</th>
<th>Treatment at Operation</th>
<th>Presence (+) or Absence (-) of: PLP</th>
<th>Presence (+) or Absence (-) of: ALP</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>6017 (1)</td>
<td>Sham hypox</td>
<td>+</td>
<td>+</td>
<td>Sham hypox.</td>
</tr>
<tr>
<td>6028 (1)</td>
<td>Sham</td>
<td>+</td>
<td>+</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>6031 (1)</td>
<td>Sham</td>
<td>+</td>
<td>+</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>6001 (1)</td>
<td>Anterior hypox</td>
<td>+</td>
<td>+ fragment</td>
<td>Incomplete anterior hypox.</td>
</tr>
<tr>
<td>6003 (1)</td>
<td>&quot;</td>
<td>-</td>
<td>+ fragment</td>
<td>Incomplete full hypox.</td>
</tr>
<tr>
<td>6008 (4)</td>
<td>&quot;</td>
<td>+</td>
<td>+ small</td>
<td>Incomplete anterior hypox.</td>
</tr>
<tr>
<td>6021 (8)</td>
<td>&quot;</td>
<td>+</td>
<td>+ not intact</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>6007 (1)</td>
<td>Hypox</td>
<td>-</td>
<td>-</td>
<td>Full hypox.</td>
</tr>
<tr>
<td>6013 (4)</td>
<td>&quot;</td>
<td>-</td>
<td>-</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>6022 (4)</td>
<td>Anterior hypox</td>
<td>+ small</td>
<td>-</td>
<td>Anterior hypox.</td>
</tr>
<tr>
<td>6023 (4)</td>
<td>Hypox</td>
<td>+</td>
<td>-</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>6011 (8)</td>
<td>&quot;</td>
<td>-</td>
<td>-</td>
<td>Full hypox.</td>
</tr>
<tr>
<td>6002 (12)</td>
<td>&quot;</td>
<td>-</td>
<td>-</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>6029* (1)</td>
<td>Sham hypox</td>
<td></td>
<td></td>
<td>Not kept.</td>
</tr>
<tr>
<td>6026* (1)</td>
<td>Anterior hypox</td>
<td></td>
<td></td>
<td>Not kept, died on day of operation.</td>
</tr>
<tr>
<td>6006* (1)</td>
<td>Hypox</td>
<td></td>
<td></td>
<td>Gross examination at death, no PLP or ALP</td>
</tr>
<tr>
<td>6025* (4)</td>
<td>Anterior hypox</td>
<td>+</td>
<td>+ not intact</td>
<td>Incomplete anterior hypox</td>
</tr>
<tr>
<td>6014* (4)</td>
<td>Hypox</td>
<td></td>
<td></td>
<td>Gross examination at death, no PLP or ALP</td>
</tr>
<tr>
<td>6027* (4)</td>
<td>Anterior hypox</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot; &quot;</td>
</tr>
<tr>
<td>6004* (8)</td>
<td>Anterior hypox</td>
<td>+</td>
<td>+</td>
<td>Incomplete anterior hypox.</td>
</tr>
</tbody>
</table>

* Did not survive until Day 18 post-estrus.

PLP = posterior lobe of pituitary.

ALP = anterior lobe of pituitary.

( ) = days post-estrus of treatment.
Table 17. Body weight (kg) and adrenal and thyroid gland weights of possums after sham hypophysectomy, incomplete hypophysectomy or hypophysectomy (hypox) performed on Days 1, 4, 8 or 12 post-oestrus. Autopsy was performed on Day 18 post-oestrus.

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Treatment</th>
<th>Body weight (kg)</th>
<th>Weights (mg) at autopsy of:</th>
<th>Pre-op</th>
<th>Autopsy</th>
<th>Adrenals</th>
<th>Thyroids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>mg/kg</td>
<td>mg</td>
<td>mg/kg</td>
<td>mg</td>
<td>mg/kg</td>
</tr>
<tr>
<td>6017</td>
<td>(1) Sham</td>
<td>2.50</td>
<td>2.70</td>
<td>224</td>
<td>83.0</td>
<td>114</td>
<td>42.2</td>
</tr>
<tr>
<td>6028</td>
<td>&quot;</td>
<td>2.05</td>
<td>1.95</td>
<td>117</td>
<td>60.0</td>
<td>56</td>
<td>28.7</td>
</tr>
<tr>
<td>6031</td>
<td>&quot;</td>
<td>2.60</td>
<td>2.25</td>
<td>259</td>
<td>115.1</td>
<td>109</td>
<td>48.4</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>2.40</td>
<td>2.30</td>
<td>200</td>
<td>86.0</td>
<td>93.0</td>
<td>39.8</td>
</tr>
<tr>
<td>± SEM</td>
<td></td>
<td>0.20</td>
<td>0.22</td>
<td>52.7</td>
<td>54.1</td>
<td>7.6</td>
<td>10.6</td>
</tr>
<tr>
<td>6001</td>
<td>(1) Incomplete hypox</td>
<td>1.90</td>
<td>1.95</td>
<td>143</td>
<td>72.8</td>
<td>90</td>
<td>46.2</td>
</tr>
<tr>
<td>6003</td>
<td>&quot;</td>
<td>2.25</td>
<td>2.55</td>
<td>159</td>
<td>62.4</td>
<td>185</td>
<td>72.5</td>
</tr>
<tr>
<td>6008</td>
<td>&quot;</td>
<td>2.60</td>
<td>2.70</td>
<td>160</td>
<td>59.3</td>
<td>100</td>
<td>37.0</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>2.25</td>
<td>2.40</td>
<td>154.0</td>
<td>64.8</td>
<td>125</td>
<td>51.9</td>
</tr>
<tr>
<td>± SEM</td>
<td></td>
<td>0.20</td>
<td>0.23</td>
<td>5.5</td>
<td>4.1</td>
<td>30.1</td>
<td>10.6</td>
</tr>
<tr>
<td>6021</td>
<td>(8) Incomplete hypox</td>
<td>2.30</td>
<td>1.90</td>
<td>143</td>
<td>75.3</td>
<td>104</td>
<td>54.7</td>
</tr>
<tr>
<td>6007</td>
<td>(1) Full hypox</td>
<td>1.90</td>
<td>2.05</td>
<td>-</td>
<td>110</td>
<td>-</td>
<td>53.7</td>
</tr>
<tr>
<td>6013&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&quot;</td>
<td>1.85</td>
<td>1.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6022</td>
<td>(4) Anterior hypox</td>
<td>2.45</td>
<td>2.40</td>
<td>141</td>
<td>58.8</td>
<td>148</td>
<td>61.7</td>
</tr>
<tr>
<td>6023</td>
<td>&quot;</td>
<td>2.20</td>
<td>2.00</td>
<td>154</td>
<td>77.0</td>
<td>112.4</td>
<td>56.2</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>2.10</td>
<td>1.99</td>
<td>123.5</td>
<td>57.2</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>± SEM</td>
<td></td>
<td>0.14</td>
<td>0.19</td>
<td>12.3</td>
<td>2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6011&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(8) Full hypox</td>
<td>1.8</td>
<td>1.6</td>
<td>146.8</td>
<td>91.8</td>
<td>105.6</td>
<td>66.0</td>
</tr>
<tr>
<td>6002&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&quot;</td>
<td>2.45</td>
<td>2.05</td>
<td>183.8</td>
<td>89.7</td>
<td>171.2</td>
<td>83.5</td>
</tr>
<tr>
<td>Intact females</td>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>110.5</td>
</tr>
<tr>
<td>± SEM (n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>22.2(17)</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup>Animals autopsied on Days 15, 19 and 17 post-oestrus respectively.

( ) = days post-oestrus of treatment.
(P < 0.025) in the sham-operated females than in untreated intact females, suggesting the operation itself had affected thyroid function (Table 17).

The ovaries of hypophysectomised females lacked growing follicles and were reduced in weight (P < 0.01) compared with those of sham-operated females (Table 18). In all animals the CL was enlarged 3 to 6 times compared with the size at Day 1 (about 10 mg), and although the CL of hypophysectomised females weighed less than the sham-operated controls and intact animals, the difference was not significant (Table 18). Independent of the type of operation, or the day on which it was performed, all pregnant animals had full term foetuses at autopsy. One sham-operated female gave birth on Day 18 and in another, although a neonate was not found, one uterus was more enlarged and vascular than the other and was post-partum in appearance. The third sham-operated female was not pregnant. Of the four females which were incompletely hypophysectomised one gave birth on Day 17 post-oestrus while three had dead full term foetuses either in the uterus or the median vagina. None of the hypophysectomised females gave birth. Three of these animals had full term foetuses in their uteri on Day 18. In two others operated on Day 4 nothing was recovered, although in one of these one uterus was enlarged and oedematus. The only animal operated on on Day 12 was non-pregnant (Table 18).

The autopsy results for animals which died or were killed soon after operation are shown in Table 19. Independent of the type of operation there was an increase in the weight of the CL and continued development of the embryo in the pregnant animals.
Table 18. Weights of various tissues of the reproductive tract, presence of growing follicles and stage of embryo development at autopsy on day 18 post-oestrus of possums after sham hypophysectomy, incomplete hypophysectomy and hypophysectomy.

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Uterus (g)</th>
<th>Ovaries</th>
<th>Growing Foll-</th>
<th>Stage of Embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UGS Non- pregnant</td>
<td>CL (mg)</td>
<td>CL (mg) icles</td>
<td></td>
</tr>
<tr>
<td>Sham hypophysectomy</td>
<td>6017 (1)</td>
<td>13.36</td>
<td>1.111</td>
<td>1.772</td>
</tr>
<tr>
<td></td>
<td>6028 (1)</td>
<td>12.33</td>
<td>0.738</td>
<td>0.797</td>
</tr>
<tr>
<td></td>
<td>6031 (1)</td>
<td>13.02</td>
<td>1.070</td>
<td>1.492</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>± SEM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incomplete hypophysectomy</td>
<td>6001 (1)</td>
<td>10.0</td>
<td>0.504</td>
<td>1.670</td>
</tr>
<tr>
<td></td>
<td>6003 (1)</td>
<td>16.0</td>
<td>1.401</td>
<td>3.565</td>
</tr>
<tr>
<td></td>
<td>6008 (4)</td>
<td>13.7</td>
<td>0.946</td>
<td>1.437</td>
</tr>
<tr>
<td></td>
<td>6021 (8)</td>
<td>10.6</td>
<td>0.63</td>
<td>1.362</td>
</tr>
</tbody>
</table>
Table 18 continued

<table>
<thead>
<tr>
<th>Animal</th>
<th>DL (mg)</th>
<th>HR (mg)</th>
<th>HL (mm)</th>
<th>HD (mm)</th>
<th>B (cm)</th>
<th>W (cm)</th>
<th>Final Animal Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>6007^1</td>
<td>14.8</td>
<td>0.677</td>
<td>2.05</td>
<td>140</td>
<td>43</td>
<td></td>
<td>Pregnant, dead FTF in cervix, 214 mg, HL = 6.0 mm.</td>
</tr>
<tr>
<td>6013a</td>
<td>10.4</td>
<td>0.72</td>
<td>3.12</td>
<td>70.8</td>
<td>38</td>
<td></td>
<td>Pregnant, foetus close to term in uterus.</td>
</tr>
<tr>
<td>6022a</td>
<td>12.74</td>
<td>0.83</td>
<td>2.43</td>
<td>224</td>
<td>37</td>
<td></td>
<td>Nothing recovered, right uterus enlarged, oedematous.</td>
</tr>
<tr>
<td>6023a</td>
<td>8.5</td>
<td>0.315, 0.347</td>
<td>157</td>
<td>31</td>
<td></td>
<td>Nothing recovered, uterus small.</td>
<td></td>
</tr>
</tbody>
</table>

Mean (days 1 & 4): $\text{Mean} = 173.7$, $\text{SEM} = 37.0$, $\text{SEM} = 3.5$

<table>
<thead>
<tr>
<th>Animal</th>
<th>DL (mg)</th>
<th>HR (mg)</th>
<th>HL (mm)</th>
<th>HD (mm)</th>
<th>B (cm)</th>
<th>W (cm)</th>
<th>Final Animal Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>6011b</td>
<td>7.25</td>
<td>0.71</td>
<td>1.076</td>
<td>124</td>
<td>20.8</td>
<td></td>
<td>Pregnant, dead FTF in uterus.</td>
</tr>
<tr>
<td>602c</td>
<td>9.20</td>
<td>0.838, 0.718</td>
<td>181</td>
<td>33.6</td>
<td></td>
<td>Unfertilised egg recovered.</td>
<td></td>
</tr>
</tbody>
</table>

Intact females: Mean $\text{Mean} = 303$, $\text{SEM} = 49.3$

<table>
<thead>
<tr>
<th>Animal</th>
<th>DL (mg)</th>
<th>HR (mg)</th>
<th>HL (mm)</th>
<th>HD (mm)</th>
<th>B (cm)</th>
<th>W (cm)</th>
<th>Final Animal Outcome</th>
</tr>
</thead>
</table>

HL = head length; FTF = full term foetus; a,b,c = Animals autopsied on Days 15, 19 and 17 post-oestrus respectively.

( ) = Day post-oestrus of operation. **UGS = urogenital system.**
Table 19. Autopsy results for possums which died before Day 18 post-oestrus.

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Type of Operation</th>
<th>Day Post-</th>
<th>Day Post-</th>
<th>Weight of Cl. at Autopsy</th>
<th>Stage of Embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>6029*</td>
<td>Sham hypox</td>
<td>1</td>
<td>6</td>
<td>-</td>
<td>Nothing recovered.</td>
</tr>
<tr>
<td>6026</td>
<td>Anterior hypox</td>
<td>1</td>
<td>1</td>
<td>11.8</td>
<td>Egg.</td>
</tr>
<tr>
<td>6006</td>
<td>Hypox</td>
<td>1</td>
<td>4</td>
<td>-</td>
<td>2 cleaving eggs.</td>
</tr>
<tr>
<td>6025*</td>
<td>Failed hypox</td>
<td>4</td>
<td>10</td>
<td>67.0</td>
<td>Unfertilised egg.</td>
</tr>
<tr>
<td>6027</td>
<td>Anterior hypox</td>
<td>4</td>
<td>5</td>
<td>10.0</td>
<td>Cleaving egg.</td>
</tr>
<tr>
<td>6014</td>
<td>Hypox</td>
<td>4</td>
<td>7</td>
<td>20.0</td>
<td>Cleaving egg.</td>
</tr>
<tr>
<td>6004*</td>
<td>Hypox</td>
<td>8</td>
<td>12</td>
<td>60.0</td>
<td>Vesicle, 6.0 mm diameter. Primitive streak, no somites</td>
</tr>
</tbody>
</table>

*Animals killed because of infection.
(c) **Plasma progesterone profiles.** The plasma progesterone results for animals operated on Days 1 and 4 have been combined and mean profiles presented for the three groups (Fig. 66a,b,c). In all animals, irrespective of treatment, there was a significant rise in plasma progesterone which was similar in pattern to that in cycling intact animals. Within treatment comparison of levels on Day 4 with concentrations on other days post-oestrus revealed that (1) in sham-operated animals, levels were significantly higher on days 8, 10, 12 and 14 (P < 0.005), (2) in females where hypophysectomy was incomplete levels were significantly higher on Days 10, 12 and 14 (P < 0.005) and on Days 8, 16, and 18 (P < 0.03), and (3) after hypophysectomy levels were also significantly higher on Days 8, 10 and 12 (P < 0.005), Day 14 (P < 0.01) and Days 16 and 18 (P < 0.03).

Day by day t-tests of mean progesterone concentrations on equivalent days after oestrus for sham-operated and hypophysectomised possums revealed no significant differences except on Day 14 when levels in hypophysectomised animals were lower (P < 0.025). There were no differences on any days between sham-operated females and females which were incompletely hypophysectomised.

On Day 8 one animal was successfully hypophysectomised and in another the operation failed. In the latter, the progesterone profile (Fig. 67a) was not different from the normal pattern, but in the former, progesterone levels did not increase above 2 ng/ml (Fig. 67b), and at autopsy, both were pregnant and had term foetuses (Table 18). In the one female fully hypophysectomised on Day 12 progesterone levels were similar to intact animals until the day of operation. However levels had declined from 7.2 ng/ml on Day 12 to 0.4 ng/ml on Day 14, but it was not possible to determine whether
this decline was due to operational stress or reflected the same response as in the other fully hypophysectomised animals (Fig. 67c).

DISCUSSION

Three conclusions can be drawn from these results. First, since operation on Day 1 did not prevent the formation of the CL the pre-ovulatory surge of LH must occur before this time; second, after ovulation the subsequent formation and growth of the CL does not require the presence of the pituitary; and third, progesterone produced by the CL in the absence of the pituitary is sufficient to support pregnancy to term.

The failure of parturition in all hypophysectomised, pregnant possums could be due to either the absence of the pituitary or inadequate levels of progesterone after Day 12. Even if the CL does require luteotrophic support to maintain high levels of progesterone in the closing stages of the luteal phase, these levels cannot be essential for parturition since the CL can be dispensed with after Day 11 without affecting parturition (Sharman, 1965). The results for the animals in which hypophysectomy was incomplete give support to an essential role for the pituitary in the process of parturition; plasma progesterone levels were elevated on Day 14 but parturition had not occurred by Day 18. In 3 of these incompletely hypophysectomised animals it is highly probable that pituitary function was sufficiently disrupted to prevent parturition, as only small fragments of the anterior lobe, associated with a small posterior lobe, remained in the sella turcica region after surgery.

The maintenance of high levels of progesterone after Day 12 may be important for the development of the mammary gland, independent of the progesterone requirements for pregnancy. It is
concluded, therefore, that in the possum, as in the tammar, the CL is autonomous, and does not require luteotrophic support to produce sufficient progesterone to maintain pregnancy to term.

5.4 SUMMARY AND CONCLUSIONS

In this chapter the effects of hypophysectomy on luteal function in the tammar and possum were examined.

In the lactating tammar removal of the pituitary induced reactivation of the CL and blastocyst resulting in normal growth and progesterone production by the CL and development of the embryo. Lactation ceased after the removal of either the whole pituitary or the anterior lobe and the pouch young died 7 to 21 days later.

In the possum removal of the pituitary on Days 1, 4, 8 and 12 post-oestrus did not affect either the formation and growth of the CL or the development of the embryo to term, but prevented parturition. Plasma progesterone levels were similar to intact cyclic animals until Day 12. On Day 14 levels were significantly lower indicating that the pituitary may provide a luteotrophic stimulus to maintain progesterone secretion at this time.

It was concluded that the CL of both species are autonomous with respect to progesterone production for the maintenance of the conceptus, although it is likely that hypophysial stimulation is necessary for successful parturition and lactation.
Plate 1. Diagrammatic transverse section through the head of a tammar wallaby or possum showing the principal anatomical features.
PLATE 1.

Brain
Dura mater
Infundibulum
Anterior pituitary
Posterior pituitary
Intermediate lobe
Sphenoid
Nasopharynx
Carotid artery
Plate 2. Transverse section of the sella turcica region of a tammar after sham hypophysectomy (x 10). After surgery the drill site was filled with Gelfoam sponge. (See Plate 1 for diagrammatic representation of anatomy). 1 cm = 1 mm
Animal No. 5634

Plate 3. Transverse section of the sella turcica region of a tammar after full hypophysectomy (x 10). Animal No. 5136

Plate 4. Transverse section of the sella turcica region of a tammar after anterior hypophysectomy. Note that intermediate lobe and posterior lobe are intact (x 10). Animal No. 5321
Fig. 60. Plasma progesterone in 6 lactating tammars after sham hypophysectomy and in 1 lactating tammar (4789) after posterior hypophysectomy (Experiment 1, March).
FIG. 60.

Plasma progesterone (pg/ml)

Tammar No.

4358

5149

4932

4975

5002

4802

4789

Days after operation

0 7 14 21
Fig. 61. Plasma progesterone in 5 lactating tammars after hypophysectomy (Experiment 1).
FIG. 61.

Tammar No.
4689

Plasma progesterone (pg/ml)

4969

Days after operation
FIG. 61. (cont.)

Plasma progesterone (pg/ml)

Days after operation

Tammar No. 4979

4564

4946
Fig. 62. Plasma progesterone levels (Mean ± SEM) in lactating tammars after (a) sham hypophysectomy (n = 6) and (b) hypophysectomy (n = 5 until Day 7, n = 2 on Day 14 and 21; o-o, x-x) (Experiment 1).
FIG. 62.

(a) Plasma progesterone (pg/ml)

(b) Plasma progesterone (pg/ml)

Days after operation
Fig. 63. Plasma progesterone in 5 lactating tammars after sham hypophysectomy performed during lactational quiescence (Experiment 11, April).
FIG. 63.

Plasma progesterone (pg/ml)

Tammar No. 4513

Days after operation
Fig. 64. Plasma progesterone in 4 lactating tammars after hypophysectomy (n = 3) and after adeno-hypophysectomy (n = 1, 5321) performed during lactational quiescence (Experiment 11, April).
FIG. 64.

![Graphs showing plasma progesterone levels after operation](image-url)

- Tammar No. 4896
- Tammar No. 5321
- Tammar No. 5230
- Tammar No. 5136

Days after operation
Fig. 65. Plasma progesterone levels (Mean ± SEM) in lactating tammars after (a) sham hypophysectomy (n = 5), and (b) hypophysectomy (n = 4) (Experiment 11).

▼ = days significantly different from control, P < 0.005.
FIG. 65.

Plasma progesterone (pg/ml)

Days after operation
Plate 5. Transverse section of sella turcica region of a possum after sham hypophysectomy (Refer to Plate 1 for diagram of general anatomy) (x 10). 1 cm = 1 mm. Animal No. 6028

Plate 6. Transverse section of sella turcica region of a possum after full hypophysectomy. Note that drill site is filled with Gelfoam sponge (x 10). Animal No. 6007

Plate 7. Transverse section of sella turcica region of a possum which was incompletely hypophysectomised. Note fragments of anterior pituitary (arrows) associated with the posterior pituitary (x 10). Animal No. 6004
Fig. 66. Plasma progesterone levels (Mean ± SEM) in female possums after hypophysectomy performed during the breeding season;
(a) after sham hypophysectomy (n = 3) performed on Day 1 post-oestrum,
(b) after incomplete hypophysectomy performed on Days 1 (n = 2) and 4 (n = 1) post-oestrum, and
(c) after hypophysectomy (n = 2) or adeno-hypophysectomy (n = 2) performed on Days 1 (n = 1) and 4 (n = 3) post-oestrum.
FIG. 66.

![Graph showing plasma progesterone levels (ng/ml) over days after oestrus.](image)

- (a) Plasma progesterone levels from days 0 to 20 post-oestrus.
- (b) Plasma progesterone levels from days 0 to 20 post-oestrus.
- (c) Plasma progesterone levels from days 0 to 20 post-oestrus.
Fig. 67. Individual profiles of plasma progesterone for 3 possums after operation; (a) incomplete hypophysectomy performed on Day 8 post-oestrus, (b) hypophysectomy performed on Day 8 post-oestrus, and (c) hypophysectomy performed on Day 12 post-oestrus.
FIG. 67.

(a) Plasma progesterone (ng/ml)

(b) Plasma progesterone (ng/ml)

(c) Plasma progesterone (ng/ml)

Days after oestrus

Days after oestrus

Days after oestrus
CHAPTER 6.

PLASMA PROLACTIN DURING LACTATION IN DIPROTODONT MARSUPIALS

6.1 INTRODUCTION

In contrast to eutherians, lactation in marsupials is of longer duration and is accompanied by marked changes in milk composition (Green, 1984), a several-fold increase in the size of the active mammary gland (Smith et al., 1969; Findlay, 1982; Tyndale-Biscoe, Stewart and Hinds, 1984) and increasing milk production by the active gland (Green, 1984). At birth, the marsupial young is poikilothermic and entirely dependent on a relatively stable pouch environment and a continuous or almost continuous supply of milk from the teat to which it is attached. Coincident with the maternal changes described above, the young enters a phase of rapid growth, achieves homeothermy and many of its metabolic functions become fully developed. Shortly after this time the young vacates the pouch permanently, although it will continue to take milk from its mother for some time (Tyndale-Biscoe, 1973; Sharman, 1973). This last phase of lactation is thought to be equivalent to the suckling period of eutherians such as the horse and ruminants, which are born in an advanced state of development (Sharman, 1973).

In eutherians many hormones, particularly insulin, corticosteroids and prolactin, are associated with the onset and subsequent maintenance of lactation (Ensor, 1978). During early
lactation the sucking stimulus of the neonate elicits a release of prolactin and it is the frequency of this stimulus and the consequent high levels of prolactin which are important for the maintenance of galactopoiesis. As lactation proceeds, however, both the frequency of the sucking stimulus and the release of prolactin in response to the sucking stimulus diminish and prolactin concentrations decline as weaning occurs (Cowie, Forsyth and Hart, 1980).

In marsupials the role of prolactin in lactation is unknown, although in tammars the pituitary must be present for the maintenance of early lactation (< 100 days) (Section 5.2). In this chapter, therefore, the main aims were to describe changes in plasma prolactin during lactation in the tammar and possum, and to examine the relationship between prolactin levels and the sucking stimulus of the tammar pouch young during late lactation (> 200 days) and at the time of pouch exit.

6.2 THE TAMMAR

6.2.1 PLASMA PROLACTIN DURING LACTATION IN THE TAMMAR

Introduction

For the tammar, the major changeover in milk composition of lactation occurs between 170 and 220 days (Messer and Green, 1979; Green, Newgrain and Merchant, 1980; Green, 1984) and the five-fold increase in gland weight, which is due to both hypertrophy and hyperplasia of the alveolar cells, occurs between 140 and 210 days (Findlay, 1982; Tyndale-Biscoe et al., 1984). Between 150 and 200 days the young achieves homeostasis, and vacates the pouch
permanently at about 250 days (Murphy and Smith, 1970), although lactation may continue for up to 300 days (W.E. Poole, pers. comm.).

In the non-lactating tammar, seasonal changes in plasma prolactin occur, with levels being lowest in lactational quiescence and highest in seasonal quiescence (Tyndale-Biscoe and Hinds, 1981, 1983). During seasonal quiescence, until late October or early November, the majority of females are normally lactating (Berger, 1966), but little is known of either the changes in plasma prolactin associated with lactation or their relationship to the seasonal changes. Analysis of plasmas collected by Dr Jenny Hawkins, in 1975, indicated that a marked increase in plasma prolactin occurred in lactating females after 100 days of lactation (Hinds and Tyndale-Biscoe, 1982). These higher levels were maintained in two females which successfully lactated, but loss or artificial removal of the pouch young during mid to late lactation resulted in a return to basal levels. From these preliminary results it was apparent that the pattern of plasma prolactin during lactation in the tammar contrasted markedly with the eutherian pattern in which circulating prolactin concentrations are highest in the early stages of lactation (Ensor, 1978; Cowie et al., 1980).

Therefore in order to correlate the changes in plasma prolactin with the known changes in milk composition, gland weight and growth of the young, a group of animals has been monitored during the normal sequence of events in a breeding cycle.

**Experimental design**

A group of animals (initially 12) was sampled once a week between 0800 and 0900 h from a lateral tail vein for 14 months from before birth in one breeding season until after birth in the following breeding
season. When births were expected in the first season, daily pouch checks were made and daily blood samples taken to detect changes in prolactin at parturition and oestrus. Plasmas were frozen and stored at -20°C until the end of the experiment when all of the samples from each animal were analysed in the same assay.

Each week the pouch was checked for the presence of a young and the time of pouch exit was recorded. Throughout the experiment no pouch young were deliberately removed although some were accidentally lost or died. Three of the adults also died during the year.

Results

Nine females gave birth and one entered oestrus between 20 January and 4 February (Mean ± SEM date of birth/oestrus = 29 January ± 1.6 days, n = 10). The two remaining females in the group entered oestrus between 9 and 16 February and had produced young by 16 March. In the following season the first birth or oestrus was detected in 8 of the 9 surviving animals within 1 to 3 days of the date of first activity in the previous season (Mean ± SEM date of birth/oestrus = 27 January ± 1.9 days, n = 8) (Table 20).

Six females successfully raised their first born young of the season and pouch exit occurred between 5 October and 2 November (Mean ± SEM date of pouch exit = 25 October ± 4.6 days, n = 6) when the young were 257 to 282 days old (Mean ± SEM age of young at pouch exit = 269 ± 4.1 days, n = 6). In four of the remaining 6 animals the young was born later (between 16 March and 4 May) and, although successfully raised, pouch exit occurred when the young were 222 to 259 days old (Mean ± SEM age = 241 ± 7.7 days) i.e. the later born young vacated the pouch at a significantly earlier age (P < 0.0005).
## Table 20. Status of female tannars during annual cycle

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>First Birth</th>
<th>Next Birth</th>
<th>Date of Pouch Exit</th>
<th>Age of Young at Pouch Exit (Days)</th>
<th>First Birth or Oestrus</th>
</tr>
</thead>
<tbody>
<tr>
<td>4485</td>
<td>20 Jan</td>
<td></td>
<td>5 Oct</td>
<td>258</td>
<td>19 Jan</td>
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<td>4210</td>
<td>30 Jan</td>
<td></td>
<td>25 Oct</td>
<td>268</td>
<td>30 Jan</td>
</tr>
<tr>
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<td></td>
<td>2 Nov</td>
<td>275</td>
<td>29 Jan</td>
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<td>1 Feb</td>
<td></td>
<td>2 Nov</td>
<td>275</td>
<td>31 Jan</td>
</tr>
<tr>
<td>4638</td>
<td>4 Feb</td>
<td></td>
<td>19 Oct</td>
<td>257</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Mean</th>
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<th>269</th>
</tr>
</thead>
<tbody>
<tr>
<td>± SEM</td>
<td>4.6 days</td>
<td>4.1 days</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>First Birth</th>
<th>Next Birth</th>
<th>Date of Pouch Exit</th>
<th>Age of Young at Pouch Exit (Days)</th>
<th>First Birth or Oestrus</th>
</tr>
</thead>
<tbody>
<tr>
<td>4698</td>
<td>-</td>
<td>16 Feb</td>
<td>16 Mar</td>
<td>30 Nov</td>
<td>2 Feb</td>
</tr>
<tr>
<td>86938b</td>
<td>4 Feb</td>
<td>23 Mar</td>
<td>23 Nov</td>
<td>245</td>
<td></td>
</tr>
<tr>
<td>4178</td>
<td>31 Jan</td>
<td>20 Apr</td>
<td>28 Nov</td>
<td>222</td>
<td>&lt;13 Mar</td>
</tr>
<tr>
<td>4506</td>
<td>-</td>
<td>1 Feb</td>
<td>4 May</td>
<td>23 Dec</td>
<td>31 Jan</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mean</th>
<th>4 Dec</th>
<th>241</th>
</tr>
</thead>
<tbody>
<tr>
<td>± SEM</td>
<td>7.9 days</td>
<td>7.7 days</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>First Birth</th>
<th>Next Birth</th>
<th>Date of Pouch Exit</th>
<th>Age of Young at Pouch Exit (Days)</th>
<th>First Birth or Oestrus</th>
</tr>
</thead>
<tbody>
<tr>
<td>4478</td>
<td>24 Jan</td>
<td></td>
<td></td>
<td>205</td>
<td>27 Jan</td>
</tr>
<tr>
<td>4322c</td>
<td>-</td>
<td>16 Feb</td>
<td>16 Mar</td>
<td>157</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mean</th>
<th>29 Jan</th>
<th>27 Jan</th>
</tr>
</thead>
<tbody>
<tr>
<td>± SEM</td>
<td>1.8 days</td>
<td>1.9</td>
</tr>
</tbody>
</table>

*Female died 19 Oct, young survived.*
*Female died 18 Dec, young already weaned.*
*Female died 20 Aug.*
*Pouch young died.*

*Young born later in the breeding season were significantly younger at pouch exit than those born in early February (t-test, P < 0.0005).*
than young born at the beginning of the breeding season. Of the remaining two animals, one female died in August after 157 days of lactation while in the other the young, born in late January, died 205 days later (Table 20).

Plasma prolactin levels for the individual females are presented in Figs 68 to 73. Figures 68, 69 and 70 show the profiles for the 6 females whose young were born at the end of January and successfully raised. Since these females underwent a temporally normal sequence of events they are considered to represent the typical pattern in the annual cycle. Therefore a mean profile of their prolactin levels throughout lactation has also been presented (Fig. 74). Prior to birth, plasma prolactin levels were low, and except for a sharp surge of prolactin at parturition (Chapter 4.2.2) remained at basal concentrations of less than 20 ng/ml for the first 140 days of lactation. After this time irregular fluctuations of between 40 and 100 ng/ml occurred, and by 180 to 200 days levels were consistently higher than 60 ng/ml, and generally greater than 100 ng/ml, remaining so for the next 50 to 80 days. Shortly before, or at the time of, permanent vacation of the pouch, between 250 to 280 days, levels declined rapidly, and remained low thereafter (Figs 68-70 and 74). However, in these animals white milk could be expressed from the sucked gland up to 35 days later.

In the other four animals which retained their young to weaning, similar changes in plasma prolactin levels (Figs 71 and 72) occurred during lactation. The young of these four females were born later in the breeding season (March to May) and the rise in prolactin levels occurred at a correspondingly later time in the year indicating that the changes in prolactin are independent of the season. However, in two females (4178, 4506) whose young vacated
the pouch at 222 and 238 days, earlier than any other young, peak levels were maintained at greater than 60 ng/ml for only 40 to 50 days prior to pouch exit (Figs 71 and 72). This earlier fall in prolactin levels coincided with the summer solstice, so a seasonal influence cannot be dismissed.

In the female which died in August, 157 days after the birth of her young, plasma prolactin levels had remained consistently low (Fig. 73). In the animal which lost her young after 205 days the pattern of plasma prolactin had followed the typical pattern up to this stage, but after the loss of her young levels declined to basal concentrations within three weeks, as in the previous study (Hinds and Tyndale-Biscoe, 1982).

During this stage of lactation, between 180 and 250 days, it appears to be the presence of the young which maintains the high level of circulating prolactin since, if the young is lost or removed (Fig. 73; Hinds and Tyndale-Biscoe, 1982) levels rapidly decline.

6.2.2 RESPONSE IN PLASMA PROLACTIN LEVELS DURING LATE LACTATION TO REMOVAL AND REPLACEMENT OF THE POUCH YOUNG

The influence of the sucking young during late lactation is examined in the following study by determining the response in plasma prolactin to (a) the removal of the pouch young for 24 h and (b) the return of the young to the pouch.

Six lactating females were selected in October, and the age of their pouch young determined from head length measurements and comparison with tammar growth curves (W.E. Poole, pers. comm.). At the start of the study the young ranged in age from 203 to 242 days
and could be expected to remain in the pouch for a further 20 to 50 days.

The females and their young were used at two different times. On the first occasion each young (203-242 days) was in its mother's pouch, while on the second occasion, 21 days later, four of the young had vacated the pouch.

**Experiment 1**

Blood samples were taken from the lateral tail vein at 0900 h on Days -4, -1 and 0. Immediately after the Day 0 sample had been taken, pouch young were removed and blood samples were collected 7 h and 24 h (0900 h on Day 1) later. The young were then replaced in the pouch and further samples were collected 7 h later, and at 0900 h on Days 2, 3 and 6.

**Results.** Before the pouch young were removed peripheral prolactin levels were within the range expected for this stage of lactation (> 80 ng/ml) (Figs. 75 and 76). Within 7 h of removal of the pouch young levels had decreased 2 to 10 fold and remained at this concentration until after the young were replaced. Levels then increased during the next 7 h, had returned to high concentrations by 24 h, and remained elevated throughout the next 5 days in all except one animal which had the oldest young.

The results show that between 200 and 240 days of lactation it is the presence of the young in the pouch which maintains the high plasma prolactin levels in the mother, i.e. that prolactin is released in response to the sucking stimulus of the young.
Experiment 2

In this second experiment, performed three weeks later, milk samples were also collected from the animals as part of another study. Oxytocin was used to induce milk let-down and the mammary gland was massaged to obtain the milk; otherwise the experimental protocol was the same as before.

At the beginning of this experiment only the two smallest young (224 and 244 days old) were still in their mother's pouch. At 0900 h on Day 0 the group was caught and the young separated from their mothers. Four and a half hours later, when milk had accumulated in the gland, the animals were anaesthetised (1.5 ml sodium brietal, Parke Davis, Aust.) and 0.2 IU oxytocin (Syntocin, Sandoz, Aust.) injected intramuscularly. Over a period of less than 5 minutes as much milk as possible was removed from the gland. Blood samples were collected on Days -4 and -1, at the time of removal of pouch young (Day 0), prior to induction of anaesthesia and 0.5, 2.5 and 4.5 h after injection of oxytocin. At 0900 h the next day another sample was taken and the young returned either to the pouch or to the yard with their mothers. Further samples were collected 7 h and 1, 2 and 5 days later, as in the previous experiment.

Results. In 4 of the 6 females there was a similar though not so marked response in plasma prolactin to removal and replacement of the young, as had been observed 21 days previously (Figs 77 and 78). Nevertheless the increase in plasma prolactin which occurred within 7 h of replacement of the young was not maintained during the next few days. In the 2 remaining animals plasma prolactin levels were low (< 20 ng/ml) and unchanging during
the course of the experiment. There was a small increase in prolactin in 4 of the 6 females within 30 minutes of collection of the milk samples. Whether this response was due to either the injection of oxytocin and/or the manual emptying of the mammary gland is unknown (Figs 77 and 78).

From these two experiments it is clear that there is a critical stage during late lactation when the presence of the sucking young is important for the maintenance of plasma prolactin levels. Beyond this time, after pouch exit, the sucking stimulus no longer promotes prolactin secretion and plasma prolactin levels decline to basal concentrations (< 40 ng/ml), although milk is still being produced by the gland. Indeed in this group milk was still being produced by all females 43 days after the end of the second experiment by which time the young were between 267 and 306 days old. This suggests that high prolactin levels are not required to maintain milk production after pouch exit.

DISCUSSION

The pattern of plasma prolactin during lactation in the tammar clearly differs from that described for eutherian species (Cowie et al., 1980), and can be divided into four phases. Initially levels are low (< 40 ng/ml) and stable (0 - 140 days), then they begin to fluctuate (140 - 180 days) before reaching peak levels (> 100 ng/ml) between 180 and 200 days. These high levels are maintained for 50 to 80 days (= 180 - 250 days) but decline sharply at the time of permanent pouch exit which occurs after about 240 days. During this final phase (> 240 days) levels remain low although milk is still being produced for the young.
The low levels of plasma prolactin before 140 days of lactation, which are no greater than in non-lactating females (Hinds and Tyndale-Biscoe, 1982), imply either that the sucking stimulus does not promote prolactin secretion, or that the quantity of the stimulus is insufficient at this time. This is in sharp contrast to the marked response elicited by the sucking stimulus of the eutherian neonate (rats - Amenomori et al., 1970; cow - Karg and Schams, 1974; goats - Butt et al., 1972; Hart, 1974; sheep - Lamming et al., 1974; marmoset - McNeilly, Abbott, Lunn, Chambers and Hearn, 1981; pig - Bevers et al., 1978). Prolactin and/or other anterior pituitary hormones, however, are essential for continued galactopoiesis at this stage in the tammar since lactation fails in all females after full or anterior hypophysectomy and the young die 7 to 21 days after operation (Hearn, 1974; this study, Chapter 5).

The effectiveness of the low levels of plasma prolactin may be due to differential development of specific receptors for prolactin in the sucked gland (Stewart and Tyndale-Biscoe, 1982). In the last 2 weeks of pregnancy there is an increase in the weight of the four mammary glands and the number of prolactin receptors per cell increases to reach highest concentrations at birth. In the first 7 days after parturition only the number of receptors in the sucked gland continues to increase while the remaining glands regress. In the sucked gland the receptor concentration per cell reaches a maximum by about 84 days of lactation although the total number of receptors in the gland continues to increase as the gland undergoes hyperplasia and hypertrophy. Between 120 and 240 days there is a 14-fold increase in the size of the gland, with the greatest increase occurring between 140 and 200 days (Tyndale-Biscoe
et al., 1984). Coincident with this increase is the rise in plasma prolactin levels to maximum concentrations by about day 200, and the changeover in milk composition from high to low carbohydrate content and from low to high fat content (Messer and Green, 1979; Green et al., 1980). It is interesting that these changes occur after the young ceases to be permanently attached to the teat, after about 100 days (Murphy and Smith, 1970), and may reflect a change in sucking patterns from continuous to intermittent. Before 100 days of lactation the young grow slowly and daily milk intake ranges between 1 and 4 ml/day. Beyond this time there is a marked increase in milk consumption and body weight, so that by 200 days the average intake of milk is about 50 ml/day (Green, 1984). These changes in milk intake probably reflect the increasing energetic requirements of the young which, between 100 and 200 days, achieves homeostasis; its thermoregulatory and metabolic functions develop (Setchell, 1974; Paton and Janssens, 1981) and the thyroid, adrenal and pituitary glands begin to secrete their respective hormones (Setchell, 1974; Call et al., 1980; G. Wilkes, pers. comm.). Furthermore it is during this period that the incremental growth rate of the young accelerates (W.E. Poole, pers. comm.), closely paralleling the accelerated growth of the active mammary gland and the rising level of plasma prolactin.

After 200 days the high level of plasma prolactin is maintained until pouch exit at about 250 days. If the young is lost or removed during this time levels rapidly decline inferring that it is the sucking stimulus which is responsible for the high level of prolactin in the circulation. Indeed the response in plasma prolactin to removal and replacement of young at this time is similar to the response seen in lactating sows whose piglets were
removed and replaced (Bevers et al., 1978). Thus this third stage of lactation in the tammar can be equated to the early stages of lactation in eutherians. After pouch exit the response in plasma prolactin elicited by the sucking stimulus declines, but despite this, and the low levels of circulating prolactin, milk continues to be produced by the sucked gland for up to 60 more days. However, from about 220 days of lactation the young begins to eat grasses (Murphy and Smith, 1970) and by 270 days it has a well established microbial population in the forestomach (A.C.I. Warner, pers. comm.). Hence the decline in prolactin levels probably reflects the decreasing frequency of sucking by the young which is a consequence of the transition to the adult digestive pattern and diet (Paton and Janssens, 1981). This fourth phase, therefore, may be equivalent to late lactation in eutherians; in women, for example, who are feeding their infant exclusively on breast milk, the frequency of sucking episodes is high and plasma prolactin levels are high, but once supplementary food is given there is a marked decline in sucking frequency and a corresponding drop in prolactin levels (Howie and McNeilly, 1982).

During the course of lactation in tammars the temporal sequence of the changes in prolactin concentration is maintained independent of when the young were born, implying that the pattern is inherent to the state of lactation, i.e. the peak concentrations observed between August and December are a function of lactation and are not related to the time of year. However, the highest concentrations at this time are almost two-fold higher than levels in non-lactating females in seasonal quiescence (Tyndale-Biscoe and Hinds, 1981, 1983) so any seasonal fluctuations may have been masked by the lactational changes.
This study has shown conclusively that the pattern of plasma prolactin during lactation in the tammar differs markedly from that described for eutherians, particularly in the early stages. It also posed the question "Is the pattern typical of other marsupials which have lengthy periods of lactation?", and led to the following study in the possum.

6.3 THE POSSUM

6.3.1 PLASMA PROLACTIN DURING LACTATION IN THE POSSUM

Introduction

During lactation in the female possum there are marked changes in milk composition (Gross and Bolliger, 1959), as well as a large increase in the weight of the suckled mammary gland after 80 days (Smith et al., 1969). Similar changes occur during lactation in the tammar (Green et al., 1980; Tyndale-Biscoe et al., 1984) and so it was of considerable interest to determine whether the pattern of plasma prolactin in the possum was similar to that described for the tammar.

Possum young remain associated with their mother for about 200 days. Between birth and 120 days they are found exclusively in the pouch, while after this time they cling to the mother's back (Dunnet, 1956; Smith et al., 1969). Weaning is thought to commence at about 130 days and lactation has ceased in most females by 230 days (Smith et al., 1969).
Methods

Lactating possums were obtained opportuney and so were at various stages of lactation when first sampled. At capture, measurements of various body parameters of the young (body weight, length of head, pes, manus, ear and tail) were taken, and the age estimated from the growth curves of Lyne and Verhagen (1957). The estimates of age from head length and from pes length seldom differed by more than 5 days.

Blood samples were collected from a lateral tail vein of the mothers twice weekly from the time of capture until weaning. Animals were then autopsied and the mammary glands and reproductive tracts dissected and weighed.

Another group of four possums was sampled to determine whether changes in plasma prolactin occurred in non-lactating females. Two of these animals had not produced a young between May and June while the breeding history of the remaining two females was unknown.

Results

Lactation was followed for 174 days after birth in one possum (6058), at which time the young died. Plasma prolactin levels were low and usually undetectable during the first 60 days and then fluctuated from basal concentrations to 45 ng/ml until 120 days. Thereafter levels were 50 ng/ml or more until the young was lost (Fig. 79).

No other animal was caught with a young less than 60 days of age. The two animals (6073, 6076) sampled from day 60 onwards showed similar fluctuations in plasma prolactin until about 120 days (Fig. 79). Thereafter peak levels of between 30 and 50 ng/ml were
observed until about day 180 in all females which lactated successfully. Beyond 180 days levels declined to basal concentrations even though the mammary gland was being suckled and the young were still associated with the mother (Fig. 79). Figure 80 presents a composite profile of prolactin levels in these five females throughout lactation.

In another female (6075), although the pattern of plasma prolactin before 120 days was similar to the other females at this stage of lactation, her young died at 129 days. Prolactin levels did not increase from then until she escaped at 178 days (Fig. 79).

The weights of the suckled mammary glands taken at autopsy (Table 21) reflect the regression of the gland as weaning occurs, although the timing of weaning varies considerably between animals (compare possums 6077 and 6076). The ovaries of all except one of these females were small and inactive; in this possum (6074) there was a CL weighing 36 mg. Therefore she had resumed cycling while weaning her young and could have produced a second young in the minor breeding season.

Plasma prolactin levels in non-lactating females showed no consistent pattern. In 2 females levels fluctuated from undetectable to more than 20 ng/ml. However these higher levels were not maintained for more than 2 or 3 sampling times. In the other 2 animals prolactin was undetectable in 17 of 23 samples and in 30 of 32 samples respectively (Fig. 81). At autopsy the ovaries and mammary glands of these animals were small and inactive (Table 21).
Table 21. Weights of mammary glands and ovaries in lactating, post-lactating and non-lactating possums.

<table>
<thead>
<tr>
<th>Animal Stage of Lactation (days)</th>
<th>Number</th>
<th>Weight of Mammary Gland (g)</th>
<th>Weight of Ovary (mg)</th>
<th>Appearance of ovaries</th>
</tr>
</thead>
<tbody>
<tr>
<td>LACTATING FEMALES</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6073 169</td>
<td>9.18</td>
<td>0.16</td>
<td>123</td>
<td>139 Inactive. Many haemorrhagic follicles.</td>
</tr>
<tr>
<td>6058 178</td>
<td>6.53*</td>
<td>0.30</td>
<td>142</td>
<td>128 Inactive. Small follicles.</td>
</tr>
<tr>
<td>6076 207</td>
<td>11.41</td>
<td>0.19</td>
<td>151</td>
<td>132 Inactive. Many haemorrhagic follicles.</td>
</tr>
<tr>
<td>POST-LACTATING FEMALES</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6077 201</td>
<td>1.86</td>
<td>0.23</td>
<td>83</td>
<td>89 Flat, no follicles visible.</td>
</tr>
<tr>
<td>6074 234</td>
<td>1.20</td>
<td>0.87</td>
<td>111</td>
<td>98 Cycling - CL on right ovary = 36 mg.</td>
</tr>
<tr>
<td>NON-LACTATING FEMALES</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6061</td>
<td>-</td>
<td>0.18, 0.23</td>
<td>129</td>
<td>142 Inactive, many small follicles, old CA.</td>
</tr>
<tr>
<td>6064</td>
<td>-</td>
<td>0.19, 0.27</td>
<td>114</td>
<td>103 Inactive, flat, no follicles visible.</td>
</tr>
<tr>
<td>6066</td>
<td>-</td>
<td>0.20, 0.24</td>
<td>104</td>
<td>113 Inactive, many small follicles, old CA.</td>
</tr>
<tr>
<td>6067</td>
<td>-</td>
<td>0.18, 0.22</td>
<td>224</td>
<td>227 Inactive, many white spotted follicles, 2 old CA.</td>
</tr>
</tbody>
</table>

*Gland regressing as young died 2 to 3 days before autopsy. CA = Corpus albicans.
Discussion

During lactation in possums, as in tammars, the pattern of plasma prolactin can be divided into four phases. In the first phase (0 - 60 days) levels remain low, in the next (60 - 120 days) they fluctuate from low to high concentrations (3 - 40 ng/ml), while in the third phase (140 - 170 days) peak concentrations of 30 - 50 ng/ml are observed. During the final phase (> 180 days) plasma prolactin declines to basal levels. Although the peak concentrations (30 - 50 ng/ml) are lower than in the tammar (> 100 ng/ml), and the time scale is different (6 months vs 9 months) the general pattern closely resembles that described for the tammar (Section 6.1.1; Hinds and Tyndale-Biscoe, 1982). Furthermore, as in the tammar, the marked changes in prolactin concentrations after 60 days correlate with the rapid increase in the weight of the mammary gland after 80 days (Smith et al., 1969), the increase in the percentage of protein, fat and total solids in the milk after 65 days (Gross and Bolliger, 1959) and the increase in body weight of the young which vacates the pouch at about 120 days (Dunnet, 1956; Lyne and Verhagen, 1957).

In non-lactating possums there were no consistent changes in plasma prolactin, implying that there are no seasonal changes in the secretion of prolactin by the pituitary between the major and minor breeding seasons (June to October). Since significant increases in plasma prolactin occurred in lactating possums but not in either non-lactating females or in one female after she lost her young, the sustained high levels of prolactin in late lactation can probably be attributed to the sucking stimulus of the young, as was demonstrated experimentally for the tammar.
6.3 SUMMARY AND CONCLUSIONS

In this chapter the pattern of plasma prolactin during lactation in the tammar was described and found to be very different from the eutherian pattern. Levels of circulating prolactin were highest in the second half of lactation and were maintained for a period of about 50 to 80 days by the sucking stimulus of the young. This stage of lactation could be equated to the early stages of lactation in the eutherian but no eutherian equivalent could be found for the low levels present in the early stages in the tammar.

The pattern of plasma prolactin in the possum during lactation was similar to that in the tammar, and it was concluded that the profile was characteristic of lactation in the diprotodont marsupials.
Figs. 68-73. Plasma prolactin levels (ng/ml) in individual tammars during lactation. Samples were collected before birth in one season until after birth in the following season. The time of pouch vacation, loss of young or death of the adult is indicated for each animal. B = birth; 0 = oestrus.
FIG. 68.

Tammar No. 4411

Tammar No. 3970

Plasma prolactin (ng/ml)

Month of the year
FIG. 69.

[Graph showing plasma prolactin levels over the month of the year for two tammar nos. 4573 and 4210. The x-axis represents the month of the year (Jan to Dec), and the y-axis represents plasma prolactin levels (ng/ml). The graph includes annotations for pouch exit (275 days and 268 days) and changes in prolactin levels.]
FIG. 70.

Plasma prolactin (ng/ml)

Month of the year

Tammar No.

Adult died, 257 days of lactation

Pouch exit 258 days

B

B

B

J F M A M J J A S O N D J
FIG. 71.

Tammar No. 4698

Plasma prolactin (ng/ml)

Month of the year

Tammar No. 86938

Plasma prolactin (ng/ml)

Month of the year

- Female died
- Pouch exit 245 days
- Pouch exit 259 days
- Young lost
FIG. 72.

Tammar No. 4506

Pouch exit 238 days

Plasma prolactin (ng/ml)

Month of the year

B Young Bert

Lost 222 days

4178
FIG. 73.

Tammar No. Young

Adult died

157 days of lactation
Fig. 74. Mean ± SEM plasma prolactin levels (ng/ml) during lactation in the tammar (n = 6). B = birth; Solid arrows indicate time of permanent pouch exit; Dashed arrow indicates when all females were post-lactating.
Fig. 75. Plasma prolactin levels (ng/ml) during late lactation in 6 individual tammars before and after removal of pouch young, and after replacement of the young in the pouch. Removal of pouch young (RPY) = solid arrow; Replacement of pouch young = dashed arrow.
FIG. 75.

![Graph showing plasma prolactin levels for Tammar No. 5097, 5033, 4959, 5474, 5346, and 5395 after different days.](image)

<table>
<thead>
<tr>
<th>Tammar No.</th>
<th>Age of young (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5097</td>
<td>242</td>
</tr>
<tr>
<td>5033</td>
<td>233</td>
</tr>
<tr>
<td>4959</td>
<td>230</td>
</tr>
<tr>
<td>5474</td>
<td>224</td>
</tr>
<tr>
<td>5346</td>
<td>223</td>
</tr>
<tr>
<td>5395</td>
<td>203</td>
</tr>
</tbody>
</table>

Days after RPY:

-4 0 2 4 6
Fig. 76. Mean ± SEM plasma prolactin (ng/ml) during late lactation (203-242 days) in tammars (n = 6) before and after removal of pouch young, and after replacement of the young in the pouch. Removal of pouch young (RPY) = solid arrow; Replacement of pouch young = dashed arrow.
Fig. 77. Effect on plasma prolactin levels of oxytocin injection given 4.5 h after removal of pouch young during late lactation (224 to 263 days) for 6 tammars. Day 0 = time of removal of pouch young; Day 1 = time young replaced in the pouch. Dashed vertical line = time of intramuscular injection of oxytocin (0.2 IU).
FIG. 77.

[Graph showing plasma prolactin levels for Tammar No. 5097, 5033, 4959, 5474, 5346, and 5395. The x-axis represents days after RPY, and the y-axis represents plasma prolactin (ng/ml). Each line corresponds to a different tammar, with age of young (days) indicated on the right side of the graph.]
Fig. 78. Mean ± SEM plasma prolactin levels for 4 lactating tammars given a single intramuscular injection of oxytocin 4.5 h after removal of pouch young. Two lactating females which did not show a response in plasma prolactin to the return of the pouch young have been presented individually. Day 0 = time of removal of pouch young; Day 1 = time young replaced in the pouch; Dashed vertical line = time of injection of oxytocin (0.2 IU).
FIG. 78.

Plasma prolactin (ng/ml) vs. Days after RPY showing changes with young removed and replaced, oxytocin injection.
Fig. 79. Plasma prolactin levels (ng/ml) in individual lactating possums (n = 6). Animals were caught at different times after the birth of their young and sampled thereafter.
FIG. 79.

Plasma prolactin (ng/ml)

Days of lactation

Possum No. 6077

Young on back

6074

Young on back

6076

Young on back

6073

Young on back

6058

Young on back

Young died

6075

Young died
Fig. 80. Mean ± SEM plasma prolactin levels for 5 possums during lactation. From 0 to 65 days, \( n = 1 \), from 68 to 120 days, \( n = 3 \), from 120 to 163 days \( n = 5 \), from 163 to 194 days \( n = 3 \), and from 200 to 233 days \( n = 1 \).
FIG. 80.

Days of lactation

Plasma prolactin (ng/ml)
Fig. 81. Plasma prolactin levels (ng/ml) in 4 non-lactating possums sampled between the major and minor breeding seasons from July to October.
FIG. 81.

Plasma prolactin (ng/mL) vs. Days

Possum No.
6064
6067
6061
6066

Days

Month of the year
CHAPTER 7.

GENERAL DISCUSSION

INTRODUCTION

The mechanisms regulating reproductive function have been examined in many eutherian species but, until recently, comparatively little was known of the endocrinology of reproduction in marsupials. In the last ten years, however, several aspects have been investigated and the results have led to a reassessment of the mechanisms involved in the control of luteal function and hypophysial-luteal interaction, maternal recognition of pregnancy, hormonal control of embryonic diapause and the regulation of lactation and seasonal breeding in marsupials (Tyndale-Biscoe et al., 1974; Tyndale-Biscoe, 1979; Renfree 1981a,b; Tyndale-Biscoe and Hinds, 1981, 1983). The significance of the contributions made in this thesis to our understanding of some of the events in marsupial reproduction and lactation are discussed in this chapter, and where possible, compared and contrasted with appropriate events in eutherian species.

MATERNAL RECOGNITION OF PREGNANCY/HORMONAL EQUIVALENCE

In most eutherian species early pregnancy is characterised by a prolongation of the luteal phase of the oestrous cycle and suppression of ovulation and this has been termed the maternal recognition of pregnancy (Short, 1969). In almost all marsupials,
however, pregnancy occupies a period less than the length of the oestrous cycle, and it does not inhibit the cyclic recurrence of oestrus and ovulation (although subsequent lactation does). The many anatomical and histological similarities between the post-oestrous phases of pregnant and non-pregnant females implied that there is a lack of maternal recognition of pregnancy in marsupials and led to the proposal that the two states may be hormonally equivalent (Sharman, 1970).

Since 1970, the hormonal equivalence hypothesis has been examined in five marsupial species, and for four of these there is no evidence of a foetal influence on early steroid hormone profiles; in the opossum (Harder and Fleming, 1981), possum, native cat and tammar (this study, Chapter 4) no differences in plasma progesterone levels were observed in the first 10 days post-oestrus in pregnant and non-pregnant females. The tammar, provides the strongest evidence for a lack of maternal recognition of early pregnancy - the early progesterone peak occurs in both the oestrous cycle and pregnancy irrespective of either the presence of a blastocyst or whether the cycles have been delayed by lactation. It is also present when reactivation of the CL is induced by either hypophysectomy (Chapter 5) or manipulation of the photoperiod (Hinds and den Ottolander, 1983). Thus the progesterone peak is considered to be a normal concomitant of CL development and is not part of a mechanism for recognition of early pregnancy, as has been proposed for a fifth species, another macropocid, the quokka. Cake et al. (1980) claimed that recognition does occur since they observed a transient rise in progesterone levels on Days 3 to 4 after RPY only in pregnant females. Another study on the quokka (Wallace et al., 1983) reported that the peak occurred in non-pregnant females as
well, but, as explained earlier (Chapter 4), in both studies inadequate numbers of animals were used and the same animals were not examined during both an oestrous cycle and a pregnancy. Clearly the occurrence of the progesterone peak in the pregnant and/or non-pregnant quokka needs to be clarified. Until this has been done, however, there is no unequivocal evidence for maternal recognition of early pregnancy in any of the marsupials which have been examined under controlled experimental conditions.

Although there is no evidence for maternal recognition of early pregnancy the foetus does have an effect in the later stages of pregnancy. In the tammar the embryo and/or its membranes induce a local, stimulatory effect on the endometrium such that, after Day 16 of pregnancy, the endometrium of the gravid uterus is significantly heavier than the non-gravid one and remains so until after parturition (Renfree and Tyndale-Biscoe, 1973). The transfer of embryos to the uterus contralateral to the CL demonstrated that the embryo effect on the endometrium was independent of the CL (Renfree and Tyndale-Biscoe, 1973). Foetal influences are also observed around the time of parturition in the macropodids and the opossum. In the opossum there are changes in the oestrogen:progesterone ratio just before parturition which are not apparent in the oestrous cycle, while in the tammar parturition is accompanied by an earlier decline in progesterone levels, a surge of prolactin and an earlier onset of oestrus. Hybrid breeding experiments using grey kangaroos showed that the foetal genotype determines gestation length (Kirsch and Poole, 1972; Poole, 1975) as young resulting from crosses between male western grey kangaroos and female eastern grey kangaroos were born on Day 34, two days earlier than if the females mated with conspecific males. Thus the
marsupial conceptus exerts an influence, not at the beginning, but from the middle to the end of pregnancy. Among the eutherian species only the monoestrous carnivores resemble marsupials in that the life-span and secretory activity of the CL are similar in the fertile and infertile cycles (ferrets - Heap and Hammond, 1974; blue fox - Møller, 1973a; red fox - Bonnin et al., 1978; dog - Concannon et al., 1975). In contrast to the marsupials, however, in these species maximum levels of progesterone are reached shortly after implantation (between 10 to 20 days after ovulation); thereafter progesterone levels decline slowly and parturition (between 42 and 64 days) is not accompanied by an abrupt decrease in plasma progesterone concentrations. Nevertheless, in the dog the life-span of the CL of pregnancy is significantly shorter (about 64 days) than those of non-pregnant animals which have a more variable life-span (51 to 82 days) (Concannon et al., 1975). Thus, as in the tammar, in the dog the pregnant uterus and/or foetus may trigger changes that effect the termination of CL function at the end of pregnancy.

**AUTONOMY OF THE MARSUPIAL CL**

Earlier studies on the effects of hypophysectomy in the tammar indicated that the function of the CL, after reactivation, may be autonomous (Hearn 1973, 1974; Tyndale-Biscoe & Hawkins, 1977). The current findings support this view since after reactivation induced by hypophysectomy the CL produced progesterone, without apparent luteotrophic support, such that levels in the peripheral circulation were not different from those in animals undergoing pregnancy after RPY. This independence of the tammar CL is in agreement with the observed lack of LH receptors in luteal
tissue (Stewart & Tyndale-Biscoe, 1982), the ability of the CL to produce and secrete progesterone in vitro in the absence of added gonadotrophins, and the inability of either LH or prolactin to affect this rate of steroidogenesis in vitro (Sernia et al., 1980; Hinds et al., 1983). Thus, in the tammar CL, the changes in the progesterone secretion rate around Day 5 (Hinds et al., 1983) which result in the transient peripheral progesterone peak, may occur because the CL has an intrinsic ability to regulate its progesterone production rather than an ability to respond to stimulation by extrinsic factors of hypophysial origin. However, although the tammar CL appears to be autonomous, two other mechanisms may maintain progesterone secretion in the absence of the pituitary. Either LH has a very high affinity for its luteal receptor and is irreversibly bound in the absence of sufficient levels of circulating hormone such that it can exert a luteotrophic effect for an extended period after hypophysectomy, or the pre-ovulatory surge of LH may provide the single stimulus essential for maintenance of the CL of pregnancy/oestrous cycle in marsupials.

In many eutherian species hypophysectomy performed before mid-pregnancy results in abortion because the CL requires luteotrophic support from the pituitary to maintain progesterone production at this time (see Chapter 1). However the effects of hypophysectomy on luteal function during the oestrous cycle are more varied and could be interpreted as an ability of the CL to function autonomously. In the dog, hypophysectomy induces luteolysis but the rate of regression of progesterone secretion after hypophysectomy on Day 10 of the luteal phase is much slower than after hypophysectomy on Day 34 or later (Concannon, 1980). In the guinea-pig, growth of the CL and luteal progesterone synthesis continue after
hypophysectomy performed before Day 10 of the cycle although only about one-half of the CL persisted for at least five weeks (as after hysterectomy), and arterial progesterone levels were extremely low (Heap, Perry and Rowlands, 1967). Similarly, in the pig, hypophysectomy after the pre-ovulatory LH surge does not affect the formation and growth of CL which are maintained for about the length of one oestrous cycle (21 days). However, both luteal progesterone content and plasma progesterone concentrations are reduced by about one-half (see Anderson and Melampy, 1967 for review).

*In vitro* studies also support the view that, after formation, the porcine CL is autonomous during the early stages of its life-span (Hunter, 1981). Progesterone production *in vitro* by CL collected at 6 1/2 days of pregnancy is high and cannot be stimulated by the addition of either gonadotrophin or cyclic AMP. In contrast, in Day 12 1/2 CL, progesterone production was lower and could be stimulated by LH and cyclic AMP to levels similar to 6 1/2 day CL. For both the guinea-pig and pig, it is thought that the growth of the CL and luteal progesterone production depend on a single luteotrophic stimulus at the time of ovulation. The current results in the tammar, could be interpreted similarly. However in contrast to the pig and guinea-pig, in which plasma progesterone concentrations were low after hypophysectomy, peripheral progesterone levels in tammars after hypophysectomy were similar to levels in intact animals undergoing pregnancies. Furthermore, in the tammar, the CL has extremely low numbers of binding sites for LH compared to the number of sites in the pregnant pig CL (a ratio of 1:163) (Stewart and Tyndale-Biscoe, 1982), injection of prolactin for 7 days after hypophysectomy delays reactivation of the CL for this interval, and, despite the absence of circulating
gonadotrophins in the intervening 7 days, the CL subsequently grows and supports a pregnancy to term (Tyndale-Biscoe and Hawkins, 1977). Also, as mentioned earlier, LH does not stimulate steroidogenesis by luteal tissue incubated in vitro on Day 1 after RPY (Sernia et al., 1980). These results support the view that the tammar CL is autonomous, but further investigation is necessary to determine whether LH can remain bound to its few CL receptors for 7 days and still exert a luteotrophic effect.

The maintenance of similar levels of plasma progesterone in intact and hypophysectomised female possums undergoing pregnancies indicates that the possum CL may also be independent of hypophysial influences. However, as with the tammar, the results obtained could also be due to retention of LH for an extended period.

In the light of these conclusions it is worth considering Rothchild's (1981) proposed model for the evolution of the regulation of the CL of mammals. He proposed that all primitive CL were autonomous and capable of producing both progesterone and prostaglandins. In his model the secretion of each hormone is regulated by positive feedback, and this characteristic, as well as the luteolytic effect of prostaglandins on progesterone synthesis, eventually induces luteal regression. However with the evolution of viviparity the CL became sensitive to luteotrophic factors, firstly of hypophysial and then of placental origin, which prolonged the life-span of the CL during pregnancy in most eutherians, with the exception of the carnivores. One of the main assumptions of the theory is that once a critical concentration of tissue progesterone is reached in the CL prostaglandin production increases, progesterone secretion declines and luteolysis proceeds. Typically then, the production of progesterone during the life-span of the CL
comprises a rising phase, a plateau phase and a regressing phase. In most pregnant eutherians either the plateau phase or the regressing phase is prolonged by extrinsic factors. In the marsupials, as in the monoestrous eutherian carnivores, none of the phases are affected by pregnancy except in the pregnant tammar, as in the pregnant dog, where an earlier regression of the CL is induced at parturition. Thus in the possum, opossum, native cat and bandicoot, the pattern of progesterone secretion and its regulation may be as proposed by Rothchild (1981), although nothing is known of either prostaglandin production or its effects in these species. However, in the tammar, the progesterone peak on Day 5, 6 or 7, which occurs before any increase in the mass of the CL, appears to result from an increased and then a decreased rate of secretion, while the subsequent rise in progesterone in mid-cycle is probably due to the increased CL mass (Hinds et al., 1983). The changes inducing the early peak do not conform with a self-regulating positive feedback system which also incorporates the luteolytic effects of prostaglandins. Possibly the tissue progesterone concentration has not reached a critical level by Day 5, and so prostaglandin production does not lead to luteal regression. Certainly tissue levels of progesterone on Day 5 are less than half those on Day 23 of the cycle (Renfree et al., 1979). Conversely, the brief increase in secretion over 2 to 4 days could be equated with the rising, plateau and regressing phases of the oestrous cycle of the rat. The intrinsic mechanisms regulating the tammar CL are clearly more complex than those proposed in the regulatory model of Rothchild (1981) and cannot be resolved without some investigations of the prostaglandin content of luteal and ovarian tissues. It will also be necessary to determine what controls the changes in secretion
rate of progesterone in the tammar CL in the absence of the pituitary.

PROLACTIN AND LACTATION IN MARSUPIALS

One of the most interesting results of this thesis has been the finding that the pattern of peripheral prolactin levels during lactation in the tammar and possum differs markedly from the eutherian pattern. Prolactin concentrations are low during the first half of lactation when the young is ectothermic and begin to increase as the young achieves homeostasis. During the second half of pouch life the homeothermic young undergoes a period of rapid growth, which coincides with changes in milk composition and milk production, and the sucking stimulus begins to promote prolactin secretion. Shield (1966) considered that the time at which the quokka young achieves homeostasis, half way through pouch life, approximates the same developmental stage of a eutherian young (such as the laboratory rat) at birth. The changes in plasma prolactin in the second half of lactation in the tammar and possum, which can be equated with lactation in the eutherians after parturition, support this view.

A feature of lactation in many macropodid marsupials is the ability to suckle two young of different ages at the same time. This phenomenon, known as concurrent asynchronous lactation, is characterised by the production of milks from adjacent mammary glands which differ in both quality and quantity. Concurrent asynchronous lactation commonly occurs in the continuously breeding macropodids such as the red kangaroo (Sharman and Calaby, 1964), and agile wallaby (Merchant, 1976) but has also been reported in possums (Smith et al., 1969). Under special conditions the tammar and
quokka can also suckle two offspring simultaneously (Messer and Green, 1979; Yadav and Eadie, 1973). While the mechanisms controlling the production of two types of milk are still unknown, low levels of prolactin which support milk production both at the beginning and the end of lactation may provide some indirect evidence of how it occurs. In the red kangaroo, for example, the young vacates the pouch at about 235 days but continues to suckle until about 360 days of age. Shortly after pouch vacation a new young is born and attaches to another teat (Sharman and Pilton, 1964). If the pattern of plasma prolactin is similar to that of the tammar, prolactin levels would be high until the time of pouch exit when they would abruptly decline. The lower prolactin levels, while continuing to maintain milk production in the older gland, also permit the establishment of the first phase of lactation in the newly suckled gland. Furthermore, since the older gland is suckled for about 130 days after the birth of the second young it is possible that the rise in prolactin levels associated with achievement of homeostasis in the young and changes in milk composition do not occur until after this time. Thus the different pattern of prolactin concentrations in macropodid marsupials may be of adaptive significance, allowing concurrent asynchronous lactation to occur.

CONCLUSION

The current study has increased understanding of some aspects of the endocrinology of marsupial reproduction, particularly in the tammar. It has shown that (i) there is no unequivocal evidence for maternal recognition of early pregnancy in marsupials, although the foetus does influence its time of birth, (ii) the function of the
marsupial CL is probably autonomous and (iii) the pattern of prolactin in lactation is different from the eutherian pattern. Clearly the mechanisms controlling reproduction in marsupials are not the same as in eutherians, but they are equally complex and sophisticated.
BIBLIOGRAPHY


APPENDICES I-IV
APPENDIX I.

Plasma progesterone profiles for four females which failed to undergo a pregnancy after removal of their pouch young.
APPENDIX 1.

<table>
<thead>
<tr>
<th>Tammar No.</th>
<th>Days after RPY</th>
<th>Days of next cycle</th>
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<tbody>
<tr>
<td>4707</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4826</td>
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</tr>
<tr>
<td>4782</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4780</td>
<td></td>
<td></td>
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APPENDIX II.

Plasma progesterone (ng/ml) in possums in which a return to oestrus after removal of a pouch young was not detected.

<table>
<thead>
<tr>
<th>Date</th>
<th>Animal Number</th>
<th>6045</th>
<th>6046</th>
<th>6049</th>
<th>Date</th>
<th>Animal Number</th>
<th>6067</th>
<th>6060</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.11.81</td>
<td>&lt;0.10</td>
<td>0.16</td>
<td>-</td>
<td></td>
<td>15.5.82</td>
<td>0.53</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>&lt;0.10</td>
<td>-</td>
<td></td>
<td>16</td>
<td>0.13</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.18</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>12</td>
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</tr>
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APPENDIX III.

Published papers which have arisen either partially or wholly from this study.


APPENDIX IV.

List of papers presented at Scientific Meetings during the period of candidature.

THE EFFECT OF PROLACTIN ON SECRETION OF PROGESTERONE IN VITRO
BY THE CORPUS LUTEUM.

APPENDIX IV.

List of papers presented at Scientific Meetings during the period of candidature.

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THE EFFECT OF PROLACTIN ON SECRETION OF PROGESTERONE IN VITRO
BY THE CORPUS LUTEUM.
THE EFFECT OF PROLACTIN ON SECRETION OF PROGESTERONE IN VITRO
BY THE TAMMAR CORPUS LUTEUM

Lynette Hinds and Jane Thompson

Presented at 'Prolactin 1980 and beyond'. A satellite workshop following the Sixth International Congress of Endocrinology, 1980.

In the tammar wallaby, the corpus luteum (CL) of post-partum ovulation is suppressed and as a result, the development of the newly fertilized ovum is arrested at the blastocyst stage. Reactivation of the CL (and blastocyst) follows removal of the suckling pouch young or injection of bromocriptine, an inhibitor of prolactin (PRL) secretion (1). These and other observations indicate that it is PRL, produced in response to suckling, which directly inhibits the CL (2). Specific binding sites for PRL have been demonstrated in the CL (3).

Following reactivation, circulating progesterone levels rise steadily. Using in vitro incubations of quiescent and reactivated CL, it has been shown that there is no change in the tissue progesterone content of reactivated CL and that the progesterone secretion rate does not change from Day 0 to Day 16, except briefly at Day 5 (4).

The aim of this study was to determine whether the rise in progesterone secretion rate in Day 5 CL could be suppressed by PRL. The effect of PRL on in vitro progesterone secretion from these CL was therefore tested.
Five females were injected with bromocriptine and their pouch young removed. On Day 5 the CL were removed and halved. Each half was incubated in Krebs-Ringer bicarbonate buffer + 0.2% glucose for 4 hrs at 35°C under 95% O₂/5% CO₂ in the presence or absence of o-PRL (50 ng/ml) and the progesterone concentration in the tissue and medium were determined by radio-immunoassay sensitive to 25 pg/ml. Previous experiments confirmed that the progesterone in the medium represents active production and secretion of progesterone by luteal tissue (4) and that the rate of secretion is linear over the 4-hour incubation period.

There was no effect of PRL on either the tissue concentration or rate of secretion of progesterone (paired sample t-test, P > 0.1 in both cases) (see Table 1).

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These results indicate that PRL does not suppress steroidogenesis in the CL. An alternative hypothesis that prolactin suppresses the growth of luteal cells is being investigated.

DEMONSTRATION OF A TRANSIENT PEAK OF PLASMA PROGESTERONE ASSOCIATED
WITH REACTIVATION OF THE QUIESCENT CORPUS LUTEUM OF THE TAMMAR

L.A. Hinds and C.H. Tyndale-Biscoe


On Day 5 after removal of pouch young (RPY) from the tammar
there was a significant increase in the rate of secretion of
progesterone from pieces of corpora lutea incubated in vitro,
compared to secretion on Days 0, 9 and 16 (1). This increase was
reflected in the peripheral plasma progesterone levels of some of
the animals. In the quokka, Setonix brachyurus there is a
significant increase in plasma progesterone on Days 3-4 after RPY.
This peak occurred during the pregnant cycle and was not apparent
during the non-pregnant cycle (2).

The experiments we now report were designed to confirm that
this transient peak is a consistent feature of reactivation in the
tammar, and if it is, whether it occurs in females undergoing a non-
pregnant cycle as well as those undergoing a pregnant cycle.

For the experiment a group of 9 lactating females carrying
quiescent blastocysts were selected early in the breeding season,
and were followed through two consecutive cycles. The pouch young
were removed to initiate the first (pregnant) cycle and at the end
of the cycle (Day 26-28) when births and post-partum oestrus
occurred the females were mated with vasectomized males. The second
series of pouch young were removed 10 days after birth to initiate a
second (non-pregnant) cycle.
Daily blood samples of 3.0 mls were collected from jugular catheters or tail veins for 10 days after each RPY, over the period of parturition and oestrus, and during the short period of lactation. Plasma progesterone was measured by radioimmunoassay (3).

In the pregnant cycle peripheral progesterone increased from basal levels (< 200 pg/ml) to a peak ranging from 350 to 1270 pg/ml on Day 5, 6 or 7. The peak lasted about one day after which levels declined to near basal. Subsequently, progesterone increased and remained high until parturition as has been described previously (4). Throughout the 10 days of lactation progesterone levels were again basal and, as in the pregnant cycle, there was a similar transient but significant increase in plasma progesterone (343 to 489 pg/ml) 5 to 7 days after RPY. For each animal the peak occurred on the same day after RPY in both the pregnant and non-pregnant cycle.

These results confirm that a transient rise in plasma progesterone occurs, and that it is coincident with the known onset of hyperplasia and hypertrophy of the reactivating corpus luteum (5). Furthermore, the absence of the blastocyst in no way affects the time or degree of this rise, in contrast to the conclusion of Cake et al. (2) for the quokka.

(2) Cake, M.H., Owen, F.J. and Bradshaw, S.D. J. Endocr. 84, 153-158 (1980).
CHANGES IN PLASMA PROGESTERONE CONCENTRATIONS RELATED TO PARTURITION, OESTRUS AND THE LH SURGE IN THE TAMMAR

C.A. Horn and L.A. Hinds


Pregnancy shortens the interval between one oestrus and the next in the tammar wallaby, *Macropus eugenii* (1). After removal of pouch young (RPY), females carrying a diapausing blastocyst gave birth 26.2 days later, and came into oestrus at 26.4 ± 0.59 (s.d.) days. If the females were mated with vasectomized males and pouch young removed oestrus occurred 30.4 ± 0.99 days later (1).

In order to determine whether the effect of pregnancy was reflected in the hormonal status of the female we have measured plasma progesterone and luteinizing hormone (LH) levels in a group of seven females over the period of parturition and/or oestrus for two consecutive cycles, each of which was initiated by RPY. In the first (pregnant) cycle the animals were bled at 8 hourly intervals from Day 25 RPY until 2 days after parturition and oestrus, and were mated with vasectomized males. In the second (non-pregnant) cycle animals were bled from Day 26 RPY until 2 days after mating was observed. Plasma progesterone (2) and LH (3) levels were measured by radioimmunoassay.
In the first cycle all seven females gave birth 26.08 ± 0.6 days after RPY. In the sample immediately prior to finding a new young in the pouch, progesterone levels were high (> 450 pg/ml) but within 8 hrs of birth and teat attachment they had dropped sharply to basal levels (< 200 pg/ml). Mating occurred 0-16 hours after birth, and the LH peak 0-16 hours after mating, a finding similar to previously reported observations (3).

In the second cycle five of the seven females mated 29.9 ± 2.15 days after RPY. In contrast to the pregnant cycle, progesterone declined gradually to basal levels over a period always greater than 24 hours. Matings generally did not occur until progesterone levels had dropped below 200 pg/ml. The relationship between mating and the LH peak was the same as in the pregnant cycle. The LH peak occurred over a period of 0-24 hours either before or after the copulatory plug was observed.

These results confirm that pregnancy shortens the oestrous cycle in the tammar, and suggest that this occurs as a result of the more rapid decline in progesterone seen in pregnant animals at parturition.

THE RESPONSE IN PLASMA PROGESTERONE TO HYPOPHYSECTOMY
OF THE FEMALE TAMMAR

L.A. Hinds and C.H. Tyndale-Biscoe


A transient peak of plasma progesterone is associated with reactivation of the quiescent corpus luteum (CL) of the tammar. The rise occurs between 5 and 8 days after removal of pouch young in both the pregnant and non-pregnant cycle (1). Hearn (1974) (2) demonstrated that hypophysectomy of lactating females induced reactivation of the quiescent CL and dormant blastocyst. These results suggested that the CL became autonomous once released from the pituitary-induced inhibition.

The following experiment was designed to determine (a) whether the occurrence of the peak of progesterone is dependent upon the presence of the pituitary and (b) whether plasma progesterone levels in hypophysectomised females differ from levels in intact females.

In early lactational quiescence five females were fully hypophysectomised and a control group of another five females underwent a sham-operation on Day 0. All females were lactating and their pouch young were reattached to the teat after the operation. Blood samples were collected from lateral tail veins on Days 0, 1, 3 to 7, 14 and 21 for determination of progesterone by radioimmunoassay (3). All animals were autopsied at the time of death or on Day 21, and the reproductive tract examined.
Both groups retained their young during the experiment although the young of the hypophysectomised group rapidly lost weight, did not remain constantly attached to the teat and died 7 to 21 days after operation. The young of the control group all remained attached and their body weights had doubled by Day 21.

Two hypophysectomised females died at Day 7, a third at Day 12, and the two other animals survived until Day 21. The latter three animals had reactivated and were carrying an enlarged vesicle and late stage foetuses respectively. There was no resumption of development of quiescent CL or blastocysts in the control group.

Four of the five hypophysectomised females showed changes in plasma progesterone typical of the pattern and concentration observed in a normal pregnancy or an oestrous cycle. Three animals showed a peak of progesterone (397, 625, 682 pg/ml) around Days 6 to 7 after operation, and levels increased in late gestation. Plasma progesterone in the control females remained at basal concentrations of about 200 pg/ml.

We conclude, therefore, that the CL of the tammar is independent of the pituitary once the pituitary-induced inhibition is removed, since it does not require any luteotrophic support for its growth or progesterone production during the reactivated cycle.

THE CORPUS LUTEUM OF THE BRUSH POSSUM DOES NOT REQUIRE LUTEOTROPHIC SUPPORT

C.H. Tyndale-Biscoe and L. Hinds


In the tammar the corpus luteum (CL) can grow and support pregnancy after hypophysectomy (1) and it is devoid of receptors for LH (2). In the possum the CL secretes greater amounts of progesterone than the tammar's from Days 4 to 12 post-oestrus (3) and the 17 day pregnancy fails if this is prevented by luteectomy before Day 10 (4). The CL of this species possesses abundant LH receptors (2), so may require a luteotrophic stimulus to support CL growth and the maintenance of pregnancy. Seventeen females were hypophysectomised on Days 1, 4, 8 or 12 post-oestrus and three sham control operations were performed on Day 1. The animals were autopsied on Day 18, one day after expected parturition. Adrenals, thyroids, ovaries and CL were weighed and embryos assessed for stage of development. The sella turcica was serially sectioned to establish the completion of hypophysectomy.

The 3 sham operated controls and 9 of the hypophysectomised females survived to Day 18 but in 3 of the latter hypophysectomy was incomplete. There were no significant differences in the organ weights (mg) of the three groups (Table 1) except that the ovarian tissue was lighter in the hypophysectomised females. The CL were not significantly different and in all groups were enlarged.
Table 1.

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All the pregnant animals carried full term foetuses at autopsy on Day 18 (Table 2). However, only the sham control females and one of the failed hypophysectomised females gave birth to a live young; in the others the term foetus was found dead in the uterus, cervix or vaginal cul de sac. In another hypophysectomised female killed on Day 15 the foetus was alive and at the normal stage of development. We conclude that the CL of the possum, like that of the tammar, does not require a luteotrophic stimulus to grow and support pregnancy but that parturition is adversely affected by hypophysectomy.

Table 2.

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PROGESTERONE AND PROLACTIN PROFILES IN 2 MARSUPIALS
WITH CONTRASTING PATTERNS OF REPRODUCTION

Lyn Hinds

Presented at Annual General Meeting Australian Mammal Society, 1982

The tammar wallaby has a gestation period (27 days) which is almost the same length as the oestrous cycle (29 days). Plasma progesterone levels range from < 200 pg/ml to about 1000 pg/ml and the pattern is bimodal. An early peak occurs on Days 5-7 while a second peak of several days duration occurs in late gestation. The pattern and concentration of progesterone are similar in the pregnant and non-pregnant cycle except that the fall in progesterone at parturition is more rapid. Prolactin levels are basal except for a pre-partum surge. In contrast, in the brush possum in which the gestation period is confined to the luteal phase (17 days) of the 25 day oestrous cycle, the plasma progesterone pattern is unimodal and similar in both cycles. Maximum levels (8-12 ng/ml) which occur between Days 12-15 are 10-fold greater than in the tammar. In addition a surge of prolactin occurs prior to oestrus as well as at parturition.

These results will be discussed in relation to the known function of the corpus luteum in the 2 species.
PLASMA PROLACTIN DURING LACTATION IN THE TAMMAR WALLABY

L.A. Hinds


In eutherians the frequency of the suckling stimulus and the subsequent high levels of prolactin (PRL) are important for the maintenance of lactation (1). However the role of prolactin during lactation in marsupials has not been determined. This study describes the changes in plasma PRL throughout lactation in the tammar wallaby.

Six tammars were bled at weekly intervals for one year during which time a single pouch young was successfully reared by each animal. Additional blood samples were collected at hourly intervals for 28 hours at 2 monthly intervals throughout lactation to determine short term changes in PRL. Plasma PRL was determined by radioimmunoassay (2).

In all females, plasma PRL levels were low (< 20 ng/ml) during the first 150 days of lactation, and then fluctuated markedly (20-100 ng/ml) up to 180-200 days. Thereafter levels were constantly greater than 100 ng/ml until the young permanently vacated the pouch at 260-280 days, when PRL declined sharply to basal levels.

There was no evidence of an inherent diurnal rhythm of PRL secretion at any stage of lactation. However the short term fluctuations which occurred in late lactation (about 200 days) were of greater magnitude than in early lactation or post weaning.
These results demonstrate that the secretion of PRL throughout lactation in the tammar differs from the eutherian pattern in that it can be divided into two phases. In the early phase, only low levels of PRL are necessary to maintain lactation as levels are no greater than those in non-lactating females (2). However, such levels are essential since lactation fails in females which have been hypophysectomised (3). In the second phase, levels of PRL are higher and appear to be induced by the suckling young as levels declined rapidly when the young was removed (2). This second stage of lactation can therefore be equated with lactation in eutherians. The change from the early to late phase corresponds to the period when marked changes occur in milk composition (4) and in the metabolism and behaviour of the young, which is becoming semi-independent (5).

THE CORPUS LUTEUM OF THE TAMMAR WALLABY DOES NOT REQUIRE LUTEOTROPHIC SUPPORT

L.A. Hinds

Presented at the 29th Congress of the International Union of Physiological Sciences, 1983

In the oestrous cycle and pregnancy of the tammar wallaby there is a transient pulse of plasma progesterone on Day 5, 6 or 7 post oestrus. The pulse occurs before the corpus luteum (CL) has increased in size, but in vitro studies indicate that it is of luteal origin since the rate of secretion of progesterone by incubated luteal tissue was higher on Day 5 than on Day 0 or 9. Addition of luteinizing hormone on Day 1 or prolactin on Day 1 or 5 had no effect on progesterone production in vitro. Furthermore after hypophysectomy performed on Day 0 the progesterone pulse was unaffected and of similar magnitude and duration as in intact animals.

These results suggest that the CL of the tammar wallaby, once formed, is independent of the pituitary since it does not require any luteotrophic support for its growth or production of progesterone during the cycle. This is in agreement with the observation that luteal cell membranes lack receptor sites for luteinizing hormone.
Plasma prolactin during lactation in the possum, *Trichosurus vulpecula*

L.A. Hinds

To be presented at Symposium on 'Possums and Gliders'
(Australian Mammal Society) 1983

Lactating possums (n = 6) were sampled twice weekly from capture until weaning. Plasma prolactin (PRL) was measured by a radioimmunoassay validated for the possum.

The pattern of plasma PRL during lactation can be divided into 4 phases. In the first phase (0-60 d) levels remain low, in the next (60-120 d) they fluctuate upwards (3-40 ng/ml), while in the third phase (140-170 d), peak levels of 30-50 ng/ml are observed. In the final phase (> 180 d) PRL declines to basal. If the young is lost in the second or third phase PRL declines.

The increase to peak levels of PRL by 120 d coincides with the several-fold increase in the weight of the mammary gland, changes in milk composition and an increase in the growth rate of the young. During lactation in the possum the pattern of plasma PRL closely resembles that in the tammar wallaby, and thus the pattern is probably typical of lactating diprotodont marsupials.