THE OESTROUS CYCLE AND MANIPULATION OF REPRODUCTION IN THE COMMON WOMBAT (*Vombatus ursinus*)

by

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Declaration

In accordance with the requirements of Monash University for the degree of Master of Reproductive Sciences, I hereby declare that this thesis contains no other material that has been accepted for the award of any other degree or diploma in any University and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the thesis.

Matt West

Dedication

In memory of the wombats of Myrtleford and Murrindindi

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Abstract

Wombats represent a dichotomy in wildlife management. The Common Wombat (*Vombatus ursinus*) and the Southern Hairy-nosed Wombat (*Lasiorhinus latifrons*) are abundant and in many areas considered an agricultural problem. In contrast the Northern Hairy-nosed Wombat (*Lasiorhinus krefftii*) is critically endangered and only 113 remain in an isolated population in central Queensland. Knowledge of reproduction in the abundant species could assist the management of wombats in agricultural areas and provide a basis for assisted breeding programs for the endangered Northern Hairy-nosed Wombat.

Common Wombats extend throughout southeastern Australia and Tasmania. The Common Wombat in Tasmania was found to be polyoestrus with an oestrous cycle length of ~33days (Peters 1977). Field observations in Tasmania and Victoria have found that the Common Wombat is capable of breeding all year round and suggest that the Common Wombat is monovular (McIlroy 1973; Green and Rainbird 1987; McIlroy 1995; Triggs 1996).

The aims of this study were to investigate the oestrous cycle of the Victorian Common Wombat (*Vombatus ursinus hirsutus*), to induce follicular growth and oocyte maturation by hormonal stimulation (superovulation treatment) and to test the viability of oocytes obtained. No previous study has investigated the oestrous cycle of the Common Wombat in Victoria or the technique of superovulation in this species. Other monovular marsupials have been superovulated using Porcine FSH and LH (Molinia *et al* 1998b, Molinia *et al* 2000) and these hormones were used in this study.

Wild caught female Common Wombats were examined to develop knowledge of the endocrine, vaginal cytological, and external morphological changes associated with the oestrous cycle and anoestrous. Following superovulation treatment, oocytes were collected from follicles by aspiration. Immature oocytes were placed into TCM 199 maturation media for IVM. The viability of mature MII oocytes was assessed by ICSI.

The oocytes were cultured and nuclear staining techniques were used to see if fertilisation had occurred.

This study found that:

The oestrous cycle could be defined and monitored in the Common Wombat by following changes in progesterone and oestradiol concentrations and vaginal cytology. The length of the oestrous cycle was ~ 47 days. Periods of high progesterone concentrations (peak 87nmol/L) alternated with periods of low progesterone (≤ 6.9nmol/L). The luteal phase lasted ~ 28 days and follicular phase lasted ~ 19 days. In cycling wombats oestradiol concentrations were slightly elevated (~ 2.1pg/ml) during periods of low progesterone. A high percentage of superficial epithelial type cells occurred in vaginal smears during the follicular phase. Parabasal-intermediate type epithelial cells predominated during the luteal phase. The latter pattern was also indicative of the cytology of anoestrous wombats.

Pouch and urogenital opening changes were unreliable indicators of the stage of the oestrous cycle. Mean rectal body temperature was ~ 34.8°C. Fluctuations in temperature were not associated with reproductive status. Adult cycling wombats (n=3) had a body weight of ≥23.5kg. Subadults (n=3) were below this weight.

Porcine FSH/LH stimulation successfully induced multiple large (≥4mm) antral follicle development in 10 of 11 wombats. A mean of 5.5 MII oocytes were aspirated from the follicles of 6 wombats, that were stimulated during the follicular phase of the oestrous cycle (n=3) or following various intervals after pouch young removal (n=3). Three subadults (n=3) and 2 anoestrous adults did not produce MII oocytes.

The yield of MII oocytes collected from FSH/LH stimulated wombats can be doubled USING 24 –30hrs IVM of immature oocytes. Immature oocytes matured more readily to the MII stage when cumulus cells remained attached, compared to naked immature oocytes. Cleavage was observed in ~5% of injected oocytes and 2 pronuclei were observed in ~5% of injected oocytes. Parthenogenesis cannot be excluded.

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Wildlife research is challenging at the best of times, and this project proved to be no exception. This project would not have been possible without the advice, assistance encouragement, support and enthusiasm of the staff and students from Monash IRD, and my friends and family; all to whom I am indebted.

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I have not named everyone, and I appreciate the help that you have all given. I have enjoyed the challenge of this project, and most importantly the opportunity to work with such a fascinating and idiosyncratic species

Thank you!

Abbreviations

ART	Assisted Reproductive Technique
COC	Cumulous oocyte complex
BSA	Bovine serum albumin
DMEM	Dulbecco's Modified Essential Medium
E_2	Estradiol (E2)
EMEM	Eagles Minimum Essential Medium
FCS	Fetal Calf Serum
FSH	Follicle Stimulating Hormone
Hepes	N-[Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
ICSI	Intra -cytoplasmic sperm injection
IVF	In-vitro fertilization
LH	Luteinizing Hormone
MIRD	Monash Institute of Reproduction and Development
MI	Metaphase I
MII	Metaphase II
NT	Nuclear Transfer
P_4	Progesterone (P4)
PA	Parthenogenetic
PB	Polar Body
PBS	Phosphate buffered solution
pLH	Porcine Luteinizing Hormone
pFSH	porcine Follicle Stimulating Hormone
PN	Pronucleus
PSOF	Possum modified synthetic oviduct fluid
SOF	Synthetic oviduct fluid
TCM-199	Tissue culture medium 199
UV	Ultraviolet

Chapter 1

Literature Review

Wombats present a dichotomy in wildlife management. In many areas, the Common and Southern Hairy-nosed Wombats are considered an agricultural problem (Stott 1998; Marks 1998b) while the Northern Hairy-nosed Wombat is critically endangered (Horsup and Davidson 1994; Horsup 1998). Manipulation of reproduction offers the means to both increase the productivity of endangered species and also to control highly abundant and pest species (Mate *et al* 1998). Accumulation of knowledge for the abundant wombat species may be beneficial for the management of all three species. It is hoped by understanding the reproduction of the Common Wombat that we will establish methods to manipulate reproductive function in order to develop assisted reproductive techniques for the critically endangered Northern Hairy-nosed Wombat. Conversely, this knowledge will potentially allow us to devise strategies to manage the perceived high abundance of the common and southern hairy nosed wombats. This chapter reviews our current knowledge of reproduction in the abundant Common Wombat and also reviews superovulation as a method for collecting mature oocytes from marsupials, that may be used for assisted reproduction.

1.1 Wombats

Wombats are large, burrowing, herbivorous marsupials (Strahan 1995;Triggs 1996; Johnson 1998). Currently, three species of wombats are recognised, and all are found in Australia (Figure1.1). The Southern Hairy-nosed Wombat (Figure 1.2) is considered common but limited and is found in fragmented populations in southern and semi-arid areas west of the Murray River in South Australia extending into Western Australia (Wells 1995; McGregor and Wells 1998; St John 1998). The Common Wombat (Figure 1.2) is also considered common and is found in a variety of habitats including sclerophyll and coastal areas of south-eastern Australia, extending from southern Queensland to South Australia, Flinders Island and Tasmania (McIlroy 1973; McIlroy 1995; Williams and Menkhorst 1995; Green and Rainbird 1978b; Triggs 1996).

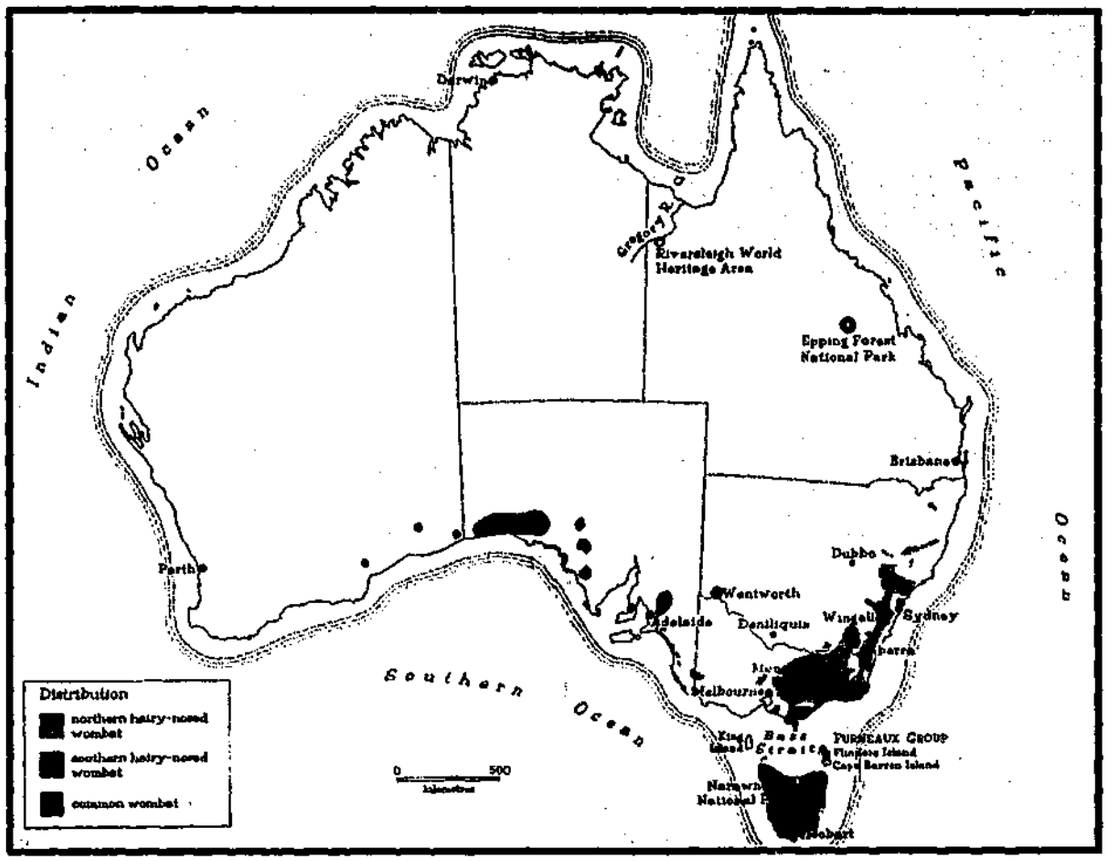


Figure 1.1. Current distribution of Wombats throughout Australia. (Modified from Woodford 2001)

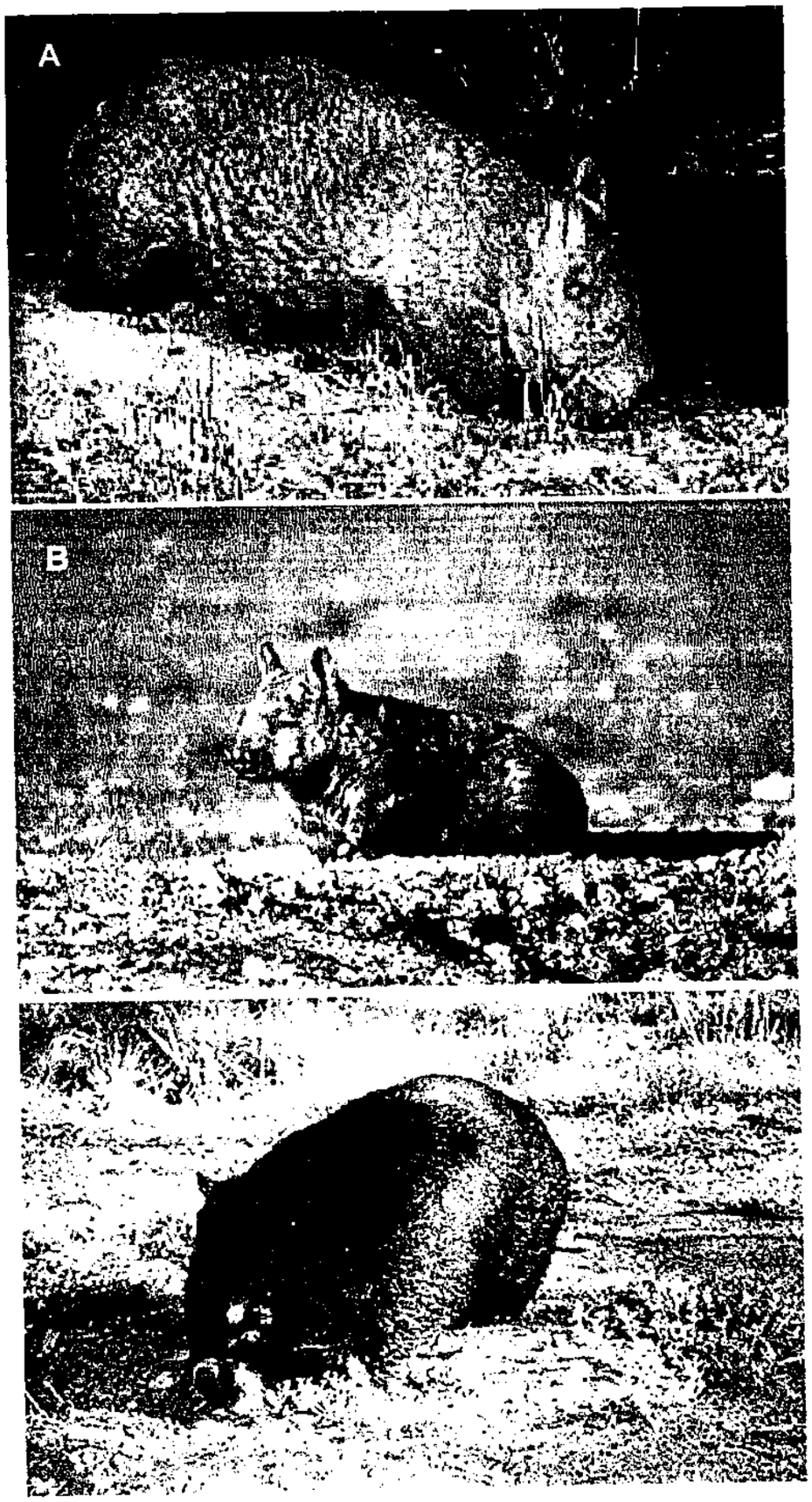


Figure 1.2. Wombat species. A: Northern Hairy-nosed Wombat (*Lasiorhinus krefftii*), B: Southern Hairy-nosed Wombat (*L. latifrons*) and C: Common Wombat (*Vombatus ursinus*).

The Northern Hairy-Nosed Wombat (Figure 1.2) is considered endangered under Queensland's Nature Conservation Act 1992, and is currently restricted to an isolated population on Epping State Forest National Park (Horsup 1997; Horsup 1998). The current population is estimated to be approximately 113 individuals (A Horsup *pers comm*), although the elusive nature of this animal has meant that clever censuring methods are required (Sloane *et al* 2000). A severely skewed sex ratio of more than twice the number of males to females (2.3:1) (Horsup 1998) restricts the rate of optimal population growth by natural breeding (Taylor *et al* 1998). Compounding problems of drought, introduced grass, predators, and restriction to a forest, are all considered to place the Northern Hairy-nosed Wombat at high risk of extinction (Johnson and Crossman 1991; Crossman *et al* 1994; Hoyle *et al* 1995; Horsup 1997; Horsup 1998).

A Northern Hairy-nosed Wombat Recovery Team was established to develop and coordinate strategies to protect the species, with the major objective of increasing the population, and preventing its extinction (Horsup and Davidson 1994; Horsup 1997; Horsup 1998). The recovery plan includes a combination of habitat management, population monitoring, education and goals for captive management and assisted reproduction in the closely related Southern Hairy-nosed Wombat (Horsup and Davidson 1994). Initial attempts to capture animals to establish a captive population have been unsuccessful, despite one female animal surviving in captive conditions for 27 years (Woodford 2001). Habitat management and assisted reproduction are the Northern Hairy-nosed Wombats best chance for survival. Several groups have been investigating assisted reproduction in the related wombat species. The Southern Hairy-nosed Wombat is the preferred species as it is phenotypically, and genetically, most similar to the Northern Hairy-nosed Wombat (Taylor *et al* 1994; Horsup 1998). All three wombat species are closely related and the Common Wombat may also provide a useful model.

1.2 Reproduction in the Common Wombat

The Common Wombat is monovular (Moritz *et al* 1998), gives birth to a single young (Boer 1998) and has a gestation length estimated to be one month (Green and Rainbird 1987a; Triggs 1996). To date, no studies have investigated fertilisation and

normal embryo development in the Common Wombat, although Hughes and Green (1998) have studied the development of prenatal wombats. Like the Koala, the Common Wombat develops in the uterus with dual placental structures, consisting of a yolk-sac placenta and a chorio-allantois (which non-invasively interfaces the uterus lumen) (Hughes and Green 1998). The prenatal Common Wombat is morphologically similar to the macropod marsupials and the Koala.

Mating behaviour, birth of the neonate, development of the pouch young and young-at-foot until they are weaned has been thoroughly described by Conder (1970), McIlroy (1973), Presidente (1982), Green and Rainbird (1987a), Taylor (1993); Triggs (1996), Boer (1998) and Marks (1998a). In short, mating appears to occur outside the burrow, the male chases the female and copulation occurs with both animals lying on their sides. After gestation, the female sits in a birth position, squatting on her hind quarters, after birth the naked neonate (~0.5g and ~15mm) crawls into the pouch from the cloaca and attaches to one of the teats. It then develops within the pouch for 6 months (~1-1.5kg) until it has fine hair and begins to peer out of the pouch. By 7 months (2.2-2.5kg) - 8 months (2.7-3.4kg) the pouch young begins to venture out of the pouch, and by 9 (3.6-5.2kg) – 10 (5-6.4kg) months the young wombat permanently leaves the pouch when it is then considered a young-at-foot (YAF) or juvenile. The YAF is weaned by 12 (7.3-11.3kg) –15 (12-19kg) months and remains with the mother until ~ 18 months (16.8 - 24kg) when it is then considered a subadult. Moritz *et al* (1998) confirm the finding by Peters (1977) that the Common Wombat enters a phase of anoestrous during lactation and post-lactation.

Previous behavioural observations by Boer (1998) suggested that oestrus was observed at approximately monthly intervals. The low body weights (18-21kg) of the wombats in his study suggested that they were originally caught in Tasmania, and is therefore consistent with the oestrous cycle length determined by Peters (1977) to be 32 - 34 days. Peters (1977) determined the oestrous cycle length of the Common Wombat by comparison of changes in vaginal cytology, pouch morphology, behaviour and body temperature. Although limited to 8 oestrous cycles, Peters (1977) found that the Common Wombat is polyestrus, and appeared to be seasonal with oestrous cycles observed between April and August with an anoestrous period between August until at least December (when the study ended). In contrast, Green

and Rainbird (1987a) found that young were produced throughout the year in Tasmania with a peak in births occurring between October and January. This has since been confirmed by Moritz *et al* (1998). Taylor (1993) also observed mating behaviour in northeastern Tasmania in December.

In Victoria, Nicholson (1963) found that wombats are born between late March and June in the Mt Buller area. Presidente (1982) found that in central Gippsland, Victoria, young were born between June - July and December. Our group has found that births occur throughout the year in the Myrtleford area of Victoria, although most births occurred between December and February (unpublished observations). McIlroy (1990) suggests that breeding dates vary along a north-south axis with breeding occurring later in the southern areas of the range.

The endocrine control of female reproduction has not yet been systematically studied in a wombat species. Peters (1977) attempted to measure progesterone and oestrogen concentrations in urine with limited success. Despite some knowledge of the general oestrous cycle in the Common Wombat in Tasmania, a similar study has not yet been undertaken on Victorian Common Wombats. Considerably more data has been collected on the control of oestrous in other marsupial species, which is reviewed in this chapter.

Several methods of assisted reproduction are currently under investigation in the Common Wombat (M Cleary *pers comm*, C Macallum *pers comm*) and the Southern Hairy-nosed Wombat (Taggart *et al* 1998; Hamilton *et al* 2000). This chapter reviews the collection of mature oocytes by the superovulation of marsupials, which may be used for assisted reproduction. A basic understanding of marsupial reproduction and the oestrous cycle is required to aid the development of these assisted reproductive techniques.

1.3 Oestrous cycle monitoring in marsupials

The oestrous cycle is the reproductive process that occurs in female mammalian species that display oestrus. It is a highly synchronized, progressive and repetitive cascade of hormonal, behavioural and underlying ovarian events (Kilen and Schwartz

1999). These ovarian events include progressive follicle growth and maturation, oocyte ovulation from the follicle and subsequent luteinization of the ruptured follicles producing the corpus lutea (Kilen and Schwartz 1999).

Knowledge of the marsupial oestrous cycle is becoming increasingly important for both the conservation of endangered species and the control of highly abundant marsupials.

Much of our current knowledge of oestrous cycles has been studied in eutherian mammals, although there is now considerable knowledge in marsupials (Tyndale-Biscoe and Renfree 1987). Studies investigating oestrous cycles have identified that in most marsupials, oestrus and ovulation occur spontaneously, most marsupials are polyestrus and monovular, and behavioural oestrus normally precedes ovulation by 1 to 2 days (Hinds *et al* 1996). However there is some variation, as many species, including those of Dasyuridae and Peramelidae families and some small possum species (such as the Honey Possum (*Tarsipes rotratus*)), are considered to be polyovular (Renfree and Shaw 1999) and recently the Koala has been shown to be an induced-ovulator, requiring the physical act of mating (Johnson et al 2000).

Areas of female marsupial reproduction that still require attention include the precise timing of mating and ovulation particularly with respect to hormonal changes (Rodger 1990, Hinds et al 1996, Molinia et al 1998). Basic reproductive knowledge of the oestrous cycle been studied in only a few marsupials species (Table 1.1; Tyndale-Biscoe and Renfree 1987). The oestrous cycle has been most thoroughly researched in the Tammar Wallaby (*Macropus eugenii*) and the Common Brushtail Possum (*Trichosurus vulpecula*).

The ratio of oestrogen to progesterone, the peaks and troughs of oestrogen and progesterone levels, the luteinizing hormone (LH) surge, follicular maturation, ovulation, corpus luteum formation and regression, associated reproductive tract changes and behavioural changes are all integral parts of the oestrous cycle of mammals including marsupials. These hormonal, morphological and behavioural changes can be monitored to study the oestrous cycle of marsupials.

Table 1.1. Examples of the length of oestrous cycle, gestation, ratio of the length of gestation to the oestrous cycle, the follicular phase and the number of oocytes ovulated at the end of each follicular phase. (Modified without permission from Table 2.2 by Tyndale-Biscoe and Renfree 1987, who compiled this table from other studies, as the authors have referenced.)

Species	Oestrous cycle	Gestation	Ratio	Follicular phase	Ovulation number
Didelphis virginiana	25.5 (22-34)	13	0.51	7-17	22
Marmosa robinsoni	25.5	13.5	0.53	16.0	20
Monodelphis domestica	32.3	13.5	0.42	14.4	?
Dasyurus viverrinus	37	19	0.51	20	7-35
Dasyuroides byrnei	60	30-31	0.51	-	11
Sminthopsis crassicaudata	31	13-16	0.47	14-16	14
S. macroura	26	12.5	0.48	-	30-40
Antechinus stuartii	-	27	-	-	11-19
Gymnobelideus leadbeateri	<30	<20	0.60	-	12
Petaurus breviceps	29	16	0.55	12	>2
Trichosurus vulpecula	25.7	17.5	0.68	8	1
T. caninus	26.4	16.2	0.61	10-11	1
Perameles nasuta	21 (10-34)	12.5	0.60	5-10	3.3
Isoodon macrourus	20.5 (9-34)	12.5	0.61	10-20	5.1
Macrotis lagotis	20.4 (12-37)	14 (13-16)	0.69	-	-
Bettongia lesueur	23	21	0.91	22	1
B. gaimardi	23.2	21.1	0.91	-	1
B. penicillata	22-23	21	0.93	-	1
Potorous tridactylus	42	38	0.90	-	1
Aepyprymnus rufescens	21-36	21-30	-	20.6	1
Setonix brachyurus	28	27	0.96	26-27	1
Thylogale billardierii	30.3	30	0.99	-	1
Petrogale penicillata	32	31	0.97	31	1
Marcropus parryi	42.2	36.3	0.86	6	1
M. giganteus	45.6±9.8	36.4±1.6	0.80	10.9±4.8	1
M. fuliginosus	34.9±4.4	30.6±2.6	0.88	8.3±5.8	1
M. eugenii	30.6	29.3	0.96	30.4	1
M. rufogriseus	31.9	29.4	0.92	29.6	1
M. agilis	32.4	29.4	0.91	29.8	1
M. rufus	34.8±0.6	33.2±0.2	0.95	34.7±0.3	1
Wallabia bicolor	32.6±3.6	35.5±2.3	1.09	24.8±3.4	1

The following is an account of previously utilised methods for monitoring the oestrous cycle in marsupials. Some methods are considered relatively non-invasive and include monitoring behaviour, urine sampling and faecal sampling. Other methods that are invasive include monitoring morphological changes via laparoscopy, monitoring body weight, pouch condition, temperature, blood sampling and vaginal smears. This review is not intended to discuss all species, or the specific methodology of the various techniques, but rather to discuss the use of each method.

1.3.1 Monitoring oestrous cycles by behavioural observations

Mating behaviour displayed by marsupials is a sign that females are in oestrus, they become receptive to the male and ovulation will occur soon after (Tyndale-Biscoe and Renfree 1987). Cyclic changes in behaviour may be observed. In the predominantly nocturnal, Common Wombats (*Vombatus ursinus*), females may become active during the day and more active during the night. This is suggested to occur at or about the time of oestrus (Peters and Rose 1979). Vocalisation has also been reported in some species such as the Koala, which is observed to bellow when in oestrus (Johnson *et al* 2000). The reproductive cycle has also been monitored in captive Common Brushtail Possums using time-lapse video surveillance and whilst it was considered effective it was also considered labour intensive (Duckworth *et al* 1998).

Monitoring oestrus by behavioural observations is advantageous in that handling of the animal is not required. However, this method usually requires the animal to be in a captive environment which may effect the reproductive cycle, especially in shy animals or environments in which natural environmental cues can not be maintained or mimicked (Rose and Jones 1996).

1.3.2 Monitoring oestrous cycles by urine analysis

A urine sample can be utilized to determine oestrus and mating behaviour in marsupials. In urine samples a significant increase in the presence of keratinised and non-keratinised epithelial cells and leukocytes occur at various stages during the oestrous cycles and sperm may be present if mating occurs (Duckworth et al 1998, Fletcher 1989). In particular a significant increase is noticed in the number of

keratinised (or cornified) epithelial cells a few days before oestrus and then a decrease several days after ovulation. This has been the general pattern observed in several other marsupials including Brown Antechinus (*Antechinus stuartii*) (Wooley 1971, Selwood 1980) Fat-tailed Dunnart (*Sminthopsis crassicaudata*) and the Stripe-faced Dunnart (*Sminthopsis macroura*) (Selwood 1987, Selwood and Wooley 1991), Kowari (*Dasyuroides byrnei*) (Fletcher 1989) and Common Brushtail Possum (Duckworth *et al* 1998).

Whilst providing similar information, urine sampling was considered easier, faster and less stressful upon the animal than vaginal smears in Common Brushtail Possums (Duckworth *et al* 1998). Urine sampling is very useful for smaller marsupials due to the size of the urogenital openings and as with Common Brushtail Possums the openings of the urethra and the vagina are very close in the urogenital sinus (Duckworth *et al* 1998).

Urine sampling can also be utilized to monitor reproductive hormones as a technique for monitoring oestrus however this method is considered labour intensive (Desauliniers *et al* 1989). Steroids were preliminarily examined in the urine of Common Wombats that were restrained in metabolic cages. Oestrogen was identified around the time of oestrus, however at other times it could not be detected (Peters 1977).

The main disadvantage of this method is that the animal requires restraining whilst the urine sample is taken and such repetitive handling may affect the reproductive cycle.

1.3.3 Monitoring oestrous cycles by faecal steroid analysis

Faecal steroid analysis offers a non-invasive method of monitoring the oestrous cycle of marsupials and assesses the excreted reproductive hormones. Monitoring reproductive hormones by this method therefore requires knowledge of the steroid metabolites that are excreted which can vary between species. This method has been successfully used in numerous eutherian species (Brown *et al* 1994), although it has not been extensively investigated in marsupials.

Faecal steroid analysis has also been attempted in some marsupial species including the female Tammar Wallaby (Connell 1999) and the male Southern Hairy-nosed Wombat (*Lasiorhinus latifrons*) (Hamilton 1997). Progesterone metabolites were identified by Connell (1999) in the faeces of Tammar Wallaby. These levels correlated to progesterone concentrations in the blood and hence may be used to monitor oestrus.

The main disadvantage of this method is the time delay encountered prior to interpretation of the oestrous cycle stage. This is due to the lag-time between hormones in the circulatory system and the metabolic products that are consequently excreted (12 hrs to 2 days in eutherians) and also the time taken for sample analysis (Schwartzenberger *et al* 1997).

1.3.4 Monitoring oestrous cycles by blood samples

Reproductive hormones circulate in the blood and as they are highly synchronized with ovarian and behavioural changes they are used for studying the oestrous cycle of marsupials. Lemon (1972) found that progesterone measured in the peripheral plasma in the Tammar Wallaby could be utilized to monitor the oestrous cycle.

Other hormones have also been studied in marsupials that may be utilized to investigate the oestrous cycle. In order to investigate post partum oestrus (and parturition) in the Tammar, progesterone, oestradiol-17β and LH have been monitored in the peripheral blood (Flint and Renfree 1982; Shaw and Renfree 1984; and Harder *et al* 1985). Blood was sampled from the lateral tail vein and hormone concentrations were determined by radioimmunoassays. This investigation clarified the control of oestrus and ovulation in the tammar. A decline in peripheral progesterone occurs followed by a rise in oestradiol-17β 8 hours later and a shift to an elevated oestradiol: progesterone ratio. Seven hours after the oestradiol peak an LH surge occurs and ovulation occurs 24 hours later. Oestrus behaviour occurred ~30hours after ovulation. These results are further supported by Horn *et al* (1985) who monitored the concentrations of follicle stimulating hormone (FSH) and LH in the peripheral circulation.

The local transfer of steroids between the ovarian vein and the uterine artery is suggested to result in a decreased concentration of steroids entering the peripheral circulation when produced in the ovaries (Curlewis *et al* 1985, Towers *et al* 1986). For this reason greater sensitivity may be observed by sampling blood from the urogenital vasculature. Towers *et al* 1986 monitored local steroid concentrations in the uterine branch of the ovarian vein of the female Tammar Wallaby. In the Tammar Wallaby, Hinds *et al* (1983) found that the highest concentration of progesterone was seen in the uterine vein 5 days after ovulation. Towers *et al* (1986) demonstrated that the highest concentration of progesterone was secreted from the ovary in which ovulation occurred, supporting that it was secreted from the active and newly formed corpus luteum. Shaw and Renfree (1984) and Harder *et al* (1984) both found that the pre-ovulatory follicle was the main source of oestradiol in the peripheral plasma.

In another species, in which oestrus has been monitored by blood hormone levels Curlewis *et al* (1985) monitored progesterone and oestradiol in the ovarian venous and peripheral plasma of Common Brushtail Possums. They found that the oestrous cycle of the Common Brushtail is characterised by a single peak of oestradiol around the time of oestrus followed by increasing levels of progesterone during the luteal phase.

In addition to the previously mentioned species oestradiol profiles have also been studied in other species including the Virginia Opossum (Harder and Fleming 1981), Red-necked Wallaby (*Macropus rufogriseus*) (Walker and Gemmell 1983) and the Koala (Johnson *et al* 2000). In all marsupial species studied so far, the highest oestrogen levels appears to be secreted towards the end of the follicular (pro-oestrus) phase with the formation of the mature Graafian follicles.

In contrast to oestrogen, variation occurs in the progesterone profile of marsupial species. Progesterone levels slowly increase after oestrus reaching a peak soon after the middle of the luteal phase and then slowly decline in the Virginia opossum (Harder and Fleming 1981) and in the eastern quoll (Hinds 1983). In other marsupials including the tammar wallaby (Lemon 1972, Hinds and Tyndale-Biscoe 1982a, Harder et al 1985) and the quokka (Cake et al 1980) the progesterone peak occurs early in the luteal phase following ovulation.

As opposed to the gas chromatography and radioimmunoassays that have been previously utilized (Curlewis *et al* 1985), more sensitive assays may provide further insight into the hormone profiles of various marsupial species during oestrous cycles. This would be particularly useful for species with very low hormone concentrations. Recent studies in marsupials have found that assay kits developed for the study of eutherian species can be used (Johnston *et al* 2000), and although validation is still required, samples can be processed more quickly.

The main disadvantage of monitoring the cyclic endocrine changes during the oestrous cycle is that blood sample collection requires, physical or chemical restraint of the animal, which may effect the duration of the cycle (Hinds *et al* 1983, Fletcher 1983).

1.3.5 Monitoring oestrous cycles by Vaginal Smears

Vaginal smears may be used to monitor the oestrous cycle, as at various stages of the cycle, differences in the populations of cells collected can be observed. In marsupials, smears are taken from the posterior vaginal sinus or the urogenital sinus (Peters and Rose 1979). Such studies investigate the presence of epithelial cells, polymorphonuclear leukocytes and sperm in daily vaginal smears, which are synchronised with ovarian and endocrine changes (Duckworth *et al* 1998).

Peters and Rose (1979) utilized vaginal cytological changes during the oestrous cycle to correlate against the basal body temperature of Common Wombats. Pro-oestrus, oestrus and post-oestrus could all be distinguished in this study. In particular the first occurrence of a fully cornified smear (composed entirely of mature squamous or superficial cells) was considered to represent the day of oestrus. This method also permits the differentiation between anoestrus and pro-oestrus (Peters and Rose 1979).

Vaginal smears have been studied in several marsupial species during oestrus including Quokka (Waring *et al* 1955, Sharman 1955), Common Brushtail Possums (Pilton and Sharman 1962), Red Kangaroo (*Macropus rufus*) (Sharman and Calaby 1964), Eastern Grey Kangaroo (*Macropus giganteus*) and Western Grey Kangaroo (*Macropus fuliginosus*) (Poole and Catling 1974), Agile Wallaby (*Macropus agilis*)

(Merchant 1976), Common Wombat (Peters and Rose 1979), Red-necked Wallaby (*Macropus rufogriseus*) (Merchant and Calaby 1981), Tasmanian Pademelon (*Thylogale billardierii*) (Rose and McCartney 1982), Grey Short-tailed Opossum (*Monodelphis domestica*) (Fadem and Rayne 1985), Yellow-footed Rock-Wallaby (*Petrogale xanthopus*) (Poole et al 1985), Wallaroo (*Macropus robustus*) Kowari (*Dasyuoides byrnei*) (Fletcher 1989), Tammar Wallaby (Poole et al 1992) and Tasmanian Bettong (*Bettongia gaimardi*) (Rose and Jones 1996). In all these species the percentage of cornified (superficial type) epithelial cells significantly increases at oestrus. The percentage of cornified (superficial type) cells then slowly decreases during the end of the follicular or start of luteal phase, and remains low into the start of the next follicular phase.

Poole *et al* (1992) suggested that following the development of a complete data set of daily smears through experience, differences between daily smears become obvious and that diagnosis of oestrus can be achieved with relatively few smears taken. A quantified analysis of vaginal smears is considered to be a simple and precise method for monitoring oestrous cycles in marsupials, however for some species the regular capture and handling of females may effect the duration of their cycle (Peters and Rose 1979, Rose and Jones 1996).

1.3.6 Monitoring oestrous cycles by body weight

Body weight has been observed to fluctuate during the oestrous cycle of the kowari (*Dasyuroides byrnei*). An increase in body weight occurs and peaks just prior to oestrus and then drops during ovulation and early luteal formation and then increases towards the end of the luteal phase and then steeply declines and the follicular phase is re-entered (Fletcher 1989). Similar changes in body weight have also been monitored in other small Dasyuridae species such as the Stripe-faced Dunnart (Hickford *et al* 2001), and its use to monitor oestrous activity is mostly restricted to the smaller marsupials.

1.3.7 Monitoring oestrous cycle by pouch and urogenital condition

In many marsupials, the pouch condition changes throughout the oestrous cycle. The pouch becomes moist and in some species such as the Eastern Quoll this is considered to coincide with oestrus (Hill and O'Donoghue 1913). The pouch of common

wombats was observed to become noticeably dirty by 10 days after oestrus and then clean again between 9 and 6 days prior to oestrus (Peters and Rose 1979). Unless in post-partum oestrus the pouch of Common Wombats appears similar in pro-oestrus and in anoestrus being shallow and having inverted teats (Moritz *et al* 1998). Changes in nipple length and mammary swelling is observed in marsupials at different stages of their reproductive cycle (Tyndale-Biscoe and Renfree 1987, Moritz *et al* 1998) however no apparent changes were observed in Common Wombats during the oestrous cycle (Peters and Rose 1979).

At oestrus, swelling of the vulva occurs in the eastern quoll (Hill and O'Donoghue 1913). At late pro-oestrus in Common Wombats the urogenital sinus became partially everted and protruded and was engorged and moist (Peters and Rose 1979). Similar observations were made in the Koala by Brown (1987); however subsequently this was not supported by Johnston *et al* (2000) finding that changes where not reliable or consistent.

In the Kowari, oestrus may be timed as vaginal bleeding occurs approximately 30 days following oestrus (Fletcher 1989) however this seems to be an anomaly amongst marsupials and is considered to represent a 'pseudo-birth' (Tyndale-Biscoe and Renfree 1987) marking the end of the luteal phase.

Other signs of oestrus may be observed when monitoring the vagina. The presence of a copulatory plug (clumped sperm) indicated that mating had taken place in the Tammar Wallaby (Molinia *et al* 1998b). The daily examination of the vagina is therefore required to determine the time of oestrus. This is even more important in the Eastern Quoll in which the copulatory plug may last for up to 14 days after copulation, which occurs at oestrus (Hill and O'Donoghue 1913). The presence of sperm around the urogenital sinus was used to estimate oestrus behaviour and mating date in the Common Brushtail Possum by Gemmell *et al* (1987). Despite these observations at oestrus, Poole *et al* (1992) considered that vaginal smears are more dependable than reliance on chance observations of mating or a retained seminal plug.

1.3.8 Monitoring oestrous cycles by basal body temperature

Peters and Rose (1979) monitored the oestrous cycle in the Common Wombat (*Vombatus ursinus*) by measurement of the basal body temperature and concluded that reproductive events are accompanied by changes in body temperature (approximate range: 33 to 36°C). In particular they noted that temperature remained low for the week following oestrus. One postulate is that raised basal body temperature during the oestrous cycle is due to a thermogenic effect of progesterone; this is supported by coinciding peaks in progesterone secretion and peaks in body temperature (Rose and Jones 1996) which are seen shortly after oestrus in the Tasmanian Bettong. In Tasmanian Bettongs, the basal body temperature remained high through out the oestrous cycle and until two days before oestrus. A small peak occurred at the time of oestrus, which is followed by an increase on day three after oestrus from which point the temperature remained high until the next 2 days prior to oestrus. Similar temperature patterns have also been seen in the Virginia opossum (Treagust *et al* 1979).

To avoid restraint of the animal, some studies have surgically implanted temperature sensitive telemetric transmitters to relay changes in body temperature in the Common Wombat (Peters and Rose 1979), the Tasmanian Bettong (Rose and Jones 1996) and the Common Brushtail Possum (Halse and Rose 1988). This has been considered to be a useful method of assessing the oestrous cycle particularly in Tasmanian Bettong. However, Halse and Rose (1988) found that basal body temperature did not indicate oestrus or ovulation in the Common Brushtail Possum suggesting that the reason for this may be that the Common Brushtail maybe less susceptible than other species to temperature changes caused by hormonal influences.

The monitoring of basal body temperature may successfully infer the stage of oestrus in some marsupials. However, for many species, the time of oestrus can only be determined in retrospect (Rose and Jones 1996).

1.3.9 Monitoring oestrous cycles by laparoscopy

Laparoscopy is perhaps the most invasive method of monitoring oestrus through observable changes in morphology and permits the study of organs *in vivo*. Its use seems restricted to gross changes in morphology at or on the surface of an organ.

This is a useful method for studying oestrous cycles as it permits the examination of the ovaries and other reproductive organs that conventionally have been studied by removal and histological analysis. Therefore, in contrast to histological analysis, laparoscopy permits observation of the progressive ovarian changes within a single animal. For this reason histological analysis is not considered a useful method for monitoring oestrous cycles.

The presence or absence of mature follicles, rupture points, corpus lutea and corpus albicantia can all be monitored by laparoscopy of the ovaries and therefore indicate stages of the oestrous cycle (Mate and Rodger 1993, Crawford *et al* 1997, Molinia *et al* 1998a). A potential disadvantage of this method is that the anaesthesia used may inhibit ovulation, although Crawford *et al* (1997) did not find that ovulation was affected, which is also supported by studies in sheep (Clarke and Doughton 1983). Laparoscopic investigations of Tammar Wallabies found that ovarian activity could be monitored by laparoscopy without disturbing normal cycling (Rodger *et al* 1993). This is supported by Crawford *et al* (1997), who monitored pre-ovulatory follicular development and ovulation in the Common Brushtail Possum, by repeated laparoscopy. Fletcher (1983) also examined Kowaris by laparoscopy to note ovarian and uterine change in hormonally stimulated animals.

The major disadvantages of monitoring the oestrous cycle by laparoscopy, are that it is invasive, that the surgical procedure must be performed on more than one occasion, and therefore it is potentially stressful to the animal. In addition, laparoscopy is also considerably more time consuming than other less invasive monitoring techniques.

1.3.10 Potential alternative methods for monitoring the oestrous cycle

Although potentially useful, methods like saliva sampling and ultrasound have not been utilized to monitor the oestrous cycle in marsupials, but have been investigated in eutherian species. Stages of the ovarian (menstrual) cycle have been successfully determined in the human and the cow (Lu *et al* 1999) by measuring oestradiol and progesterone concentrations in saliva. Oestrus cycling has also been successfully monitored in species such as the threatened Canadian Wood Bison (*Bison bison athabascae*) by using ultrasound, which permits the observation of changes in the ovary without surgery (Othen *et al* 1999).

1.3.11 Oestrous cycle monitoring - overview

All the methods discussed in this review have limitations and no single method is considered appropriate for all marsupial species. Some methods may provide an incomplete observation of the stage of the oestrous cycle and for this reason a combination of supportive methods may be required. A significant data set is required of methods prior to a determination of the stage of the oestrous cycle of a species. As there are often differences between species for a particular method, results should be considered species specific. Differences also occur between individuals as many factors can influence the oestrous cycle including stress and nutrition (Pollard and Cairncross 1977; Folman et al 1983; Domanski et al 1989; Van Niekerk and Van Niekerk 1997).

Methods of monitoring oestrous cycles in marsupials have permitted the characterization of the length of the oestrous cycle and of its components, the follicular and luteal phases. Studies have also permitted the determination of the hormonal profiles, morphological and physiological changes of the reproductive organs of many species. An even greater number of marsupials are yet to be studied, which will permit a greater understanding of marsupial reproductive function, and the utilization for management of these species.

1.4 Introduction to Superovulation in Marsupials

Superovulation is the artificial induction of ovulation by the administration of hormones to recruit and stimulate the maturation of follicles (Kemper Green *et al* 1996; Kafi and McGowan 1997; Mate *et al* 1998). Both the number of mature follicles that ovulate and the timing of ovulation are manipulated during this process. The number of follicles recruited to produce mature oocytes may be increased to exceed natural ovulation rates thereby increasing female productivity (Rodger 1990; Kemper Green *et al* 1996; Mate *et al* 1998). This follicular recruitment may be induced at times when natural ovulation would not normally occur, such as periods of anoestrus or at alternative stages during the oestrous cycle (Rodger and Mate 1988; Molinia *et al* 1998b).

Manipulation of ovulation in this manner has become an invaluable tool for assisted reproduction of many vertebrate species, (Rodger 1990; Kemper Green *et al* 1996). Whilst superovulation is currently clinically practiced for human infertility and domestic animal production, it is now being investigated for use in other animals, particularly for the preservation of endangered species (Mate *et al* 1998, Hatasaka *et al* 1997).

Superovulation of a marsupial was first used to breed species for laboratory purposes and to study early marsupial development (Nelson and White 1941; Smith and Godfrey 1970; Renfree *et al* 1988; Hayman *et al* 1990; Rodger *et al* 1992a). Marsupial superovulation is now being developed to explore artificial reproduction of endangered species and to investigate the regulation of reproduction in species considered overabundant (Rodger 1990; Mate *et al* 1998). Both lines of research rely on the collection of oocytes and significant progress is now being made by several research groups; such as the Marsupial Cooperative Research Centre (Marsupial CRC, Canberra). The goal of superovulation in marsupials is to determine simple and reliable methods to induce ovulation and to increase the yield of viable oocytes.

The first reported attempt at superovulation in a marsupial was conducted in 1941 to improve the captive breeding of the Virginian Opossum (*Didelphis virginiana*) (Nelson and Maxwell 1941, Nelson and White 1941). To date, superovulation has been investigated in eight marsupials (Section 1.4.2). Superovulation has been most thoroughly investigated in two marsupial species, the Common Brushtail Possum (*Trichosurus vulpecula*) and the Tammar Wallaby (*Macropus eugenii*).

In 1990, Rodger briefly reviewed superovulation in marsupials. It was noted that many of the early efforts since the 1940's failed to develop a practical and reliable method that yielded mature oocytes due to excessive administration gonadotrophins. In 1988, using a small sample population of Common Brushtail Possums, Rodger and Mate (1988) demonstrated that a simple superovulation method could induce ovulation. Further investigations into superovulation in several marsupial species have been undertaken by a number of investigators, who have collectively concluded that systematic studies were required to focus on the timing of administration of

exogenous hormones and how these hormones affected normal behavioural oestrus and the hormonal milieu (Hinds *et al* 1996; Mate *et al* 1998).

1.4.1 Underlying physiology and endocrinology of superovulation

The underlying physiological principle of superovulation is to recruit follicles to mature and then stimulate them to ovulate. This approach is similar to the natural follicular phase of an oestrous cycle leading to ovulation, in which most follicles developing towards a pre-ovulatory stage undergo atresia (follicular death) and ovulation of a dominant follicle occurs (Greenwald and Terranova 1988; Hillier 2001). Superovulation attempts to induce more follicles to reach the pre-ovulatory stage and to induce as many of them to ovulate as possible. Superovulation is therefore considered to 'rescue' follicles by recruiting them prior to atresia (Hirshfield 1989; Kemper Green *et al* 1996). This is almost certainly the case in monovular species in which ovulation of one oocyte occurs per cycle. Some species are, however, naturally polyovular (ovulating more than one oocyte) and the effectiveness of superovulatory methods may be difficult to judge in these species, given that the number of oocytes that are recruited may be similar to that naturally ovulated (Rodger 1990, Hinds *et al* 1996, Mate *et al* 1998).

The natural maturation of follicles and induction of ovulation involves a complex cascade of hormonal events involving a feedback system between the hypothalamic, pituitary and ovarian tissues (Greenwald and Terranova 1988; Johnson and Everitt 2000). In summary, an increase in the frequency of luteinizing hormone (LH) and follicle stimulating hormone (FSH) secretion from the pituitary coincides with the recruitment of follicles for further growth and potential ovulation (Chikazawa et al 1986, Hillier 1994). Most follicles become less sensitive to FSH and undergo atresia whilst a few become increasingly sensitive to FSH and LH and continue to mature (Drummond and Findlay 1999, Web *et al* 1999). FSH secretion regulates aromatase activity, which drives oestrogen production by the granulosa cells of the maturing follicles. Increased production of oestrogen then leads to proliferation of follicular cells and results in the development of a dominant follicle (Greenwald and Terranova 1988; Johnson and Everitt 2000). Only upon maturation of the dominant follicle and formation of the pre-ovulatory follicle, also known as the Graafian follicle, is

sufficient oestrogen secreted to act on the hypothalamus and the pituitary to produce an LH surge that induces ovulation (Caraty *et al* 1995; Hillier 2001). The LH surge is considered to stimulate meiotic and cytoplasmic maturation of the oocyte within the pre-ovulatory follicle and stimulate the final increase in follicular growth prior to ovulation (Johnson and Everitt 2000; Hillier 2001).

The integral role of the gonadotrophins, FSH and LH, in the natural female reproductive cycle is the basis for their use in the artificial manipulation of follicular recruitment and ovulation and therefore superovulation (Hillier 1994). This is also the basis for the use of other hormones with FSH or LH activity or those that stimulate the synthesis and secretion of FSH or LH. Pregnant mare's serum gonadotrophin (PMSG or PMS) or equine chorionic gonadotrophin (eCG) is produced by horses and although it has LH specific activity in the horse, in all other species tested it binds to both FSH and LH receptors producing biological effects of both (Murphy 1991). Human chorionic gonadotrophin (hCG) on the other hand, binds to LH receptors thereby causing LH biological effects (Stewart 2001). Gonadotrophin releasing hormone (GnRH also called LHRH) is also used, which stimulates the LH secretion that induces ovulation (Caraty 1995) and has been shown to induce an LH surge in the Tammar Wallaby (Evans *et al* 1980). GnRH secretion normally occurs from the hypothalamus in response to oestrogen stimulation.

Most of these studies of the natural control of ovulation its artificial manipulation, have been undertaken in eutherian species such as the cow, although an increasing number of studies are investigating marsupials species.

1.4.2 Marsupial species in which superovulation has been investigated

1.4.2.1 Common Brushtail Possum (*Trichosurus vulpecula*)

Harding (1969) conducted the first investigation into superovulation in a monovular marsupial the Common Brushtail Possum. This first study was relatively unsuccessful. PMSG (between 12.5 IU to 200 IU) was administered to animals as a daily or increasing dose for 3, 8 or 9 days with or without a single dose of 100 IU

hCG on the final day. Ovulation was induced in only three animals although a further eleven animals showed signs of premature vascularization and luteinization of follicles preventing ovulation, and therefore trapping the oocyte, which is now recognised as a sign of over-stimulation (Rodger and Mate 1988, Hinds *et al* 1996). The most successful superovulation method at that time consisted of a three day PMSG treatment followed by hCG administration on the afternoon of day three.

Ovulation was not induced when PMSG was administered alone in another study that compared various doses and treatments regimes; (Rodger and Mate 1988). PMSG doses above 10 IU were shown to induce ovarian stimulation (follicular growth) however, higher (single or multiple) doses of PMSG (200 IU or 1000 IU) were shown to over-stimulate the ovary and follicles.

Following these results Rodger and Mate (1988), investigated single doses of PMSG followed by a single or multiple dose of synthetic GnRH in order to induce ovulation. Again high doses of PMSG (50 IU) plus GnRH (100 or 175 IU) over-stimulated the ovary and follicles. Superovulation was demonstrated to be achieved by a single 10 IU PMSG injection (day 0) followed by three injections of 50 IU GnRH at 90-minute intervals, on day 3. This methodology induced multiple ovulations by the 4th or 5th day, after the PMSG injection, in 11 out of 13 (84.6%) possums treated. Superovulation was found to induce multiple ovulations in possums during most reproductive stages including immature, pregnant, lactating and cycling females. The only time the superovulation protocol was not found to be successful was during the pre-ovulatory period.

The superovulation protocol determined by Rodger and Mate (1988) was able to induce multiple ovulations even though the Common Brushtail Possum is naturally monovular. Between 8 and 24 (average 13.1) oocytes were induced to ovulate per possum (n=13), although mature MII oocytes with a polar body were only recovered from 6 animals. Bilateral ovarian stimulation occurred for two immature, juvenile possums and five adult possums (two of which had pouch young) with multiple ovulations occurring on both the ovaries. Additional information regarding the hormonal milieu influencing the reproductive system prior to artificial hormonal

stimulation was overlooked in this study, and would have been of particular interest in the animals in which it failed or resulted in poor stimulation.

When possums were administered a similar PMSG/GnRH protocol to Rodger and Mate (1988), giving 4 doses of (50 μg) GnRH instead of the 3 doses, less oocytes were ovulated (cited by Hinds *et al* 1996 as unpublished observations from Rodger, Gibson and Molinia). 6.1 oocytes ovulated per possum (n = 17) compared to 13.1 oocytes in the study of Rodger and Mate (1988).

In an attempt to identify a simpler superovulation method, Glazier and Molinia (1998) conducted a series of experiments using PMSG to induce follicular recruitment and compared the use of GnRH or various concentrations of LH to stimulate ovulation. An average of 3.9 ± 1.1 oocytes were ovulated when possums were induced using 15 IU PMSG followed by 4 x 50 μg injections of GnRH at 90 minute intervals, 72 hours later. Following injection with 15 IU PMSG, single LH injections of varying doses (between 0 and 10mg) were trialled, administered at 72 hours which was the same time point as GnRH administration. An optimal dose of 4.0 mg LH was determined, which induced ovulation of the greatest number of oocytes yielding an average of 9.5 ± 2.8 oocytes from each of six possums. This was the only dose of LH that induced a significantly ($P < 0.005$) greater number of oocytes than by using GnRH as the ovulatory stimulus (Glazier and Molinia 1998).

Compared to the study of Rodger and Mate (1988), a higher dose of PMSG (15IU as opposed to 10IU) and a longer regimen (4 rather 3 X GnRH) was administered by Glazier and Molinia (1998) who found that they needed to compensate for the greater body weight of their possums. Although the possums utilized in each study were regarded as the same species, those captured in New Zealand and used by Glazier and Molinia were of larger body weight (2 –2.5 kg) than those captured in Australia. There was however, some evidence to suggest that over-stimulation occurred as a result of this different regime.

A simpler superovulation protocol requires only 2 injections when using the PMSG/LH protocol, as opposed to the 4 to 5 injections required by the PMSG/GnRH

protocol. This improved method also resulted in an increased rate of ovulation (Glazier and Molinia 1998).

Molinia *et al* (2000) compared an FSH/LH superovulation regimen to that of the optimised PMSG/LH regimen. FSH/LH was found to induce a greater number of ovulations than PMSG/LH in seasonally anoestrous possums. However less oocytes were considered mature following FSH/LH treatment. The optimal dose of FSH/LH was found to be 8 x 3mg (porcine) FSH over 4 days followed by 4mg (porcine) LH on day 5, ovulation occurred approximately 30hrs later. McLeod *et al* (1999) has also found that (ovine) FSH can stimulate follicular development, and it was successful in both anoestrous and cycling possums.

Several studies have demonstrated that ovulated oocytes can be fertilised by artificial insemination following both PMSG/LH and pFSH/pLH (Molinia *et al* 1998a; Molinia *et al* 2000; Jungnickel *et al* 2000), although as yet, no live young have been produced.

Jungnickel *et al* (2000) repeated the simplified superovulation method developed by Glazier and Molinia (1998) primarily to investigate sperm transport within the female tract following artificial insemination. This study also provided insight into the timing of ovulation following pLH administration. In contrast to Glazier and Molinia (1998), who administered 4mg pLH 72 hours following PMSG, the superovulation method administered 4mg pLH at 78 hours following PMSG priming. Using this protocol, significant follicular growth occurred prior to 33 hours after pLH administration and ovulation was found to occur between 30 and 42 hours. An average ovulation rate of 5 oocytes per possum occurred. The results of this study suggested that the ovulation of the multiple recruited follicles did not occur simultaneously, but rather over a period of time.

1.4.2.2 Tammar Wallaby (*Macropus eugenii*)

Superovulation has been most thoroughly studied in the Tammar Wallaby. Success in this species has been preceded by thorough investigation of its reproductive physiology (Flint and Renfree 1982; Shaw and Renfree 1984; Harder *et al* 1984; Harder *et al* 1995; Tyndale-Biscoe *et al* 1983; Tyndale-Biscoe and Renfree 1987).

The first documented attempt at superovulation of the Tammar Wallaby was by Renfree *et al* (1988). In 1979, they attempted to induce ovulation with a single treatment of PMSG during the follicular phase, 23 days following pouch young removal (ovulation normally occurs 27 days after pouch young removal). Twelve animals were administered between 5 and 125 IU PMSG and examined by laparotomy on day 30. Higher doses were suggested to cause greater stimulation and all animals (n=6) receiving 50 IU PMSG or more died within 1 month (possibly as a result of over-stimulation). Multiple ovulations were induced in one animal that had received 25 IU PMSG identified by the presence of four new corpora lutea.

Renfree *et al* (1988) successfully superovulated 5 out of 9 Tammar Wallabies. Nine animals received PMSG (either 50 or 75 IU) on day 23 following pouch young removal, anti-PMS (100 µl) on day 26 and GnRH (LHRH) (5 µg) on day 27. The stimulation protocol still resulted in a low ovulation rate of 2.2 oocytes per animal (n=5), assessed by newly formed corpus lutea.

In another experiment, Renfree *et al* (1988) demonstrated that following administration of exogenous hormones, Tammar Wallabies would display mating behavior (3 of n=4) and could produce 40-cell blastocysts after mating (1 of n=3). In this experiment four animals were administered 50IU PMSG on day 23 (after removal of pouch young (RPY)) and an unstated dose of anti-PMS on day 26 plus either 5µg GnRH (n=2) or 50IU hCG (n=2) on day 26. Although 3 animals mated (on day 27) only one hCG treated female was found to carry 4 uterine 40-cells blastocysts (on day 30).

Measurement of the plasma hormonal levels (LH, progesterone and oestradiol 17β) during attempts at superovulation led Renfree *et al* (1988) to consider that the optimum dose of PMSG was between 15 or 25IU when administered at day 22 (after RPY). Four Tammars were administered 15IU PMSG on day 22 and on day 23 (after RPY), 100µl anti-PMS on day 25 and 3 x 50µg LH on day 26, at 09.00, 13.00 and 18.00 hours. Two of the 4 animals were successfully superovulated with an average of 3.5 blastocysts recovered per Tammar (n=2).

In agreement with Renfree *et al* (1988), Rodger *et al* (1993) more specifically suggested that 20IU PMSG was the most effective dose to artificially stimulate the Tammar. A small group of animals was used in this preliminary study that compared doses of 10IU (n=3) and 20IU (n=3) PMSG followed by three injections of 30μg GnRH (at 3 hour intervals, 2 or 3 days after PMSG) on days 22 or 23 RPY. The hormonal regime was not however particularly successful and in retrospect 15IU PMSG may have been more effective, given that 20IU PMSG caused very large cystic follicles and 10IU PMSG induced smaller follicles that weren't cystic.

After testing the superovulation methodology on a range of days Rodger *et al* (1993) determined that the injection of PMSG should be given 20 days after removal of pouch young followed by GnRH treatment 2 or 3 days later to induce ovulation. GnRH was found to be unsuccessful if administered in a saline solution (producing an LH surge of short duration) but if administered in suspension with olive oil ovulation could be induced (in response to an LH surge of longer duration).

Another important finding by Rodger *et al* (1993) was that a corpus luteum on an ovary inhibits follicular growth in that ovary throughout the oestrous cycle. This was the main reason postulated for low ovulation rates during superovulation of wallabies in this study, particularly as this sort of inhibition is not observed in the Common Brushtail Possum, as documented by Pilton and Sharman (1962). In contrast much better ovulation rates are observed on both the ovaries in the possum in response to similar a PMSG/GnRH ovulation protocol (Roger and Mate 1988, Roger *et al* 1993).

FSH and LH have also been used to respectively 'prime' and 'induce ovulation' in the Tammar. Early studies found that Tammar Wallabies ovulated in response to the FSH/LH administration during anoestrous (during lactational or seasonal quiescence) and 21 days after pouch young removal (cited by Hinds *et al* 1996 as unpublished an observation form Gibson, Smedley and Rodger).

Molinia *et al* (1998b) further investigated the use of FSH/LH as a method of superovulation. An advantage of the FSH/LH regime was that FSH was found to

bilaterally stimulate the ovaries in comparison to the unilateral recruitment of follicles (on the non-corpus luteum-bearing ovary) when PMSG was used (Rodger *et al* 1993, Molinia *et al* 1998b). Injected exogenous FSH has therefore been suggested to override the inhibitory effects of the corpus luteum located on one of the ovaries (Molinia *et al* 1998b). The FSH/LH protocol achieved ovulation both during cycling and non-cycling periods. In this study, ovulation was induced in the Tammar in response to eight 6mg injections of FSH at 12 hour intervals, over four days, followed by a single injection of 4mg LH on day five. 48 hours after LH administration all animals, not killed prior to this time, ovulated between 3 to 25 eggs (judged by ovulation sites). Molinia *et al* (1998b) reported that approximately 7 to 13 eggs could then be recovered from the reproductive tract. Although the maturation of these oocytes was not discussed in this paper, it was suggested that they were mature and capable of fertilisation as found by Molinia *et al* (1998a). Using the same FSH/LH protocol, Molinia *et al* 1998a successfully fertilised the superovulated oocytes of Tammar Wallabies by artificial (laparoscopic intrauterine) insemination.

When compared the FSH/LH regimen produced a greater yield of oocytes than treatment with PMSG/GnRH or PMSG/LH (Molinia *et al* 1998b). Another advantage, over other protocols, was that no signs of over stimulation were observed when FSH/LH was administered at doses of 4-6mg porcine FSH and 4-7mg of porcine LH. 4mg of FSH was considered to be the optimal dose. Even though the 6mg dose of LH had been adopted it was, suggested that an optimal dose of LH was yet to be established (Molinia *et al* 1998b)

The main disadvantage of the FSH/LH method was that it required intensive administration of the exogenous hormones; 9 injections over 5 days, as opposed to the two injections over 4 days by PMSG/LH. Another disadvantage of this method was that lower numbers of animals naturally mated following stimulation with FSH/LH as opposed to the higher numbers mating following stimulation with other exogenous hormones (Molinia *et al* 1998b). For this reason it was suggested that understanding the hormonal milieu during superovulation was important to establish factors that influence natural mating behaviour, so that artificial insemination would not be required. Molinia *et al* (1998b) also suggested that after superovulation it would be

important to investigate whether or not a suitable hormonal milieu was created to support normal fertilisation and embryo development, especially of multiple oocytes.

Peripheral blood samples have since been collected from FSH/LH stimulated Tammar Wallabies to examine hormonal levels in seasonal and lactational quiescence (Jungnickel and Hinds 2000). Progesterone, LH and FSH were all found to be similar to the concentrations observed during the pre-ovulatory phase of the natural oestrous cycle. Oestradiol concentrations were much higher in response to FSH/LH treatment, and this was considered to indicate that some degree of overstimulation was occurring. This confirmed the suggestion by Molinia *et al* (1998b) that the hormone regimen was still not optimal.

1.4.2.3 Brush-tailed Bettong (*Bettongia penicillata*)

Hayman *et al* (1990) utilized superovulation to induce multiple ovulations in the monovular Brush-tailed Bettong in order to study chiasmata formation in the oocyte during the stages of meiosis. The PMSG/GnRH superovulation method utilised in this study had been previously developed for the Common Brushtail Possum (by Rodger and Mate 1988). The initial PMSG treatment was administered eight days following pouch young removal. The animals were killed 20 hours after the first dose of GnRH and then the ovaries were collected. The superovulation technique induced ovulation in 5 of the 6 animals. 83 oocytes were recovered from the 5 animals used, suggesting that approximately 16.6 oocytes were recovered from the ovaries of each animal.

In another study, which also stimulated Brush-tailed Bettong with PMSG/GnRH, 5 ovulated oocytes were recovered from a single animal (Rodger *et al* 1992a). Ovulation had occurred by 43 hrs after the first GnRH injection. PMSG/GnRH therefore increased the natural oocyte yield in this monovular species, although a larger sample size is needed to delineate the potential induced ovulation rate.

1.4.2.4 Fat-tailed Dunnart (*Sminthopsis crassicaudata*)

Female Fat-tailed Dunnarts are polyestrus spontaneous polyovulators, and as such they already have a high ovulation rate. After natural mating up to 24 embryos may be

observed in the reproductive tract, and they have a cycle length of 31 days (Smith and Godfrey 1970, Bennett *et al* 1990). Smith and Godfrey (1970) conducted the first experiments to induce ovulation in this species, and successfully induced ovulation in both mature and immature (22-24 weeks old) animals utilising PMSG or hCG or a combination of the two hormones. An average of 31 oocytes were ovulated per animal. Despite this success, two main problems were encountered. Eggs were retained within the oviducts for abnormally long periods after induced ovulation, which is suggested to have detrimental implications for normal fertilisation and embryo development. In addition, many follicles failed to ovulate and as a result formed luteinized follicles (trapping the oocytes) that were indistinguishable from the corpus luteum until they were sectioned, and oocytes were found. Smith and Godfrey (1970) observed mating behaviour in both stimulated adult and immature dunnarts, however no evidence of fertilisation was observed.

A second study investigating induced ovulation of Fat-tailed Dunnarts similarly also reported follicular luteinization with entrapped oocytes, in 10 out 16 animals studied (Rodger *et al* 1992b). This was a suggested consequence of over-stimulation. Smith and Godfrey (1970) had administered between 2.5 and 20 IU PMSG (mostly 10 IU) in comparison to the range of 0.2 to 5.0 IU PMSG trialled by Rodger et al (1992b).

Rodger et al (1992b) conducted a series of experiments to conclude that ovulation can be induced using a single intraperitoneal injection of PMSG. Although many other marsupials seem to require secondary GnRH stimulation, it was found to be unnecessary in the Fat-tailed Dunnart. In this study, the optimal dose rate of PMSG was within the range of 0.5 to 1.0 IU. Furthermore, the lower doses of 0.5 to 0.75 IU PMSG did not seem to over stimulate the ovaries.

This has been the only superovulation study of a marsupial that has produced live young, however the success rate was low. Only one of 6 animals produced a live litter (8 young). This may suggest that an inappropriate hormonal milieu was created as a result of the superovulation protocols used to support embryo development. Particularly as the Fat-tailed Dunnart can naturally support the gestation of an average of 8 embryos to term (Bennett *et al* 1990). The study did indicate that some oocytes are capable of normal embryonic development following superovulation.

1.4.2.5 Stripe-faced Dunnart (*Sminthopsis macroura*)

Female Stripe-faced Dunnarts are polyovular and polyestrus with a mean cycle length of 23.25 days (Godfrey 1969, Woolley 1990). A single intraperitoneal injection of 1.3 IU PMSG is reported to induce follicular growth and ovulation in both cycling and non-cycling animals (cited by Hinds *et al* 1996 as unpublished observations of Merry, Johnson and Selwood), which was similar to the findings for the Fat-tailed Dunnart.

Recently, Hickford *et al* 2001 demonstrated that normal embryos were produced from mated Stripe-faced Dunnarts, stimulated with a one to two injection of equine serum gonadotrophin (eSG also called PMSG); as compared to embryos produced naturally. Ovulation could be induced in animals during anoestrous, and during the pre-ovulatory and post-ovulatory periods of the oestrous cycle. The optimal dose was reported to be 0.087 IU PMSG yielding ~21 oocytes per ovary compared to ~ 14 oocytes per ovary of natural cycling animals. Hickford *et al* 2001 considered that if the stimulated animals had been permitted to give birth, the normal number (mean of 6, range: 1 to 8) of pouch young would have been produced, unless fetal mortality occurred in the last 1-2 days of pregnancy.

1.4.2.6 Kowari (*Dasyuroides byrnei*)

Fletcher (1983) used an intensive method to induce ovulation in the seasonal, polyovular Kowari during anoestrous (one month prior to their breeding season). Animals were injected once daily with (10 or 20 IU) PMSG for ten days and then given a single dose of 500 IU of hCG on day eleven. Ovulation was induced, as determined by ovulation points and multiple newly formed corpus lutea present approximately 7 days following administration of hCG, however oocytes (n=11) were only able to be collected from the tract of one animal that had received 10 IU PMSG. No deleterious effects to ovarian function were reported. Although changes observed in the vaginal cytology and body weight were consistent with naturally oestrus females, mating success was reported to be low. This has been the only study in this species and further experiments are required to develop an optimal stimulation regimen. Progesterone concentrations were examined in this study and slightly higher

levels were observed than control (saline injected) animals, and the rise was consistent with the time of corpora lutea formation.

1.4.2.7 Virginian Opossum (*Didelphis virginiana*)

The Virginian Opossum is a seasonal breeder and polyovular; ovulating an average of 16 oocytes during a natural cycle (Hartman 1923; Reynolds 1952). This is a far greater ovulation rate than observed naturally in most other marsupials, and rarely observed as a result of superovulation. In this case the aim of Nelson and White (1941), was to induce multiple ovulations during the seasonal period when the Virginian Opossum's were in anoestrus. This was first achieved using a series of subcutaneous injections of FSH and FSH plus LH (Nelson and White 1941). Success was monitored by the presence of ovulation points on the ovaries and by the collection of eggs (although the number was not recorded). Depending on the number of doses administered and the time elapsed prior to examination of the reproductive tracts, eggs could be collected from either the fallopian tubes or the uterus.

A method for superovulation in this species was then tentatively adopted, as it had been successful in (at least) 8 animals. Although the success was difficult to judge as neither the numbers of oocytes recovered nor the numbers of ovulation points were discussed. The method consisted of 7 injections of 2.5mg FSH given as an evening injection on day 1 and administered in the morning and evening on days 2 to 4. This was followed by six injections of 2.5mg FSH plus 100RU LH, administered in the morning and evening on days 5 to 6. Eggs could then be collected from the uterus approximately 24 hours after the last injection.

1.4.2.8 Grey Short-tailed Opossum (*Monodelphis domestica*)

Rodger *et al* (1992a) attempted to superovulate the Grey Short-tailed Opossum to study the stages of follicular antrum formation and oocyte growth in this species. . The superovulation method utilized was a single subcutaneous injection of 1.0IU PMSG followed by a single intramuscular injection of 20μg GnRH (LHRH) three days later. Interpretation of the results suggests that 5 oocytes had ovulated from 6

Grey Short-tailed Opossums suggesting an ovulation rate of less than one per animal and that this particular superovulation protocol was not successful in this species.

It has since been reported that a single dose of PMSG followed 2 to 3 days later by three consecutive doses of GnRH, at 1.5 hour intervals. Ovulation was induced in 3 out 4 animals (cited by Hinds *et al* 1996 as unpublished observations from Mate, Robinson and Rodger). Although the number of eggs ovulated from these animals was not recorded 4-cell embryo's were recovered. A third method of superovulation involving a single dose of between 0.5 and 5.0 IU PMSG failed to induce ovulation, even though ovarian stimulation was observed (cited by Hinds *et al* 1996 as unpublished observation from Mate, Robinson and Rodger).

1.4.3 Comparison of superovulatory techniques for marsupials

Several hormonal regimes have now been tested in marsupial species to induce superovulation. Generally a single or multiple administrations of a gonadotrophin (PMSG or FSH respectively) is required to stimulate follicular maturation. A single gonadotrophin (LH or hCG) or gonadotrophin releasing hormone (such as GnRH) is then required to induce ovulation of the recruited follicles. This generalized methodology has been used to induce superovulation in most marsupials discussed, with the exception of two species, the Stripe-faced Dunnart and the Fat-tailed Dunnart. In these two species a single administration of a PMSG was sufficient to induce follicular recruitment and ovulation. No single superovulation regimen has therefore been developed for all marsupials.

Although this generalized methodology seems to be useful in most species the doses and particular hormones required are specific to the species and individual. Further more, it may be that species or individuals of larger body weight require higher doses (Glazier and Molinia 1998). Although, Rodger (1990) suggested that the reason for the failure of earlier studies was almost certainly due to the high levels of gonadotrophin used. It is now recognized that even moderate doses can cause degenerative changes in the ovary and inhibit ovulation (Rodger 1990, Rodger and Mate 1988). Marsupials appear to be more sensitive to PMSG treatment than

eutherian species and as such require smaller doses than eutherians of a similar body weight (Rodger and Mate 1988, Hayman *et al* 1990, Rodger *et al* 1992b).

Difficulties in the interpretation of results have occurred due to the size of sample populations used, which have often been small and contained individuals of varying age, weight and reproductive status. An important factor appears to be the reproductive status of the animals used. Superovulation regimes have been found to be less effective in animals during particular reproductive periods. Both pre-ovulatory follicles and corpus lutea have been found to reduce induced ovulation rates depending on the hormone regimen used (Rodger and Mate 1988, Roger *et al* 1993; Molinia *et al* 1998b). Possums in the pre-ovulatory period of an oestrous cycle were found to respond poorly to hormonal administration (Rodger and Mate 1988) and corpus lutea reduced ovulation rates in the corpus lutea-bearing ovary in Tammars (Roger *et al* 1993).

The success of marsupial superovulation protocols have largely been judged by the ability to increase the natural yield of ovulated oocytes, and the ability to induce ovulation at times when it would not normally occur. Ovulation rates have typically been determined by the number of ovulation points, the number of corpus lutea on an ovary, and/or the number of oocytes that are recovered from the reproductive tract. Ultimately the success of a superovulation regimen is assessed by the production of live young. So far this has only been achieved in one species, the Fat-tailed Dunnart (Rodger *et al* 1992b) and further studies are required in all species.

Other studies that have permitted mating after stimulation or have used artificial insemination (AI), and recently ICSI, have been used and allude to oocyte viability by the fertilisation and formation of embryos or blastocysts (Renfree *et al* 1988; Glazier and Molinia 1998, Hickford et al 2001; Magarey and Mate 2000). According to Molinia *et al* (1998a), inseminations resulting from copulation would reduce cost and permit a simpler method of testing oocyte viability. In order to increase breeding success following superovulation further studies are required to particularly investigate the differences in behaviour, morphology, and endocrinology from those of naturally ovulating animals (Rodger 1990; Hinds *et al* 1996; Mate *et al* 1998).

Reproductive function and its manipulation has only been studied in a few marsupial species. In contrast, many eutherian species including humans and cows, are thoroughly understood and superovulation is now routinely used (Kafi and McGowan 1997; Trounson *et al* 2001; Fielden and Hayman 1981) It is an effective primary technique for oocyte production for assisted reproductive methods, although this required intensive research. Further research into superovulation may therefore offer the means for the development of assisted reproductive techniques for endangered marsupials (Rodger 1990; Mate *et al* 1998).

1.5 Aims

The aims of this study were to:

1. Characterize the oestrous cycle of the Common Wombat in Victoria
2. Investigate a method of ovarian stimulation using pFSH/pLH administration to collect mature oocytes from Common Wombats
3. Examine the effects of pFSH/pLH treatment on some aspects of the reproductive endocrinology, vaginal cytology and external reproductive anatomy of Common Wombats
4. Collect oocytes from pFSH/pLH primed Common Wombats, mature them *in vitro* if required, and assess their viability by intracytoplasmic sperm injection.

Chapter 2

General Materials and Methods

2.1 Animals

2.1.1 Animal capture and use

A total of 11 female Common Wombats (*Vombatus ursinus*) were captured for this study (Table 1.1). Three wombats (6,13 and 14) were cage trapped in the Glenburn/ Murrindindi area of Victoria and 8 wombats (7, 62, 63, 64, 67, 68, 69 and 70) were cage trapped or netted in the Myrtleford area of Victoria. All wombats were captured in areas in which they had been declared 'unprotected wildlife' under the 1984 Victoria Wildlife Act. Capture was undertaken in accordance with Natural Resources and Environment, Flora and Fauna Research Permit Numbers 10001049 and 10001495.

Capture of wombats with a net, required one person to drive slowly with a spotlight to locate wombats out of their burrows. Upon locating a wombat another person with a large hand net moved quietly and quickly towards the animal and then captured it by throwing the net over the wombat. If the wombat escaped the net, an attempt was made to follow the animal to its burrow, in the event that it might be cage trapped.

Cage trapping involved locating a wombat entering a burrow at night. A cage trap was then immediately placed over the burrow entrance and wedged into place so that it could not be easily pushed aside. All other entrances to the burrow were blocked with timber. Traps were checked at dawn the following day and then at a maximum of 8 hours intervals. The trap remained over the burrow for a maximum of 4 nights or until a wombat had either entered the trap or dug out past the trap and escaped. Immediately after capture by net or cage trap, the wombat's weight was estimated and it was anaesthetised with 4mg/kg Zoletil (100mg/ml) by intramuscular injection. Upon anaesthesia the wombat was weighed, and its sex was determined, by the presence of a

scrotum or pouch, and its health was assessed, particularly looking for signs of sarcoptic mange.

The monitoring of reproductive status of all 11 Common Wombats began at capture. Two animals (wombats 6 and 13) were acquired from Dr Lee Skerratt, which had been experimentally infected with sarcoptic mange. Monitoring of these wombats began following recovery from mange.

Table 1.1. Female Common Wombats used in this study, noting initial observations at time of capture or acquisition*, including site of capture age group, body weight and observations of breeding activity and health.

Wombat	Capture Date	Site of Capture	Age group	Body Weight	Other observations
6	14/8/00*	Murrin	Adult	21	sarcoptic mange
7	14/9/00	Myrtle	Subadult	20	
13	3/7/00*	Murrin	Adult	24	
14	20/9/00	Murrin	Adult	27.5	lost YAF
62	2/8/01	Myrtle	Adult	28	PY
63	6/8/01	Myrtle	Adult	27.9	PY
64	6/8/01	Myrtle	Adult	29	weaned a YAF
67	8/8/01	Myrtle	Adult	27	PY
68	8/8/01	Myrtle	Subadult	21.3	
69	8/8/01	Myrtle	Adult	27.5	
70	8/8/01	Myrtle	Subadult	19.4	

PY denotes pouch young; YAF denotes young-at-foot
Murrin = Murrindindi; Myrtle = Myrtleford.

2.1.2 Animal housing

Upon capture, wombats were held for a maximum of two days (minimum of 4 hours) in temporary enclosures at the field site. The 6 meters square temporary enclosures, were constructed on a concrete base with 2 meters high timber walls. A clean 60L oil drum, lined with straw, was provided for animals to sleep in and take cover. Fresh grass, lucerne hay and water was provided *ad libitum*. Animals were transported to Melbourne University, Werribee, or Monash University, Clayton, in a small timber and straw-lined

cage (~9 m^2). All wombats were offered fresh grass, upon arrival at both housing locations, until they were observed to consume other foods offered.

Captured wombats (wombats 6,7,13,14) were housed at The University of Melbourne, Veterinary School, Werribee, between July 2000 and 28 June 2001. Wombats were housed individually in concrete pens (9 square metres) with a nest box (500mm x 1000mm) lined with straw for bedding. Food and water was provided *ad libitum*. Feed consisted of lucerne hay, and a mix of lucerne chaff, oats and Barastoc Blue Ribbon Maintenance Horse Mix (Appendix 1; Ridley Agriproducts Pty Ltd). Fresh grass was offered on alternate days.

After the 28 June 2001, captured wombats (wombats 62,63,64,67,69,70), and three of the wombats (wombats 6,7,14) previously housed at Werribee, were housed at Monash University, Central Animal Services, Clayton. At Monash University, wombats were provided with either short-term or long-term housing. This meant that wombats housed for less than 1 month (short-term) were kept in small enclosures (~7.5 m^2) and wombats held for longer periods of time (long-term) were housed in larger enclosures (15 m^2). Both small and large enclosures were made of wire mesh with a concrete floor and all had a nest box (1m x 1m). Large enclosures had a sandpit (3m x 2m x 500mm) containing 2 cubic meters of sand. Wombats in small enclosures had access to a sandpit, which was shared with 2 other wombats so that each animal was given access on a 12-hour rotation. When the space was available at Monash University, wombats were permitted access to two enclosures.

Food and water was provided *ad libitum*. Feed consisted of predominately fresh grass supplemented with lucerne hay, and a mix of lucerne chaff, oats and Barastoc Blue Ribbon Maintenance Horse Mix. Straw bedding, and tree logs and branches were provided.

2.1.3 Anaesthesia

At each examination, wombats were anaesthetised with a single intramuscular injection of 4mg/kg Zoletil (100mg/ml) (Virbac Australia) to permit monitoring of the reproductive status (Section 2.2). At this dose, anaesthesia was induced for up to 25 minutes, and complete consciousness was regained by 2 - 4 hours, assessed by the ability to move about steadily and behave in a normal manner.

Prior to surgery, anaesthesia was induced by a single intramuscular injection of Zoletil at a dose of 8mg/kg (~2ml), which induced deep anaesthesia for 40 - 60 minutes to permit monitoring of the reproductive status, and preparation of the animal for surgery. Anaesthesia was then maintained during the laparotomy procedure by 5% isoflurothane (Euthal; Delvet Pty Ltd) /oxygen inhalation administered via a mask over the wombat's muzzle (covering the mouth and nose).

Animals were monitored constantly until they had recovered completely from anaesthesia or until euthanasia following surgery.

2.1.4 Monitoring of health

Problems encountered with captive wombats include nutritional and infectious diseases, and ecto- and endo-parasitic infections as outlined by Presidente (1982). Defecation, food consumption, general changes in coat appearance and behaviour were monitored daily. Wombats were thoroughly checked for signs of poor health and body condition at each examination of reproductive status.

Wound or nail-bed infections were treated by flushing with peroxide, and antibacterial spray, combined with systemic antibiotic treatment. Un-infected wounds were treated with a topical antibacterial spray.

The nails of wombat's were clipped regularly (at 3 month intervals) when housed at Melbourne University as they did not have access to areas in which they could dig and naturally wear the nails down.

2.2 Monitoring of reproductive status

Eight captive Common Wombats were examined 2 - 3 times per week, at intervals of 1 - 4 days to monitor reproductive status. At each examination, wombats were anaesthetized (Section 2.1.3). Monitoring at each examination involved the measurement of body temperature and body weight, description of the pouch and urogenital sinus, collection of a blood sample and a vaginal smear, and changes in behaviour were noted.

2.2.1 Body temperature

At each examination, body temperature was assessed once using a digital thermometer, inserted into the rectum to a depth of 25-30mm. In addition, the temperature was taken three times at 5 minute intervals during 37 examination periods, to verify that temperature did not vary during the examination period

2.2.2 Body weight

Whilst wombats were housed at Melbourne University, their body weight was determined using calibrated sheep scales, permitting measurements to an accuracy of ±0.1kg. In the field and when wombats were moved to Monash University, their body weight was determined using hanging scales lifting the wombat off the ground in a hessian sack, permitting measurements to an accuracy of ±0.1kg.

2.2.3 Pouch examination

The pouch was described using the following terms: "dry" or "moist", "dirty" or "clean". The pouch opening was described as "open" or "incomplete" or "contracted". A photograph was taken to support the description. The length of the teats and whether or not they were lactating was recorded. Mammary swelling was also noted.

2.2.4 Urogenital opening examination

At each examination the urogenital sinus was described using the following terms: "protruded" or "inverted" and "dry", "moist" or "exuding mucus". These terms

categorised the external appearance of the urogenital sinus and a photograph was taken to support the description. Measurements of the urogenital sinus were made using callipers to determine how far the urogenital sinus was extended or protruded from the cloaca.

2.2.5 Vaginal smear analysis

Vaginal smears were taken by quickly dipping a glass tube/cannula (10cm length, 7.5mm outer diameter, 5mm inner diameter) containing a cotton swab (150mm length, with a 3.5mm diameter swab) into Phosphate Buffer Saline (PBS)(GibcoBRL). The glass tube was then inserted into the urogenital sinus to a depth of 40mm. The cotton swab was pushed through the glass tube into the posterior vaginal sinus, rotated 6 times, then withdrawn back into the glass tube. The glass tube and cotton swab were then withdrawn from the urogenital sinus. The cotton swab was rolled each of two slides which were air dried, stained and mounted. The first slide was stained using Shorr's (1941) stain (Appendix 2). The second slide was stained using Diff-Quik® stain (64851; Haleco Drug Company). Slides were then mounted using Depex mounting medium (Gibco BRL) (Appendix 2).

The two stains utilised were compared to establish which was the more efficient indicator of the oestrus cycle activity. To compare the stains and ensure a range of vaginal cell types were examined, vaginal smears stained with both stains, were analysed over two oestrous cycles (determined by progesterone concentrations), one cycle from each of two wombats (wombats 6 and 13). After the 9 April 2001, one vaginal smear slide was collected at each examination and stained with Diff-Quik.

All vaginal smears were evaluated under light microscope (mag. x100), using criteria described by Hughes and Dodds (1968), Peters (1977) and Holst (1985) to distinguish between cell types. Epithelial cells were classified as either basal, parabasal, small or large intermediate, or superficial cells with or with out a nucleus. 100 epithelial cells were randomly counted and cell types were recorded for each slide. The number of leucocytes per 100 epithelial cells was counted. The proportions of cell types were compared.

Epithelial cells were grouped according to morphology as parabasal-intermediate type cells (including basal, parabasal and small or large intermediate cells), and a second group as superficial type cells (superficial cells with or without nuclei). These groups are expressed in this study as a percentage of total epithelial cells.

As only 100 epithelial cells were counted per vaginal smear, the variation in the cell count was determined, by counting a representative sample of 10 vaginal smears 5 times each. The differences in cell counts were then compared.

2.2.6 Blood Sampling

At each examination, ~ 4mls of blood was collected from the peripheral circulation. Blood was sampled alternately from each leg of the wombat, so that blood was not collected from the same vein on consecutive examinations. Samples were taken from either the left or right cephalic vein or the left or right saphenous vein using a 22 gauge 1 inch needle and 5ml syringe. The blood sample was immediately injected into a 5ml vacuum-sealed heparinized tube (Sarstedt) and subsequently centrifuged at 6000 rpm for 5 min. The plasma supernatant was then removed, and transferred into two 1.5ml eppendorf tubes so that each eppendorf tube contained 1000 to 1300μl of plasma. The plasma was then frozen at –20°C until hormonal assays were performed.

2.2.7 Behavioural Observations

The behavioural of individuals was observed whilst wombats were in captivity and general changes were recorded at times of monitoring of the reproductive status.

2.3 Assays

2.3.1 Progesterone Analysis

Progesterone levels in the plasma samples were determined using a Chiron Diagnostics ACS:180 Progesterone Assay (Chiron Healthcare Pty Ltd). The progesterone kit assay is

an automated direct competitive immunoassay using chemiluminescent technology and an acridinium ester-labelled mouse monoclonal anti-progesterone antibody. The assay is highly specific having a cross-reactivity of less than 0.95% with other compounds (Appendix 3). The Progesterone assay measures progesterone concentrations within the range of 0.11 ng/ml (0.35 nmol/L) and 60 ng/ml (190nmol/L). Single samples were assayed for progesterone analysis at the Department of Biochemistry, Monash Medical Centre, and Clayton on an automated system. The assay requires a minimum plasma sample of 200µl, and being a direct assay, no extraction is required.

2.3.2 Progesterone Assay Validation

The Chiron Diagnostics ACS:180 Progesterone Assay was validated for use in the female Common Wombat by undertaking tests for linearity (serial dilution), and within and between assay variation. Linearity was tested by serially diluting 3 plasma samples with progesterone concentrations of between 80.3 and 123.8nmol/L. Each plasma sample was serially diluted (1:2, 1:4, 1:8, 1:16 and 1:32) with Multi-Diluent 3 (Chiron Healthcare Pty Ltd). All serially diluted samples were assayed along with the original samples within a single run, and assessed for their ability to dilute in linearly.

Within assay variation was tested by assaying 3 progesterone samples 5 times in one run. Between assay variation was tested by assaying the same 3 progesterone samples in each of 5 independent assay runs.

In addition to the assay validation steps, the Department of Biochemistry, Monash Medical Centre, Clayton ran standards to determine within and between assay variations. The average within assay co-efficient of variation was 4.1% (n = 240) and the average between assay co-efficient of variation was 8.8% (n = 192).

2.3.3 Estradiol Analysis

Estradiol concentrations were examined during the final oestrous cycle, defined by changes in progesterone, from each of the three wombats (wombat 6, 13 and 14) and during the superovulation period of all wombats. Estradiol concentrations were

determined by a modification of the method described by Prendiville *et al* (1995) using the commercially available Estradiol MAIA® manual kit (Biochem Immunosystems).

Extraction of Estradiol: The extraction of estradiol from wombat plasma was required before RIA could determine the concentrations. The extraction procedure was as follows:

- 100ul of sample (including quality controls (QCs)) were added to glass extraction tubes in duplicate and 2ml of diethyl ether was added
- samples were vortexed for 15 minutes with a 2 minute rest every 5 minutes
- Tubes were placed in a methanol/water bath at -4°C for 30 mins
- The solvent layer containing the extracted estradiol was decanted into glass assay tubes and placed into a Savant (Selby Biolab) rotary evaporator for 3 hours or until all solvent had evaporated
- Samples were reconstituted in 200µl assay buffer (0.01M PBS, pH 7.0, with 0.1% gelatin and 0.1% sodium azide), vortexed, covered and stored at 4°C overnight

Internal plasma QCs measuring low, medium and high levels of estradiol were extracted to determine inter-assays CV. Ovariectomised (OVX) samples were extracted as a sample to determine the extraction recoveries that were between 80-85% for the assays conducted. Due to the lack of wombat OVX plasma available, bovine OVX plasma was used.

Estradiol RIA protocol: Estradiol standards were prepared by serial dilution in PBS from a top dose of 50pg/ml to 0.195pg/ml. The primary antibody (rabbit antiserum) was diluted 1:6 in PBS as recommended and the volume was prepared according to the number of assay tubes. The tracer was diluted to approximately 13,000 cpm in PBS. The assay protocol was as follows:

- Extracted samples were removed from storage and allowed to reach RT
- 200ul of each standard was added to the assay tubes in duplicate

- 50µl primary antibody was added to each tube, vortexed and incubated at RT for 1 hour
- 50µl tracer (lypholized ^{125}I labelled estradiol – 13,000cpm) was added to each tube, vortexed and incubated at RT for 3 hours 15 minutes
- 300µl second antibody (goat-anti rabbit coupled to magnetic particles) was added, vortexed and incubated for 20 mins at RT
- bound and free fractions were separated by placing tubes in a magnetic rack for 20 mins at RT
- unbound tracer was decanted leaving the double antibody complex as a pellet
- the pellet was washed with distilled water and radioactivity was measured

Radioactivity was counted for 2 mins in a gamma counter and evaluated using the RIACalc program that uses a spline transformation of the binding data to determine estradiol concentrations. Concentrations were adjusted according to the extraction recovery and volume of sample for each assay.

2.3.4 Estradiol Assay Validation

The Estradiol MAIA assay was validated for use in the female Common Wombat by undertaking tests for serial dilution, spiking recovery, and within and between assay variation (Appendix 4). Linearity was tested by serially diluting 2 plasma samples with progesterone concentrations of between 11.4pg/ml and 17.5 pg/ml. Each plasma sample was serially diluted (1:2, 1:4, 1:8, 1:16 and 1:32) with buffer. All serially diluted samples were assayed along with the original samples within a single run, and assessed for their ability to dilute in linearly.

Two plasma samples with estradiol concentrations of between 2.16pg/ml and 7pg/ml were spiked by adding 0, 5, 15 and 25 pg/ml estradiol (standards supplied with kit) in directly to 100µl of sample + 100µl buffer. The spiked samples were extracted. All neat and spiked samples were then assayed and the final observed concentrations of estradiol were compared against the expected concentrations.

Within assay variation was tested by assaying 3 estradiol standard samples 6 times in one run. Between assay variation was tested by assaying the 2 estradiol samples in each of 2 independent assay runs.

2.4 Superovulation Methodology

2.4.1 Gonadotrophins for Superovulation pFSH and pLH

Porcine FSH (pFSH) (Folltropin-V; Vetrepharm) was stored at room temperature as a 400mg purified lyophilized pFSH powder until required. Just prior to the first injection the pFSH was reconstituted by adding 20ml of Folltropin-V diluent to the 400mg of powder pFSH, making a stock solution of 20 mg/ml. The pFSH stock solution was stored below 5°C and used within 4 days. Porcine LH (pLH) (Lutropin-V; Vetrepharm) was stored at room temperature as a 25mg purified lyophilized pLH powder until required. Just prior to the first injection the pLH was reconstituted by adding 5ml of Lutropin-V diluent to the 25mg of powder pLH, making a stock solution of 5mg/ml. The pLH stock solution used immediately.

2.4.2 Superovulation (days 1 to 5)

Common Wombats were stimulated with porcine FSH (pFSH) (Folltropin-V; Vetrepharm) and porcine LH (pLH) (Lutropin-V; Vetrepharm). Day 1 was the assigned as the first day of pFSH injection. Eight 6mg pFSH were administered at 12-hour intervals over 4 days; given intramuscularly into the left hind limb. On day 5, 12 hours after the last pFSH injection a single subcutaneous injection of 4mg pLH was administered.

2.4.3 Ultrasound examination of ovaries

A trans-abdominal ultrasound technique was developed for the Common Wombat. In summary the animal was restrained in a dorsally recumbent position at an angle of 40° to 50° with the head at the lower end. The area between the hip and ribs on each of the

lateral sides of the animal was shaved. The wombat was then examined using 5 – 7.5 MHz transducer trans-abdominal probes. Recorded images were compared with results of laparoscopy and laparotomy. Preliminary studies suggested that the food supply to animals needed to be reduced prior to trans-abdominal ultrasound to permit visualization of the ovaries.

2.4.4 Preparation for surgery

Laparoscopy was performed on two wombats (wombat 6 and 13) in an attempt to recover the oocytes after the first ovarian stimulation with exogenous hormones. Laparotomy was performed on day 6 of all other superovulation attempts.

Surgery was undertaken 25 –27.5 hours after the pLH injection. Anaesthesia was induced (Section 2.1.3). The area between the pouch and sternum was shaved free of hair and scrubbed with isopropyl alcohol. Anaesthesia was then maintained during the laparoscopy procedure by and isoflurothane/ oxygen inhalation administered via a mask over the wombat's muzzle (covering the mouth and nose). The wombat was given a 1ml (0.3mg) intramuscular injection of Temgesic (active ingredient Buprenorphine Hydrochloride; Reckitt and Colman Ltd), an analgesic to reduce pain following the laparoscopy. The wombat was then transferred to a sterile surgical theatre for the operation.

2.4.5 Oocyte Retrieval via Laparoscopy (day 6)

Laparoscopy was performed by Dr Glenn Edwards and Dr Sam Snelling using routine surgical methods at the University of Melbourne, Werribee Veterinary School surgical clinic. In summary the animal was restrained in a dorsally recumbent position at an angle of 40° to 50° with the head at the lower end. A small incision in the abdominal wall was made to facilitate the endoscope trochar, which was inserted with a cannula, and the abdominal cavity was insufflated with air. The trochar was then removed and replaced with the endoscope. A second incision in the abdominal wall was made and a pair of manipulating forceps inserted. During laparoscopy, ovarian morphology, including an assessment of the follicles, corpus lutea and corpus albicans if present, and uterine

morphology was assessed using the descriptions by Moritz *et al* (1998). Upon completion, incisions were sutured. The wombat was given 1.5ml subcutaneous (s.c.) injection of Clavulox Broad Spectrum Antibiotic (active ingredient Amoxycillin; Animal Health) as a precautionary measure against infection. Clavulox (1.5ml s.c.) injections were given at 3 day intervals until the wounds healed.

2.4.6 Oocyte Retrieval via Laparotomy (day 6)

During laparotomy sterile technique was used. A midline incision was made in the abdominal wall exposing the viscera of the abdominal cavity. Forceps were used to clamp any severed blood vessels. The intact intestine was partially removed from the abdominal cavity to expose the central and left regions of the abdomen exposing the reproductive tract and left and right ovary. Each ovary was gently removed from the bursa, which held it to the dorsal surface of the abdominal wall in the left or right inguinal region of the abdomen. A photograph was taken to record the appearance of the ovary *in situ*. Forceps were used to clamp the ovarian vasculature and oviduct just below the ovary. The ovary was then immediately harvested using surgical scissors cutting above the forceps and placed in sterile PBS at 35°C.

Measurements of the ovary, follicle, corpus lutea, corpus albicans and corpus haemorrhagica were made with sterile callipers and a photograph was taken the ovary *ex situ*. The ovary was then immediately placed into 40ml of PBS at 35°C in a 50ml Falcon tube and placed in a transportable incubator at 35°C.

Whilst still under anaesthesia, upon removal of both ovaries, the wombat was immediately euthanaised by injection of Pentobarbitone (Forthane; Abbott Australasia Pty Ltd) into the heart vertical. The heart beat and breathing rate of the wombats were monitored closely until they ceased when the wombat was considered dead.

2.4.7 Follicle Aspiration during Laparoscopy

All follicles >1mm diameter were punctured with a 19G 1.5in needle and 10ml glass syringe and flushed with pre-warmed TCM-199 follicular aspiration media (Appendix 5).

Collected aspiration media was immediately transferred into a 100mm falcon tissue culture dish and searched under a light microscope for the presence of oocytes (with or without surrounding cumulus cells). Aspired oocytes were then washed by transferring them through pre-warmed TCM-199 aspiration media and then into a 4-well dish containing pre-warmed and equilibrated TCM-199 maturation media (Appendix 5), and placed into an incubator at 35°C and 5% CO_2.

2.4.8 Follicular Puncture after Ovarian Harvest

Ovaries, submerged in warm PBS were removed, and placed individually into a 100mm falcon tissue culture dish containing 2ml of TCM-199 aspiration media (Appendix 5). Under a dissecting microscope, using a pair of jewellers forceps and a 19G 1.5in needle, follicles were gently teased open, expelling the follicular fluid into the aspiration media. This fluid was then searched for an oocyte. If an oocyte was found, it was transferred to a 35mm falcon tissue culture dish containing 1ml TCM-199 aspiration media, using a pulled glass pipette. This process was then repeated until all follicles had been teased open.

All oocytes were washed in pre-warmed TCM-199 aspiration media and then transferred to pre-warmed and equilibrated TCM-199 maturation media (Appendix 5) and placed into an incubator at 35°C and 5% CO_2.

2.4.9 Oocyte Decision Assessment

Some oocytes were recorded with or without cumulus cells attached. An attempt was made to remove cumulus cells from oocytes by pipetting the cumulus oocyte complex up and down through a finely pulled glass pipette. Cumulus cells remained intact with the oocyte if they could not be removed using this method. Cumulus-free oocytes were examined under light microscope and measured using an eyepiece graticule calibrated against the stage scale.

Oocytes with a polar body were selected for ICSI. Naked oocytes without a polar body and cumulus oocyte complex's were defined as immature and kept in TCM-199 maturation media (Appendix 5) for *in vitro* maturation.

2.4.10 Oocyte *In Vitro* Maturation

All immature oocytes were maintained in vitro in TCM-199 maturation media (Appendix 5) in an incubator at 35°C and 5% CO_2. Oocytes were examined under light microscope at 6 – 8 hrs intervals. Cumulus cells were attempted to be removed at 12, 24, 30, and 48 hours after collection. ICSI was performed on all oocytes that reach MII stage after 24 hours. All remaining oocytes were stained using the DNA-specific dye Hoechst 333432 48 to 96 hours after collection.

Chapter 3

The Oestrous Cycle of the Common Wombat in Victoria

3.1 Introduction

This study characterizes the oestrous cycle of the Common Wombat as assessed by vaginal cytology, body temperature, anatomy of the pouch and urogenital sinus, and progesterone and estradiol concentrations in the peripheral circulation. Although vaginal cytology and body temperature of the Common Wombat have been previously investigated in Tasmania (Peters and Rose 1979), this study did not correlate these parameters with endocrine profiles, such as progesterone and oestradiol concentrations which provide a more definitive indication of oestrous cycle length. This basic knowledge is required when considering the development of methods to manipulate reproduction in this species.

3.2 Materials and Methods

3.2.1 Animal Capture and Use

Data was collected from eleven wild caught female Common Wombats (*Vombatus ursinus*), referred to as wombats 6, 7, 13, 14, 62, 63, 64, 67, 68, 69, and 70 (Section 2.1; Table 2.1).

3.2.2 Experimental design

Progesterone and estradiol concentrations in the peripheral circulation, body weight, body temperature, vaginal cytology, pouch and urogenital opening conditions were studied by collection of consecutive samples over a period of 2 years. These parameters were used to define the reproductive status of all animals examined, and to characterise the oestrous cycle.

3.2.3 Monitoring of Reproductive Condition

Body temperature, and body weight was measured (Section 2.2.1 and 2.2.2), the pouch and urogenital opening were examined (Section 2.2.3 and 2.2.4) and a vaginal smear and blood sample was taken (Section 2.2.5 and 2.2.6).

3.2.4 Sample Analysis

(Sections 2.2.5, 2.3 and Appendix 2)

3.2.5 Statistical Analysis

As samples were not collected every day it was difficult to determine the exact time of transition between two values which differed. To allow for this, estimated cycle or phase lengths were determined from the midpoints in time between two observations indicating that a transitional change had occurred. The mean cycle or phase length was defined as the mean of a series of cycle or phase lengths estimated in this way. The range of cycle or phase lengths was defined as the minimum to the maximum cycle or phase lengths. Data are given as the mean ± standard error of the mean (SEM) (range: minimum to maximum). It was assumed that all samples collected were random. Differences in the lengths of the oestrous cycle, as defined by progesterone levels, or vaginal cytology were assessed by one-way univariate ANOVA. Differences in the lengths of basal or high progesterone phases were assessed by one-way multivariate ANOVA, to allow for relatedness of phase lengths and the error in sampling technique. For the same reasons, the One-way multivariate ANOVA was also used to assess differences in the high and basal vaginal cytology phases.

To test the statistical assumptions, Levene's Tests to assess equality of error variances and Shapiro-Wilk Tests to assess normality, were conducted. Confidence intervals and prediction intervals were calculated to determine the estimated range of minimum and maximum lengths of the cycles defined by progesterone levels, or vaginal cytology. A one-tail T-test was used to determine if the cycle length for Common Wombats in Tasmania (Peters and Rose 1974) varied from the cycle length of the Common Wombat in Victoria. Peak progesterone concentrations were compared for each cycle of an individual wombat and between wombats by repeated measures ANOVA. The difference in the estimated lengths of cycles

determined by estradiol concentrations and progesterone concentrations and vaginal cytology, were compared using a paired-samples T-test. Statistics were calculated on SPSS and Minitab computer programs.

3.3 Results

3.3.1 Progesterone profiles

Three subadult wombats (wombat 7, 67, 70) had progesterone concentrations ≤6.8nmol/L and these concentrations were defined as basal (Table 3.1). Four adult wombats (wombats 6, 14, 62 and 63) had basal (≤6.9nmol/L) progesterone concentrations for > 3 months, and were described as being anoestrus. The longest basal progesterone periods were 8 months in an adult wombat following loss of a young-at-foot (wombat 14) and 11 months in a subadult wombat (wombat 7). Basal progesterone concentrations did not vary significantly (P>0.1) between individuals or between subadult (n=3) and adult anoestrous (n=7) wombats and the results were pooled (Table 3.1). The overall basal progesterone concentration in these six wombats was 2.9nmol/L ± 0.8 with all measurements ≤ 6.9nmol/L.

Table 3.1. Progesterone concentrations of anoestrous wombats.

Wombats	Age group	Mean ± SEM	Range: min-max
7,67,70	Subadult	3.0nmol/L ± 0.2	0.6 to 6.8nmol/L
6, 14, 62, 63, 64, 69	Adult	2.8nmol/L ± 0.9	0.6 to 6.9nmol/L
Overall		**2.9nmol/L ± 0.8**	**0.6 to 6.9nmol/L**

Note: Wombat 13 in not included in this table, as it was the only wombat without an anoestrous period.

Progesterone concentrations above 6.9nmol/L were defined as high and representative of luteal activity. Twelve consecutive phases of high progesterone were monitored from 3 adult wombats (Figure 3.1; Table 3.2).

Although a 4th phase of high progesterone was observed in wombat 14 it was not monitored from start to finish. Therefore the duration of only 12 of the 13 of phases of high progesterone could only be determined. All phases of high progesterone

were observed to increase from basal concentrations, reach a peak and then return to basal concentrations (Figure 3.1). Phases of high progesterone concentrations were regularly interrupted by a phase of basal progesterone. An oestrous cycle was defined as a phase of basal progesterone concentrations followed by a phase of high progesterone concentration (Figure 3.2).

In order to determine the length of the oestrous cycle for a wombat, by progesterone concentrations:

- the start point was defined by the midpoint in time between a preceding high progesterone concentration and the first sample with basal concentrations, and
- following a period of basal progesterone concentrations and a second period of high progesterone concentrations, the end point was defined by the midpoint in time between the last sample of high progesterone concentrations and the subsequent return to basal concentrations.

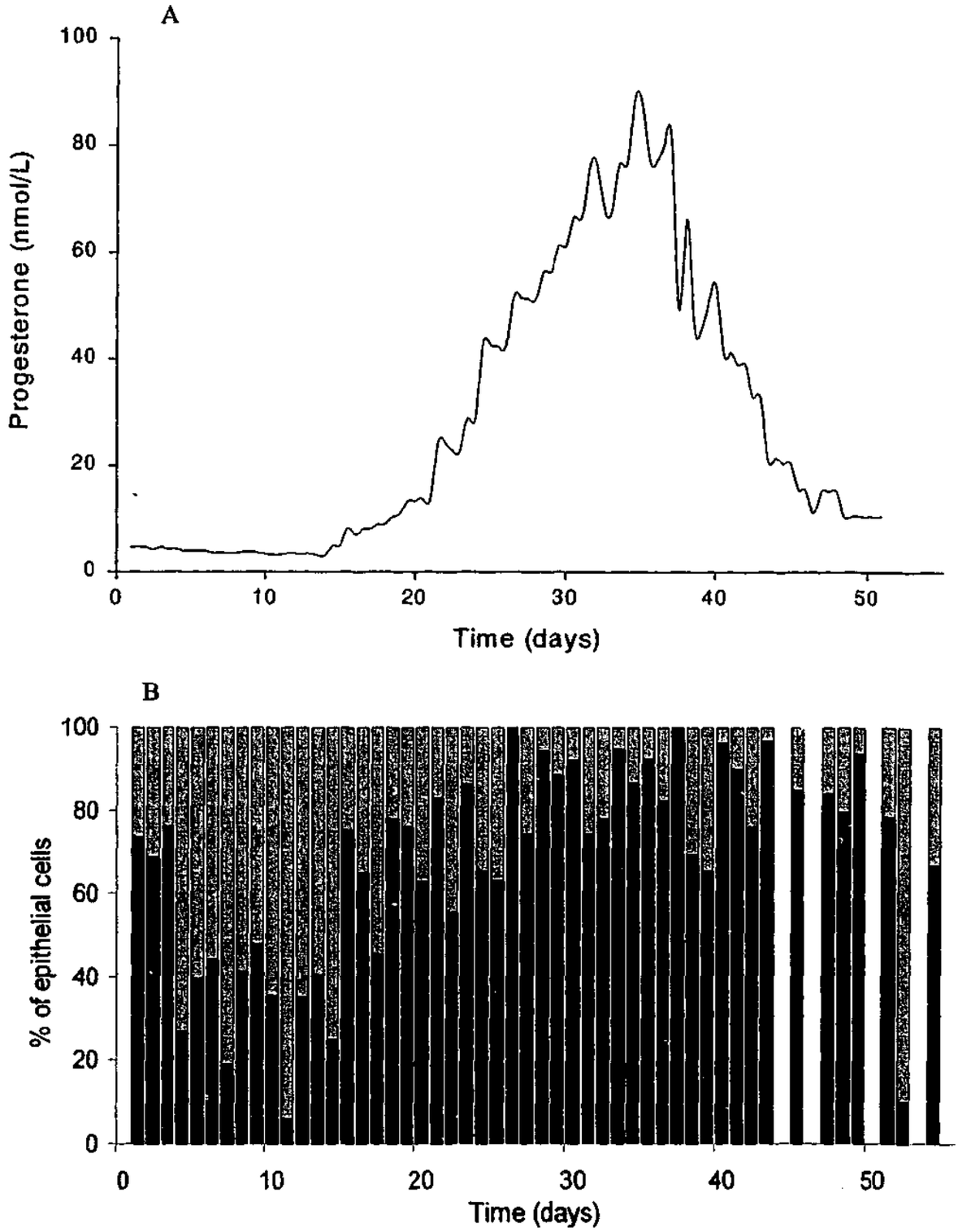
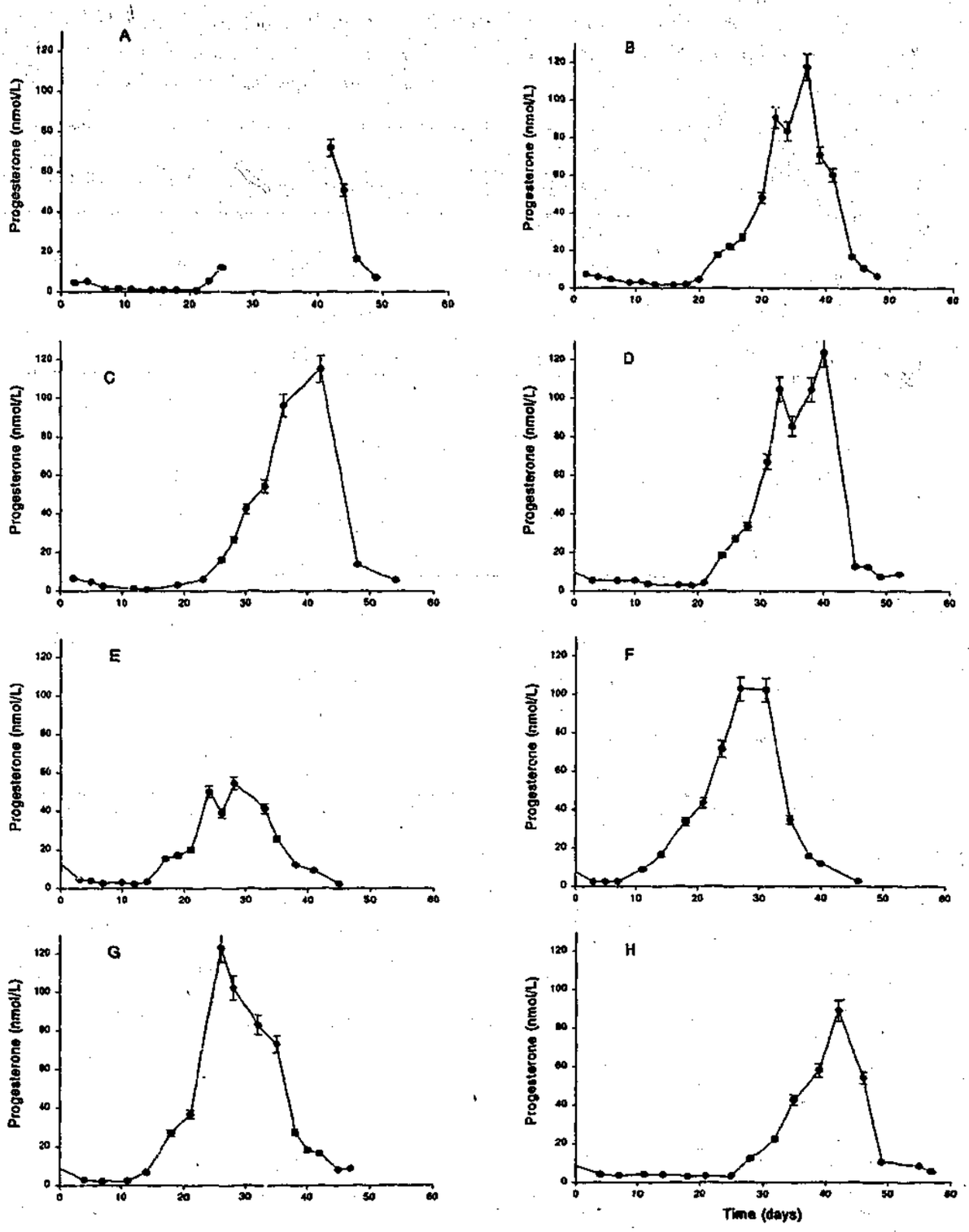


Figure 3.2. Mean progesterone (A; n=9) and vaginal cytology (B; n=12) profiles of 3 Common Wombats. Day 1 is the first day of basal progesterone concentrations (≤ 6.9nmol/L) following a preceding luteal phase (consisting of high progesterone concentrations). Solid black columns represent parabasal-intermediate type epithelial cells, and grey columns represent superficial type epithelial cells.



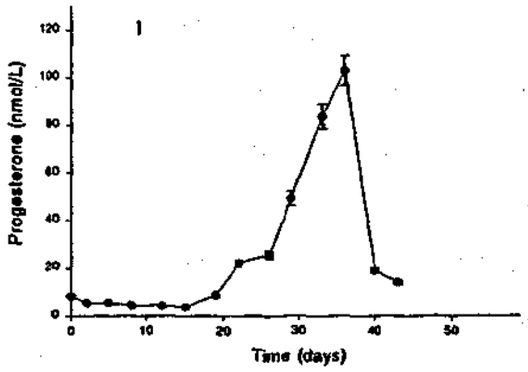


Figure 3.1. Progesterone profiles of 3 Common Wombats during the 9 monitored oestrous cycles. A, B, C, D respectively denote consecutive cycles 2, 3, 4 and 5 of Wombat 13; E, F, G, denote cycles 2, 3 and 4 of Wombat 6; H, I denotes cycles 3 and 4 of Wombat 14. Day 1 is the first day of basal progesterone concentrations (≤6.9nmol/L) following a previously monitored luteal phase (having high progesterone concentrations). Samples were not collected between days 26 – 41 of cycle 2 of Wombat 13 shown in A. Error bars denote the within assay variation (CV =6%).

Table 3.2. Lengths of the Follicular (n=9) and Luteal (n=12) Phases and the Oestrous cycles (n=9) of 3 Common Wombats (6, 13, 14) as defined by changes in progesterone concentrations. Range is given in parentheses.

Wombat	Cycle	Duration		
		Follicular Phase	**Luteal Phase**	**Oestrous Cycle**
6	1*	-	26.5 (22-31)	-
	2	15 (12-18)	27.5 (25-30)	42.5 (39-46)
	3	8 (5-11)	34 (30-38)	42 (38-46)
	4	15 (11-19)	27.5 (25-30)	42.5 (39-46)
13	1*	-	30.5 (29-32)	-
	2	23 (22-24)	26 (25-27)	49 (48-50)
	3	18.5 (17-20)	25.5 (24-27)	50 (47-53)
	4	23.5 (22-25)	26.5 (23-30)	50 (47-53)
	5	22.5 (19-29)	34 2-(31-37)	56.5 (52-61)
14	1*	-	-	-
	2*	-	21 (18-24)	-
	3	24.5 (22-27)	29.5 (28-31)	54 (52-56)
	4	19.5 (18-21)	25 (22-28)	44.5 (42-47)
Overall		**18.8 (5-29)**	**27.8 (18-38)**	**47.2 (38-45)**

* Cycles from which a full data set was not collected, preventing determination of the phase or cycle duration.

Using this definition, the mean duration of 9 follicular phases (periods of basal progesterone concentrations) was 18.8days ± 1.8 (range: 5 to 27days). The mean duration of 12 luteal phases (periods of high progesterone concentrations) was 27.8days ± 1.1 (range: 18 to 38days).

The lengths of the follicular and luteal phases did not vary significantly (P=0.119) within or between each of the 3 wombats (Table 3.2). The mean length of the oestrous cycle, determined for the 3 wombats, was 47.2days ± 1.8 (range: 35 to 60days). There was not a significant difference in the length of the oestrous cycle within or between the 3 wombats (P=0.153).

The mean peak progesterone concentration was 87nmol/L ± 5.3 (range: 41.6 to 123.8nmol/L). Peak progesterone concentrations varied significantly (P<0.05) when compared for each cycle of the 3 wombats, suggesting that two cycles (Cycle 2 of

wombat 6 and cycle 2 of wombat 14) peaked significantly lower than the other 7 cycles examined (Table 3.3).

Table 3.3. Mean peak progesterone levels (nmol/L) for each cycle of each wombat, determined by the difference between the two highest measured concentrations (min - max).

Wombat	Cycle #	Mean P_4 peak	SEM	min	max
6	1	70.1	6.3	63.8	76.4
	2	52.6	2.4	50.2	55.0
	3	102.6	0.1	102.5	102.8
	4	112.8[a]	10.3	102.5	123.1
13	1	117.6[ab]	0.3	117.3	118
	2[*]	-	-	-	-
	3	104.1	13.3	90.8	117.5
	4	105.9	9.5	96.3	115.4
	5	114.3[a]	9.4	104.9	123.8
14	1[δ]	78.3	28.6	49.7	106.9
	2	45.2	3.6	41.6	48.8
	3	73.6	15.5	58.1	89.2
	4	93.2	9.5	83.7	102.8

[*] Peak not observed
[δ] 2nd half of luteal phase was not monitored so peak may have not been sampled
[a] denotes significantly different ($P<0.05$) from wombat 14 cycle 2
[b] denotes significantly different ($P<0.05$) from wombat 6 cycle 2

Progesterone concentrations increased 3 – 7.5 days after estradiol concentrations returned to basal (Section 3.3.2). Progesterone concentrations peaked on average 15.6 ± 0.9 days (range: 9 –19 days) after the increase in progesterone concentrations (n = 8 cycles) at the start of the luteal phase. The peak occurred midway through the luteal phase.

3.3.2 Estradiol profiles

No difference was found between the estradiol concentrations of 12 consecutive samples from an anoestrous adult wombat (wombat 14) and 16 samples from 6 other anoestrous wombats. These estradiol concentrations were defined as basal. Mean basal estradiol concentrations were 0.42pg/ml ± 0.05 (range: 0 to 0.86pg/ml).

Estradiol concentrations above basal concentrations were defined as elevated (>0.86pg/ml).

Elevated estradiol levels were identified at times of basal progesterone concentrations in all three cycles examined (Figure 3.3). In one cycle (from wombat 6) a second phase of elevated estradiol concentrations was observed when progesterone concentrations had begun to decline during the high progesterone phase. Elevated estradiol concentrations lasted for 7.7days ± 2.13 (range: 1 to 17 days). Estradiol concentrations increased, during the oestrous cycle to a mean of 2.11pg/ml ± 0.22 (range: 1.18 to 3.67pg/ml). At other times during the oestrous cycle the estradiol concentrations were not different from the basal concentrations, and had a mean of 0.29 pg/ml ± 0.04 (range: 0 to 0.86pg/ml). Estradiol levels began to increase when progesterone levels returned to basal levels following the high progesterone phase. A return to basal estradiol concentrations was observed 3 – 7.5 days before the next increase in progesterone concentrations was observed.

Analysis of estradiol levels in these 3 wombats allowed an estimate of the cycle length to be determined, finding a mean length of 43.5 days ± 2.51 (range: 38.5 to 46.5). This result was not found to be significantly different to the length of the same oestrous cycles when determined by progesterone concentrations (t=-1.154; P=0.656).

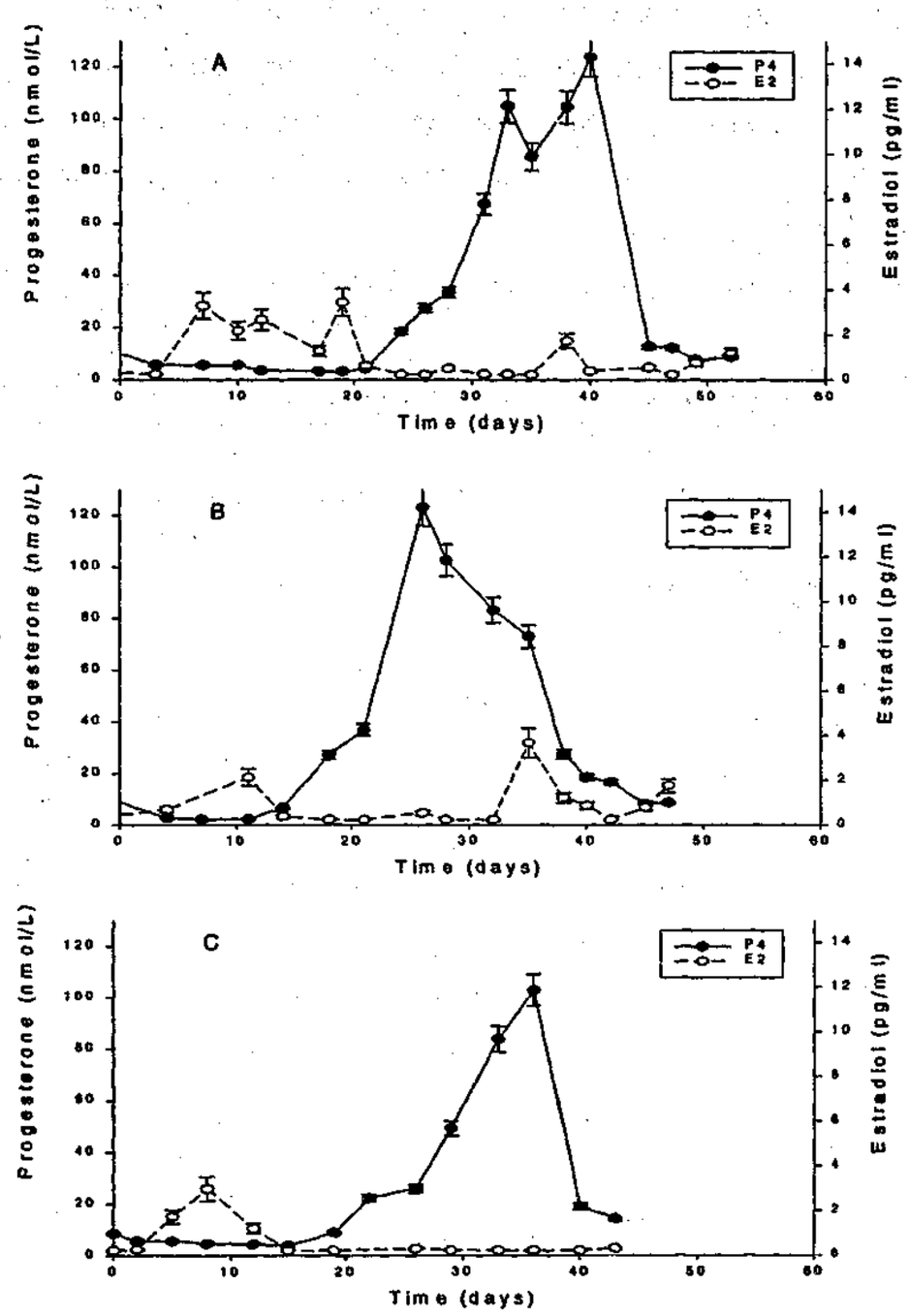


Figure 3.3. Estradiol (E2) profiles of 3 Common Wombats compared to their respective progesterone (P4) profiles. A denotes cycle 5 of Wombat 13; B denotes cycle 4 of Wombat 6; C denotes cycle 5 of Wombat 14. Error bars denote the within assay variation. Day 1 is the first day of basal progesterone concentrations (≤ 6.9nmol/L) following the previous luteal phase (consisting of high progesterone concentrations).

3.3.3 Vaginal Cytology

Variation was identified in the cell count when a representative sample of vaginal smears was counted repeatedly (Table 3.4). The variation was lowest when one group of cell types was present in very low or very high abundance. Variation increased as the proportion of cell types was close to 50%.

Table 3.4. Variation in 5 repeated counts of 10 representative vaginal smears, shown as the mean ± SEM (range: min to max).

Smear no.	Parabasal-intermediate type cells	Superficial type cells
1	99 ± 0.55 (97 to 100)	1 ± 0.55 (0 to 3)
2	96.6 ± 1.3 (92 to 99)	3.4 ± 1.3 (1 to 8)
3	76 ± 1.3 (73 to 80)	24 ± 1.3 (20 to 27)
4	67.6 ± 3.2 (59 to 78)	32.4 ± 3.2 (22 to 41)
5	54 ± 2.7 (46 to 62)	45.6 ± 2.7 (38 to 54)
6	40.2 ± 2.5 (33 to 48)	59.8 ± 2.5 (52 to 67)
7	36.8 ± 2.9 (30 to 46)	63.2 ± 2.9 (54 to 70)
8	29.2 ± 2.7 (21 to 36)	70.8 ± 2.7 (64 to 79)
9	3.8 ± 1.1 (1 to 7)	96.2 ± 1.1 (93 to 99)
10	1.2 ± 1.1 (0 to 3)	98.8 ± 1.1 (97 to 100)

The proportion of epithelial cells and leucocytes found in vaginal smears varied throughout the monitoring period, and broad patterns of change could be observed (Figure 3.4). Vaginal cytology was examined in 8 wombats during periods of anoestrous, and 3 cycling wombats (Table 3.5). During anoestrous, vaginal smears contained predominantly parabasal-intermediate type cells (mean 75.6% ± 1.8 of the total epithelial cells). In all but one smear (0.7%; n=136), > 54% of parabasal-intermediate type cells were observed, The one smear had a greater percentage of superficial type cells than parabasal-intermediate type cells. Generally, superficial type cells formed a mean of 23% ± 1.7 (< 46%) of the total epithelial cells. The percentages of total epithelial cells were not significantly different between subadult and anoestrous adult wombats (P>0.1).

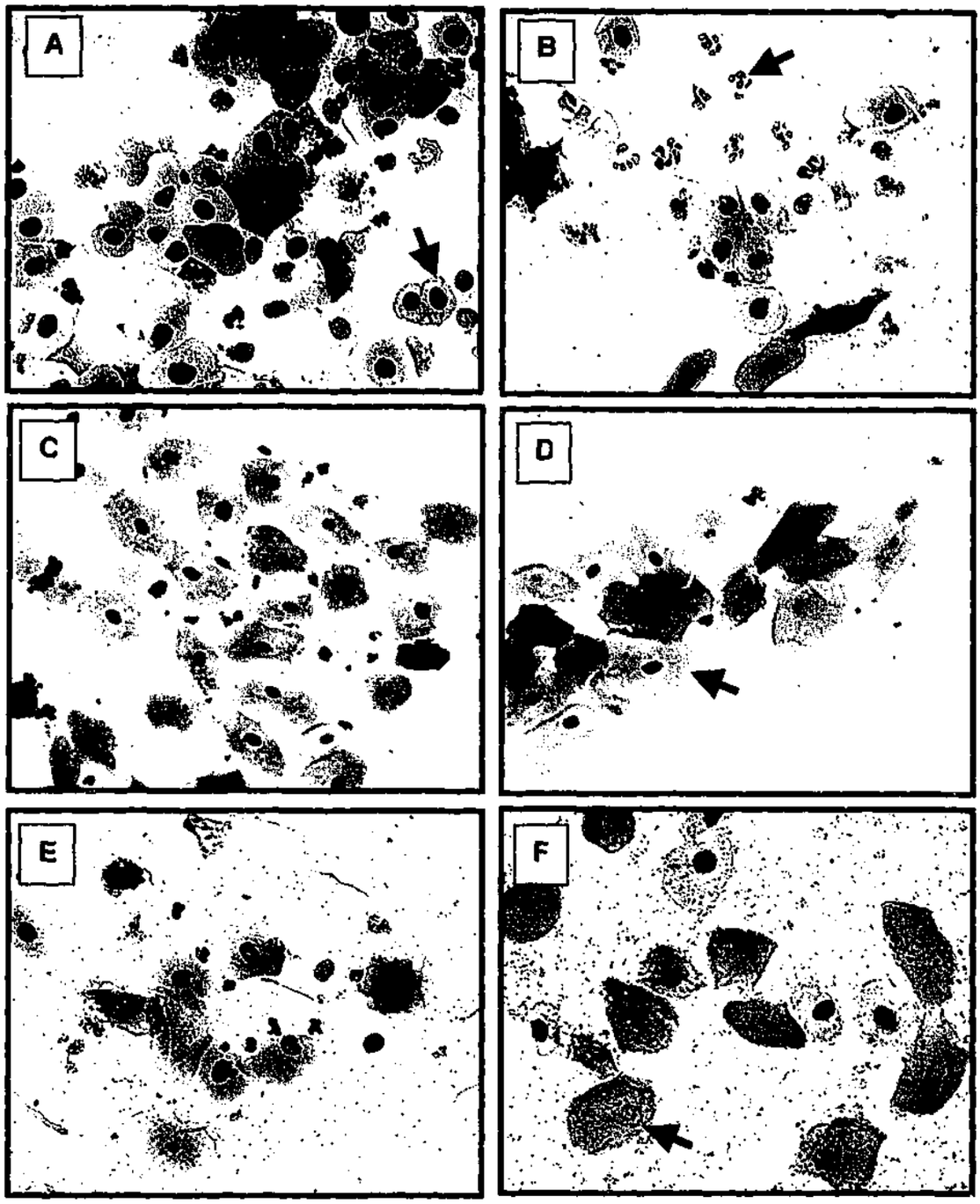


Figure 3.4. Vaginal Smears of the Common Wombat. A displays parabasal epithelial cells (arrow), predominately small and large intermediate epithelial cells; B displays intermediate type cells and leucocytes (arrow); C displays superficial epithelial cells with pyknotic nuclei; D displays superficial epithelial cells with pyknotic nuclei (arrow) and some superficial cells with almost absent nuclei; E displays intermediate type cells, superficial epithelial with pyknotic nuclei and without nuclei, and some leucocytes; F displays predominately superficial epithelial with pyknotic nuclei and without nuclei (arrow) and some intermediate type cells. A and B were typical of the luteal phase and subadult and anoestrous wombats. C, D, E and F were typical of the follicular phase. Images were taken at x200 magnification under brightfield microscopy.

Table 3.5. The percentages of epithelial cells and leucocytes during the oestrous cycle (n=12) and anoestrous periods. Data is presented as the mean ± SEM.

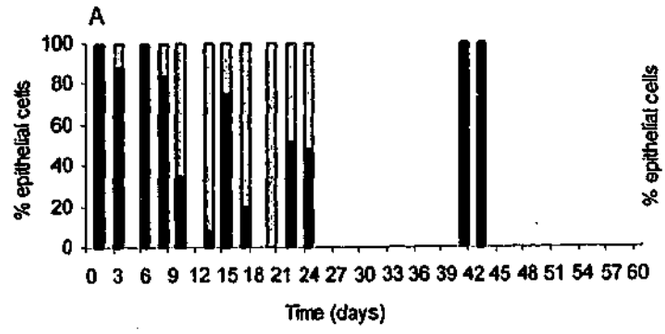
Reproductive Status	No. of wombats	% epithelial type cells		% smears with leucocytes	Leucocytes/ 100 epithelial cells
		Parabasal-Intermediate	Superficial		
Anoestrous Subadult	1	75.6% ± 1.8*	23% ± 1.7*	4.5%	12
Anoestrous Adult	7	>54%	<46%	40.5% ± 7.7	240.4 ± 91
Cycling Adult (high superficial)	3	27.3% ± 3.3	72.3% ± 3.3	62.7% ± 15ϕ	2161.5 ± 1340.5ϕ
Cycling Adult (basal cytology)	3	88.8% ± 1.1	10.5% ± 1.1		

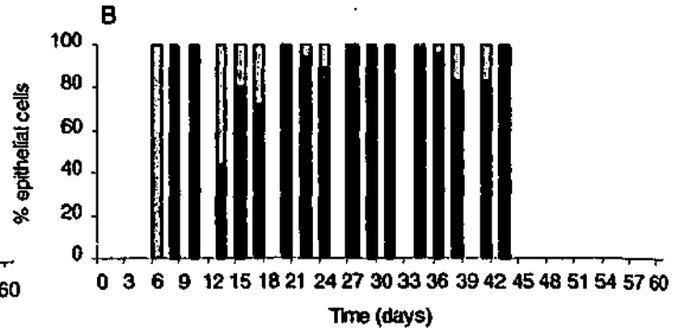
As no significant difference was found (P>0.05):
ϕ leucocyte data was pooled for cycling adult wombats; and
* epithelial cell data was pooled for anoestrous subadult and adult wombats

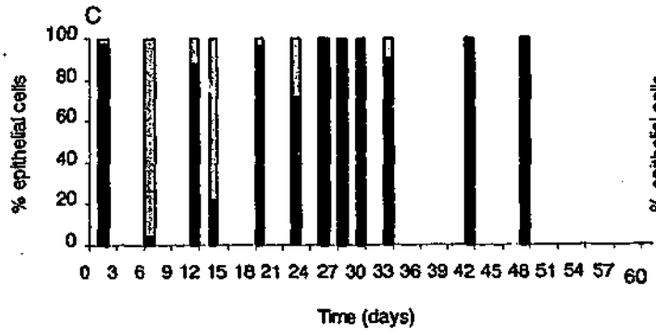
Wombats in anoestrous were used to define the following basal vaginal cytology.

- parabasal and intermediate type cells formed between 54% and 100% of the total epithelial cell population, and
- superficial type cells formed between 0% and 46% of the total epithelial cells.

During the oestrous cycle, as defined by progesterone concentrations, two phases of vaginal cytology were identified. During periods of high progesterone there was a slightly higher mean percentage of parabasal-intermediate type cells (mean 88.8%) than in anoestrous wombats (mean 75.6%; Table 3.5). This was followed by a second phase when parabasal-intermediate cells formed a much smaller percentage of the total epithelial cells. During the second phase, superficial type cells formed greater than 46% of the total epithelial cells. In 10 of the 12 phases, the superficial type cells peaked to nearly 100% of the total epithelial cells, although generally, a mean percentage of 72.3% ± 3.3 superficial type cells occurred (Figure 3.5). These 10 phases occurred during periods of basal progesterone concentrations and therefore the follicular phase of the cycle. The peaks of superficial type cells of the other two cycles examined were not as clearly associated with follicular phase (Figure 3.5).


A
% epithelial cells
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48 51 54 57 60
Time (days)


B
% epithelial cells
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48 51 54 57 60
Time (days)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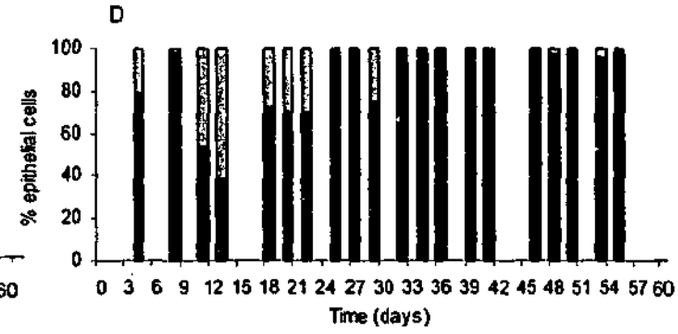
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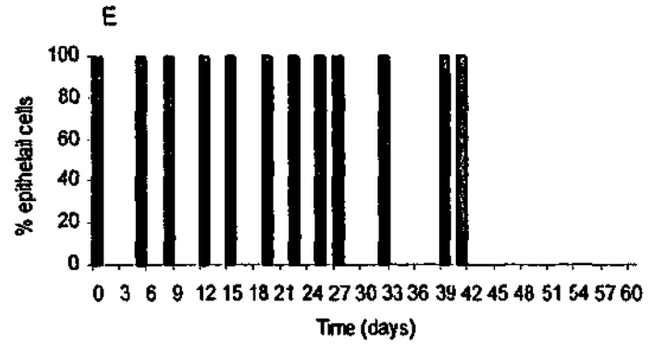
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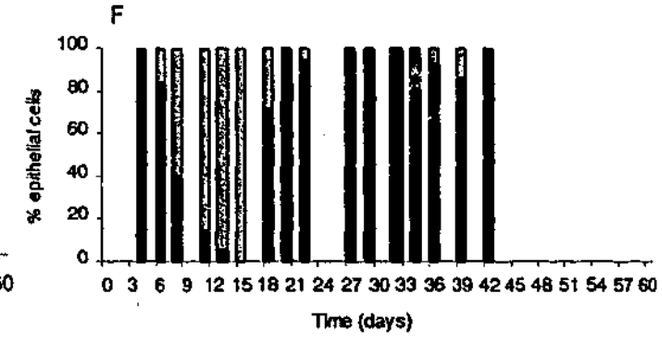
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0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48 51 54 57 60
Time (days)

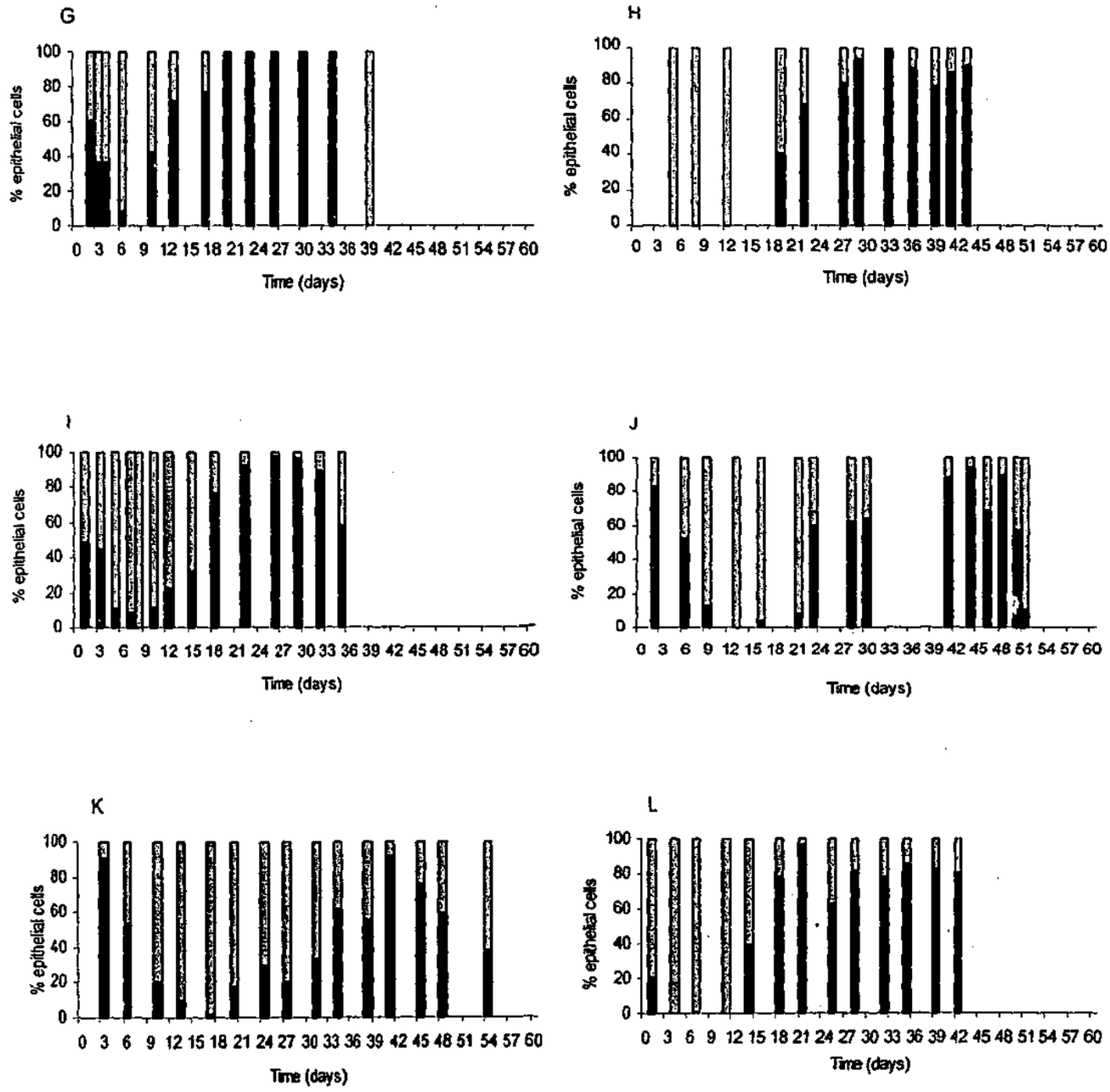


Figure 3.5. Vaginal Cytology of Common Wombats. A, B, C, D and E respectively denote cycles 1, 2, 3, 4 and 5 of wombat 13; F, G, H, I, J denote cycles 1, 2, 3, 4 and 5 of Wombat 6; K and L denote cycles 3 and 4 of Wombat 14. Solid black columns indicate the percentage of parabasal – intermediate type epithelial cells and grey columns indicate the percentage of superficial type cells. Day 1 is the first day of the follicular phase as defined by basal progesterone concentrations.

In 6 of the 12 cycles examined, the percentage of epithelial cells fluctuated during the phase of a high percentage of superficial cells, but remained greater than 46% of the total epithelial cells (Figure 3.5). In the other cycles examined the percentage of epithelial cells increased to a peak and then decreased, prior to returning to a phase of basal vaginal cytology.

The general pattern was repeated in consecutive oestrous cycles, and was observed in 91.7% of cycles (n=12). In the one cycle (wombat 13) in which this general pattern was not observed, a shift towards a high percentage of superficial cells was observed in the first half of the luteal phase (Figure 3.2).

In another cycle (wombat 14, cycle 1), in which a full data set was not collected, the general vaginal smear pattern did seem to match progesterone results. However, the duration of a high percentage of superficial cells lasted for an extended period of time 46days (range: 43 to 49days).

During the follicular phase of the oestrous cycle, a high percentage of superficial cells was sustained for a mean of 12.4days ± 2.3 (range: 2.5 to 28days). The phase of basal vaginal cytology lasted an average 32days ± 2.4 (range: 18.5 to 47days). The length of the oestrous cycle, using vaginal cytology, was 45.2days ± 2.3 (range: 32 to 55days). No significant variation was found in the cycle length as defined by vaginal cytology, within individuals or between wombats (P=0.523). Similarly, no significant variation was found in the duration of each of the phases of basal vaginal cytology (P=0.174) or high percentages of superficial type cells (P=0.402).

Leucocytes were rarely found (4.5%) on the vaginal smears of subadult wombats, and when they were found, they formed a small proportion of the total cells (Table 3.5). Leucocytes were identified more regularly on the vaginal smears (40.5% ± 7.7) of adult anoestrous wombats and represented a greater proportion of the total cells. In one adult anoestrous wombat, in two consecutive smears, nearly all cells were leukocytes (≥ 50000 leucocytes per 100 epithelial cells) and very few epithelial cells were observed.

High numbers of leucocytes were also found regularly in the vaginal smears of cycling adult wombats (**Table 3.5**). In the cycling adult wombats, 62.7% ± 15 (range: 35-89%) of smears contained leukocytes. Of the cycling adult smears that contained leucocytes, there was a mean of 290 ± 59.4 (range: 2 to 4167) leucocytes per 100 epithelial cells. No specific pattern was observed with respect to the number of leucocytes throughout the two phases of the oestrous cycle.

Mucus was another characteristic that could be identified on vaginal smears, however, clear, often copious mucous was more easily recognised upon the withdrawal of the vaginal swab prior to rolling it on a slide. In cycling adult wombats, mucous regularly coincided with the follicular phase. Mucous was also found during 7 luteal phases, however it was mostly restricted to the first 1 - 2 days as progesterone concentrations were increasing. On 3 occasions, mucous was present for 1 - 3 days in the middle of a phase of high progesterone.

Mucous was also observed for a period of 12-14 days in one anoestrous adult (wombat 14) that had basal progesterone concentrations and basal vaginal cytology, 6 months prior to the onset of the first oestrous cycle. Mucous was observed in this animal on 1 day, 2 months prior to the onset of the first oestrous cycle. Mucous was also observed in 2 adult wombats (wombat 62 and 63) in the 3 to 7 days following removal of pouch young, and again for one day, ~ 3.5 months after the removal of their pouch young. A copious, opaque to yellowish mucous, was also observed irregularly, for periods of 1 to 5 days, in an anoestrous adult (wombat 69) that had an unusual swollen urogenital sinus, although leucocyte counts were similar to other anoestrous wombats.

3.3.4 Vaginal Cytology (Shorr's Vs DiffQuick)

When vaginal smears were compared after staining with Shorr's Stain (n=37) or DiffQuick (n=37) fewer parabasal-intermediate type cells were recognized on the smears stained with Shorr's Stain. This meant that more epithelial cells were counted as superficial type cells when stained with Shorr's Stain. On average 12.4% ± 2.1 (range: -8 to 54%) more parabasal-intermediate type cells were counted on smears stained with DiffQuick. Leucocytes were also found less frequently on the

smears stained with Shorr's Stain. Leucocytes were found on 21.6% of smears stained with Shorr's Stain and 48.6% of smears stained with DiffQuick.

The major reason for the difference was that Shorr's Stain did not stain the nucleus of cells as well as DiffQuick. Secondly, the cytoplasm of the parabasal-intermediate type epithelial cells generally did not stain as brightly as the cytoplasm of the superficial type epithelial cells. Leucocytes stained poorly in Shorr's stained smears. Despite this difference, the same pattern of change was observed with both stains, enabling the same conclusions to be drawn.

3.3.5 Body temperature

The examination period, for each wombat, lasted up to 20mins. Body temperature, measured every 5mins, fluctuated slightly during the examination period. During the examination period, body temperature was observed to increase by an average of:

- 0.04°C (range –0.4 to 0.3°C) after 10 to 15mins; and a further
- 0.1°C (range –0.2 to 0.3°C) at the end of the examination (20mins).

The mean rectal body temperature of the 11 female wombats was 34.8°C ± 0.2. However, body temperature was observed to vary between 32.1°C and 37.6°C. Fluctuations in rectal body temperature were not found to be associated with changes in progesterone or estradiol levels, or vaginal cytology. When body temperature was examined over the lengths of the oestrous cycle no distinguishing pattern of change was identified that allowed a specific period of cyclic activity to be determined.

3.3.6 Body Weight

The average body weight of the 3 subadult animals was 26.3 kg ± 0.5 (range: 17.8 to 23.7kg). Body weight was observed to increase in one subadult wombat (wombat 7) from a low of 17.8kg ± 0.1 to a high of 23.7kg ± 0.1 over 11 months.

The average body weight of cycling wombats was 26.3kg ± 0.8 with a minimum observed weight of 23.5kg ± 0.1 (Table 3.6). Fluctuations in the body weights of cycling adults did occur through out the oestrous cycle and over the duration of this study although this was not related to oestrous cycle stage.

Table 3.6. Body weights of adult cycling wombats.

Wombat	Body weight (kg)				
	n	Mean	SEM	Min	Max
6	89	25.5	0.07	23.5	26.8
13	106	25.6	0.08	23.5	27.7
14	43	27.9	0.04	27.0	28.5

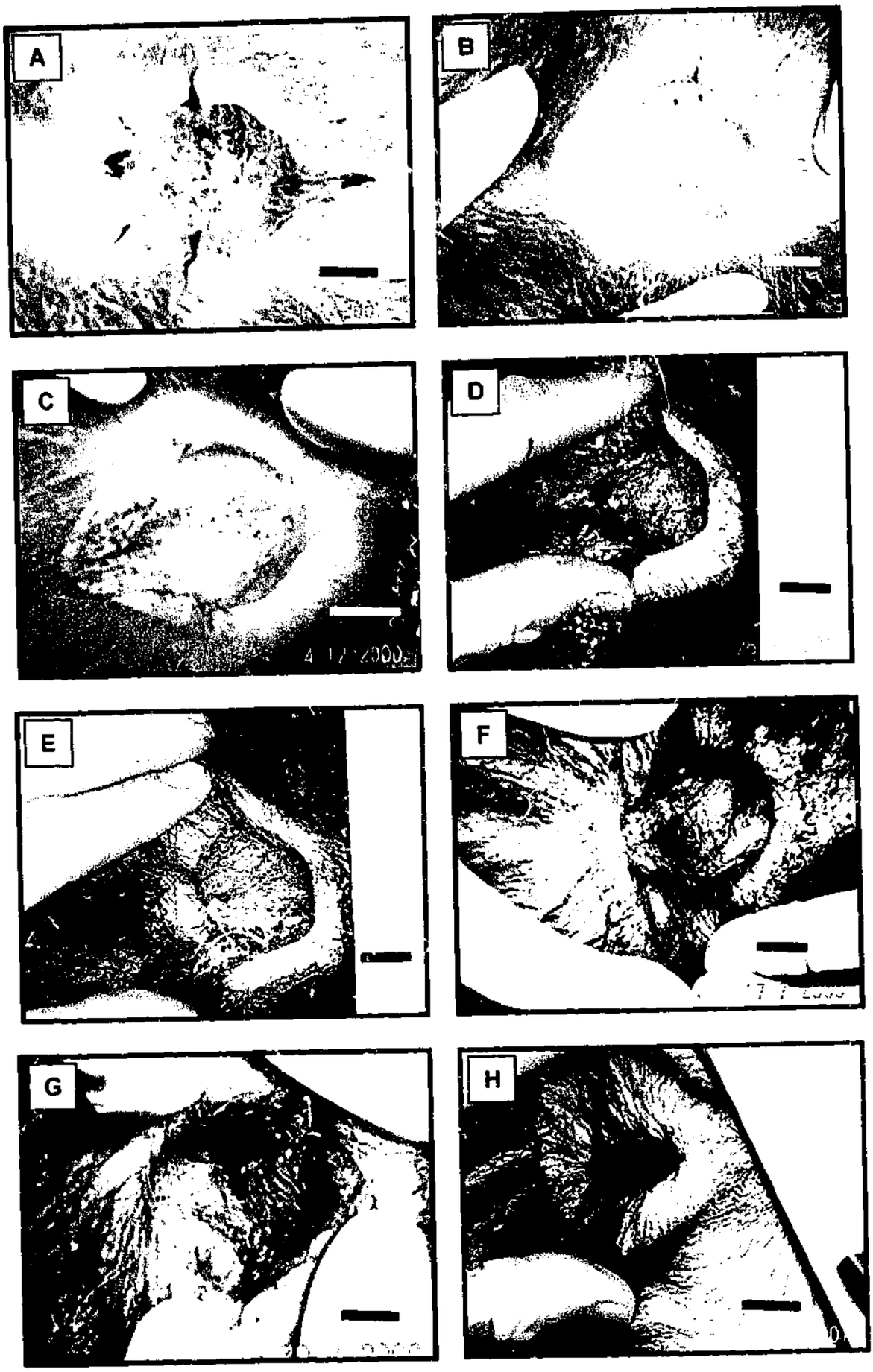
Anoestrous in adult wombats was associated with varying body weights (range: 20.6 to 27.9kg). One adult anoestrous wombat (wombat 6) had a low body weight 21.2kg ± 0.2 (range: 20.6 to 22.3kg) whilst showing clinical signs of sarcoptic mange. During this time, progesterone levels remained basal. Body weight began to increase as the clinical signs of mange disappeared following treatment with Ivermectin (IVOMEC®). The female wombat was found to begin cycling as soon as the clinical signs of mange had disappeared and body weight had increased to 23.5kg ± 0.1.

3.3.7 Pouch condition

The pouch of the Common Wombat is located on the external surface of the lower central (or hypogastric) region of the abdomen. The pouch is anterior to the urogenital sinus opening which is located near the base of the remnant tail (Figure 3.6). The pouch appearance in adult cycling wombats was compared with the pouch of subadults and adults in anoestrus.

Adult cycling wombats: The appearance of the adult pouch during the oestrous cycle was distinctly different from that of anoestrous adults or subadults. Adult cycling wombats had a distinct rim forming a circular opening to the pouch. The pouch opening was tightly contracted in adult cycling wombats so that despite anaesthesia, some effort was required to expand or open it (Figure 3.7).

Figure 3.6. External reproductive anatomy of the Common Wombat (*Vombatus ursinus*). 1: Ventral surface of the Common Wombat showing the Cloaca (C) and the Pouch (P) (Scale bar represents 100mm). 2: Close up of the cloaca the common opening of the rectum and urogenital sinus (Scale bar represents 20mm). 3: Close up of the pouch (Scale Bar represents 25mm).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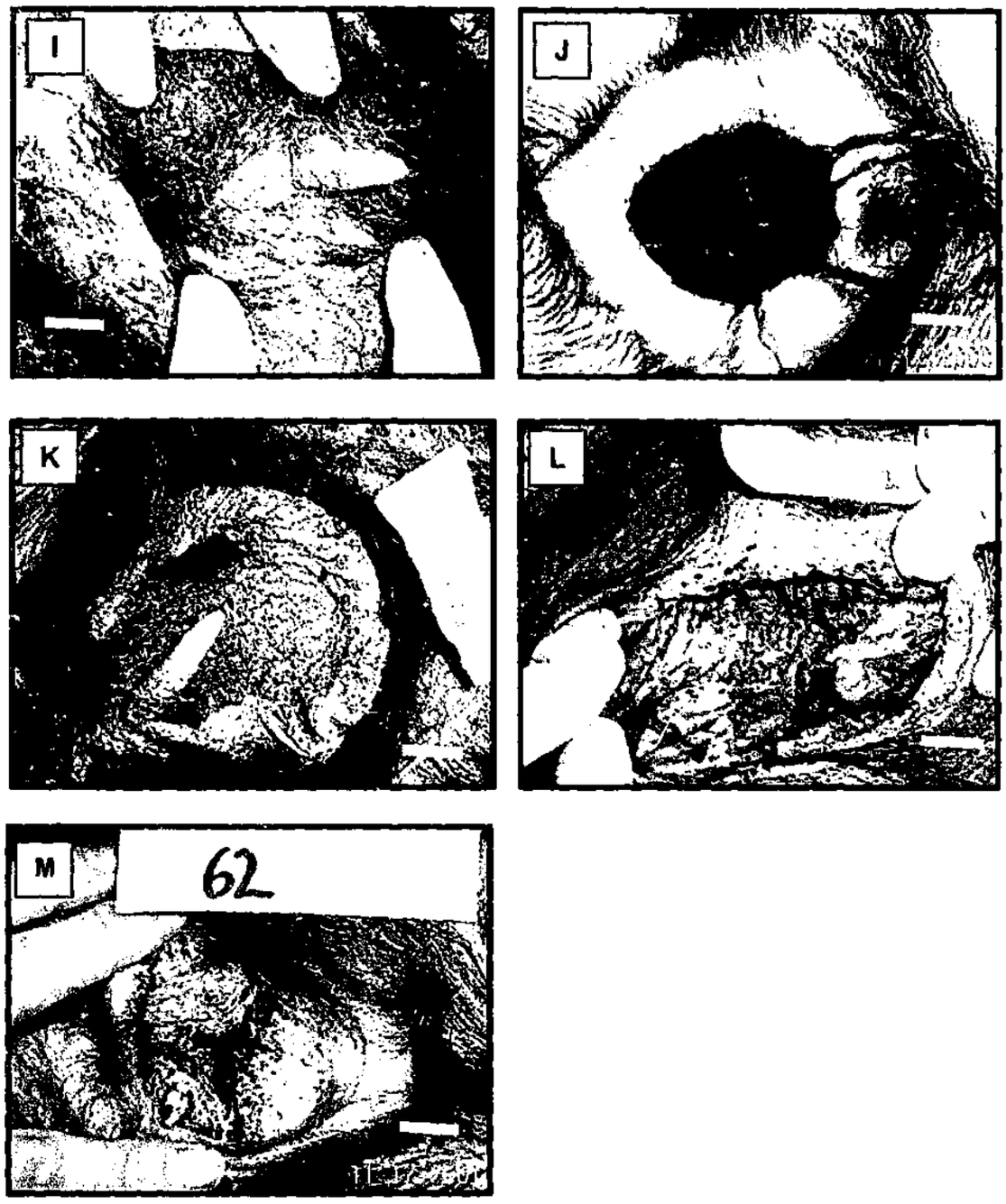


Figure 3.7. Different pouch conditions of the Common Wombat. A, B, and C show variations in the pouch of the subadults, and the teats are visible in B; D – H show different stages of the cycling adult pouch: D shows a moderately moist, mildly dirty pouch, E shows a clean moderately moist pouch, F shows a very dirty, very moist pouch, G shows the nipple of a cycling adult, H shows the pouch opening of the cycling adult which was often difficult to open. I shows the pouch of a lactating adult that had lost a young at foot, and J shows the pouch regression observed of the same pouch 6 weeks after the previous photo (I); K shows the pouch of an adult wombat after 2 weeks after removal of the pouch young, L shows the pouch and teat regression and formation of scale in the pouch 5 weeks after pouch young removal and M shows the anoestrous post lactational pouch 3 months after pouch young removal, which was also typical of other anoestrous adults. G shows the teat of the cycling adult, Scale bars indicate 10 mm.

The pouch of cycling adults was consistently larger than subadult wombats, with an internal diameter of 550 to 700mm wide, and 200 to 400mm deep. No mammary swelling was observed in adult cycling wombats, and the teat colour was comparable to the rest of the pouch. The length of the teats was found to vary between 4 to 7mm (mean: 5mm) throughout the oestrous cycle.

In the three cycling wombats, no consistent changes were observed that indicated a particular stage of the oestrous cycle. The pouch of cycling adults varied significantly in cleanliness and the amount of pouch secretion (Figure 3.7). The pouch was mostly very moist, although at irregular intervals during the oestrous cycle, the pouch secretions dried up, to mildly moist to dry. This mildly moist to dry condition lasted for periods of 1 to 5 days. As observations of the pouch were not made every day it is unclear if the pouch remained in this condition over the period.

The pouch of one adult cycling wombat (wombat 13) consistently changed from very dirty to mildly dirty or clean towards the end of the luteal phase, as determined by declining progesterone levels. The pouch of the other two cycling wombats was usually mildly to moderately dirty in appearance (wombat 6: 2 cycles of 4; Wombat 14: 1 cycle of 4). On one occasion, towards the end of the luteal phase, the pouch was found to be moderately to very dirty in appearance (wombat 14: 1 cycles of 4).

Anoestrous subadult wombats: In contrast to cycling adults (n=3), the pouch of anoestrous subadult wombats (n=3) was mostly dry and clean in appearance and was very shallow. In subadults the pouch opening at the anterior end was continuous with the abdomen, and the posterior half of the pouch had a rim forming a semi-circular opening. The posterior rim of the pouch was relaxed on some examinations and partially contracted on other examinations. When the posterior rim was contracted, a shallow cavity was formed in the posterior region of the pouch so that the teats were covered.

The posterior region of the subadult pouch was mildly moist on 28.9% of examinations (n=69) however there was no consistent pattern of change, and was often dry on subsequent examinations. The teats of the subadult, were similar in

length to the cycling adults, but pointed and darkly pigmented at the tip. Like cycling adults, subadults did not have any mammary swelling.

Anoestrous adults: Pouch young (between 4 and 6 months of age (Triggs 1996)) were removed from 3 adult wombats (wombats 62, 63, 67). Immediately after removal the pouch was found to be very deep, mildly to moderately moist and clean. One teat was very extended (range 64 to 98mm) and lactating, with associated mammary swelling. The other teat was very small, 5mm in length, and not lactating, and no mammary swelling occurred.

The progressive changes in the pouch, following pouch young removal, were monitored for two wombats (wombats 62, 63). Within one week after pouch young removal, the pouch became dry and a thick dry layer of dermal tissue had formed (Figure 3.7). After two weeks the extended teat had started to regress and only slight lactation was evident, and the dry dermal layer had become scaly. Pouch scale was shed from the posterior region of the pouch initially. The pouch scale had been completely shed in one animal (wombat 63) 1.5 months after pouch young removal, however in the other wombat the scale was shed slowly over 3 to 5 months.

Areas of the pouch were clean immediately after the pouch scale had been shed, and these areas became increasingly more moist in the days following. After the scale had been lost completely, the pouch appeared similar to that of cycling adults, and had a similar irregular pattern of change in the amount of pouch secretion and clean or dirty appearance.

The pouch shape of adult wombats, following pouch young removal, varied from that of cycling adults. During the 2 months following pouch young removal the pouch became less deep, and it remained open. The rim that formed the opening to the pouch began to regress so that at the anterior end became continuos with the abdomen, as observed in anoestrous subadults, although the pouch did not become as shallow.

Another distinguishing feature, from adult cycling wombats, was the differing lengths of the teats, following pouch young removal. By 4 - 5 months the extended teat had almost completely regressed to be ~ double the length (9 to 9.5mm) of the smaller teat (~5mm). The time taken for the teat to regress was dependent on its initial length. So the less extended teat (64mm) of wombat 63 regressed more quickly than the longer teat (94mm) of wombat 62. The extended teat of wombat 63 had almost completely regressed by 6.5 to 7 months after pouch young removal and was observed to be similar in appearance and length, within 1 to 2mm, to the non-extended teat.

Wombat 14, had, and was caught beside a burrow that contained a newly dead young-at-foot (estimated to be 10 to 12 months of age), and was found with one slightly lactating teat but without pouch young or young-at-foot. It was presumed that the young belonged to this adult, given the proximity to the adult's capture, reduced lactation of the teat, the pouch appearance. The pouch depth was too shallow to permit entry of a large pouch young, but would have been suitable for a young-at-foot to suckle. One month after capture, the pouch began to loose scale and by two months the scale had been completely shed. Milk could be expressed from the teat for one month after capture, and during this time it began to regress. The teat, which was initially 35mm in length, had completely regressed 6 months after capture.

Unlike the adult wombats (wombats 62 and 63) from which younger pouch young had been removed, the pouch opening of wombat 14 reduced after one month and retained a complete rim (Figure 3.7). The opening to the pouch remained partially contracted and partially open for 10 months after capture. The opening to the pouch became more contracted in the month leading up to the animal's first cycle since loss of the young. The exterior region of the pouch constantly had a thick brown to black wax attached firmly to the hair. This wax was also inside the anterior region of the pouch. No wax was found inside the pouch when the animal returned to cyclic activity.

Despite low progesterone concentrations, and consistent anoestrous type vaginal cytology, one adult wombat (wombat 69), that was found to have a similar pouch to

the adult cycling wombats. This pouch condition, observed for 2 months in wombat 69 was not observed in other anoestrous adults, except in wombat 14 just prior to return to oestrous activity.

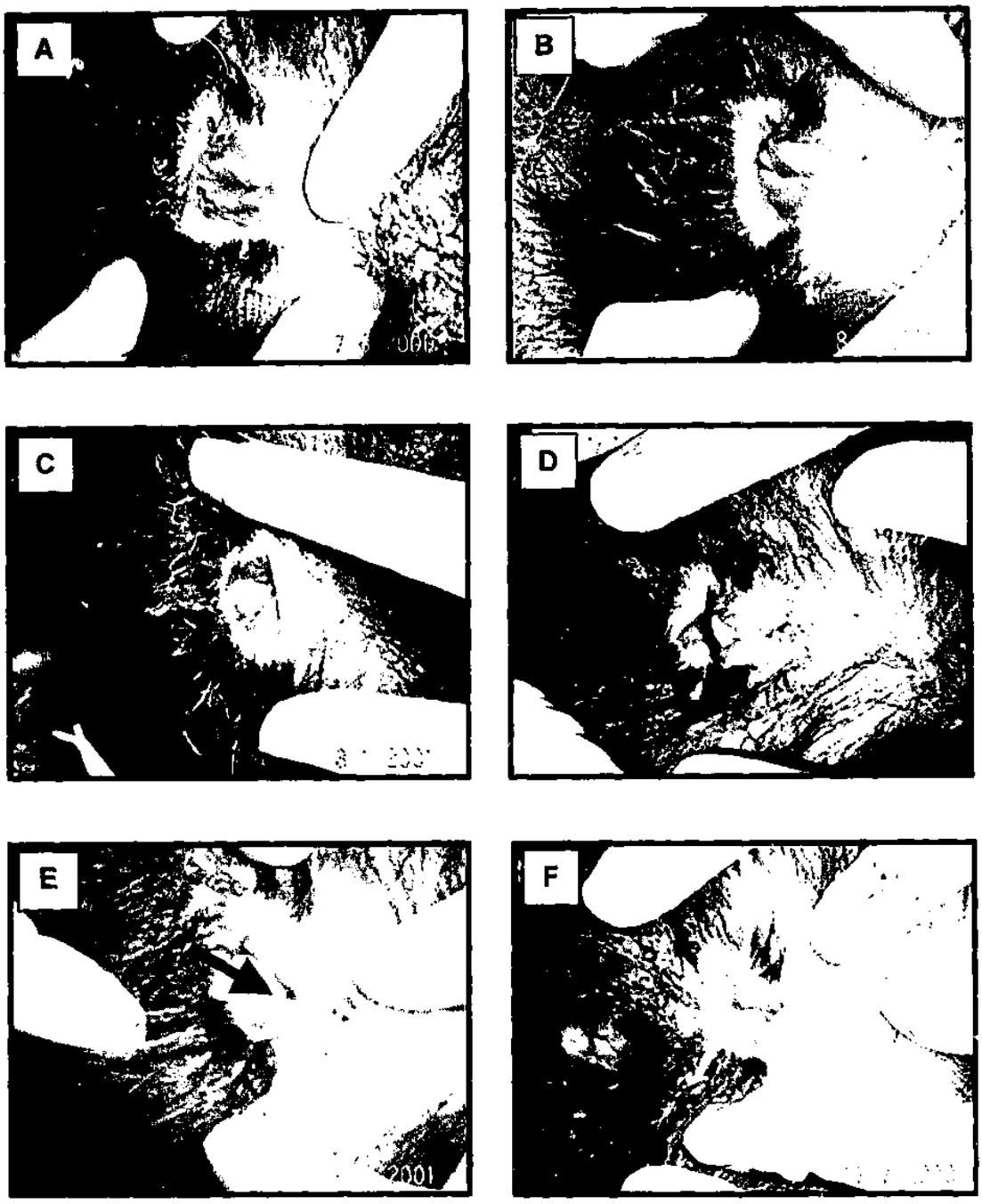
3.3.8 Changes in urogenital opening

Anoestrous subadult wombats: The urogenital opening of subadult wombats remained inverted to mildly protruded throughout the study. Occasionally the urogenital opening had a moist appearance, however this did not appear to follow a particular pattern, and may have simply been associated with urination. Mucous was not observed on the exterior surface of the cloaca, urogenital opening, or the hair surrounding the cloaca of subadult wombats. As the body weight of a subadult approached that of adult wombats ($\geq$ 23.5 kg $\pm$ 0.1) the urogenital opening became occasionally mildly to moderately protruded.

Adult cycling wombats: The urogenital opening of 3 adult wombats became protruded and turgid at intervals throughout the oestrous cycle. Similarly, the exterior surface of the cloaca and urogenital opening was periodically covered in a clear or opaque to yellowish mucous, but these changes did not necessarily coincide.

An attempt was made to quantify the swelling of the urogenital opening, and even though general measurements could be made, they were considered to be unreliable. Observations of the swelling of the urogenital opening were therefore kept to descriptions. As descriptions were not quantifiable, the vaginal opening was described as being either "inverted - mildly protruded" or "moderately - very protruded".

Through multiple observations, a general pattern was recognized in adult cycling females (Figure 3.8) with an initial period in which the urogenital opening was predominately, moderately - very protruded and often associated with external mucous coinciding with basal progesterone levels. Secondly there was a period in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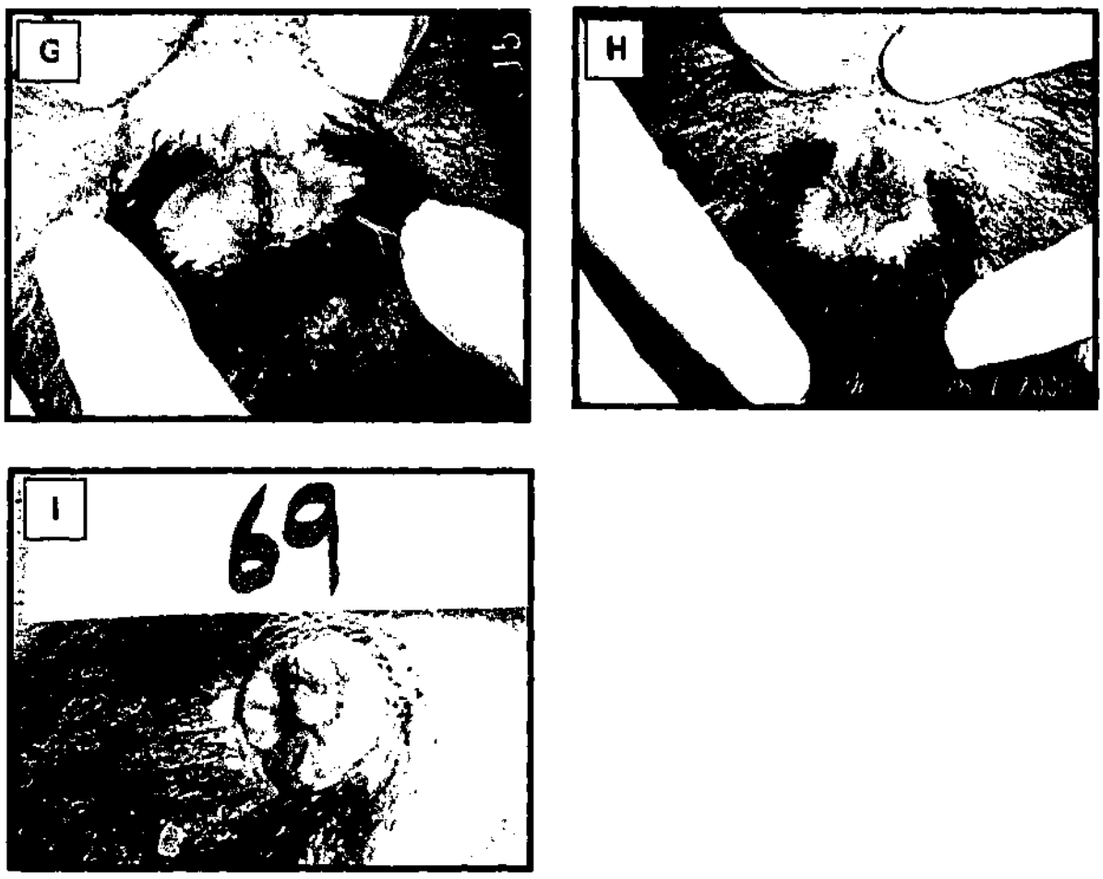


Figure 3.8. The cloaca and urogenital opening (UO) of the Common Wombat. A and B shows the protruded UO of cycling adults, typical or the follicular phase, and clear viscous mucous can be seen in A. C and D respectively show the inverted UO of a subadult and cycling adult (typical of the luteal phase). F shows the inverted UO of an anoestrous adult. E shows the cloaca of a cycling adult with a protruded UO and the arrow point to the urogenital sinus. G and H respectively show the caudal view of the subadult and cycling adult (during the follicular phase) wombat. I show the abnormally enlarged and swollen urogenital opening of wombat 69 during anoestrous. Most photos are arranged so that the animal's tail is to the left of the photo (as can be seen in C labeled T), excluding G and H where the tail is located towards the bottom of the photo.

which the urogenital opening was predominantly inverted - mildly protruded and mucus was rarely visible when progesterone levels were high.

Despite this general pattern, variations in the condition of the urogenital opening were observed. During all of the phases (n=13) when basal progesterone concentrations occurred, there were brief observations when the urogenital opening was inverted - mildly protruded. This urogenital state was mostly restricted towards the very start or end of a period of basal progesterone concentrations. During 30.7% (4/13 cycles) of luteal phases the urogenital opening was briefly moderately - very protruded. This observation was restricted towards the very start or very end of a period of high progesterone levels.

Mucus on the external surface of the urogenital opening was observed during 61.5% of follicular phases and 53.8% of luteal phases, although in contrast to the follicular phase, mucus usually only lasted for a single observation during the luteal phase. Mucous was not detected during the other phases.

The appearance of the urogenital opening in other adult wombats, including those that had pouch young removed, lost young at foot or had recently weaned a young wombat, did not follow any observed trend. Wombat 69, had a swollen urogenital opening that often was covered in an opaque mucous and unusual scar tissue on her tail, although it was not known if these observations were linked.

3.3.9 Behaviour

Although behavior was not intensively monitored throughout the study, at times of sample collection, behaviour could be broadly divided into "more aggressive" and less aggressive". More aggressive behavior was recorded following capture until the animals became accustomed to the new environment, when less aggressive behavior was observed. More aggressive behavior was also observed during follicular phases in cycling wombats. This aggressive behavior included increased resistance to being handled. Significant behavioural differences were observed between individuals and it was not considered to be a consistent indicator of estrous, or other cyclic activity.

3.3.10 Change in reproductive status over study

The reproductive status of wombats changed during the study (Figure 3.9). Oestrous cycles were observed in all months of the year (Table 3.7). Wombats began to cycle after extended periods of anoestrous following mange (wombat 6) or lactation (wombat 14). Another wombat (wombat 13) was cycling when first examined and continued to cycle for 9 months, until the study ended. A forth wombat (wombat 69), had high progesterone concentrations at the time of capture and appeared to be cycling at capture, and became anoestrous 1 month after capture.

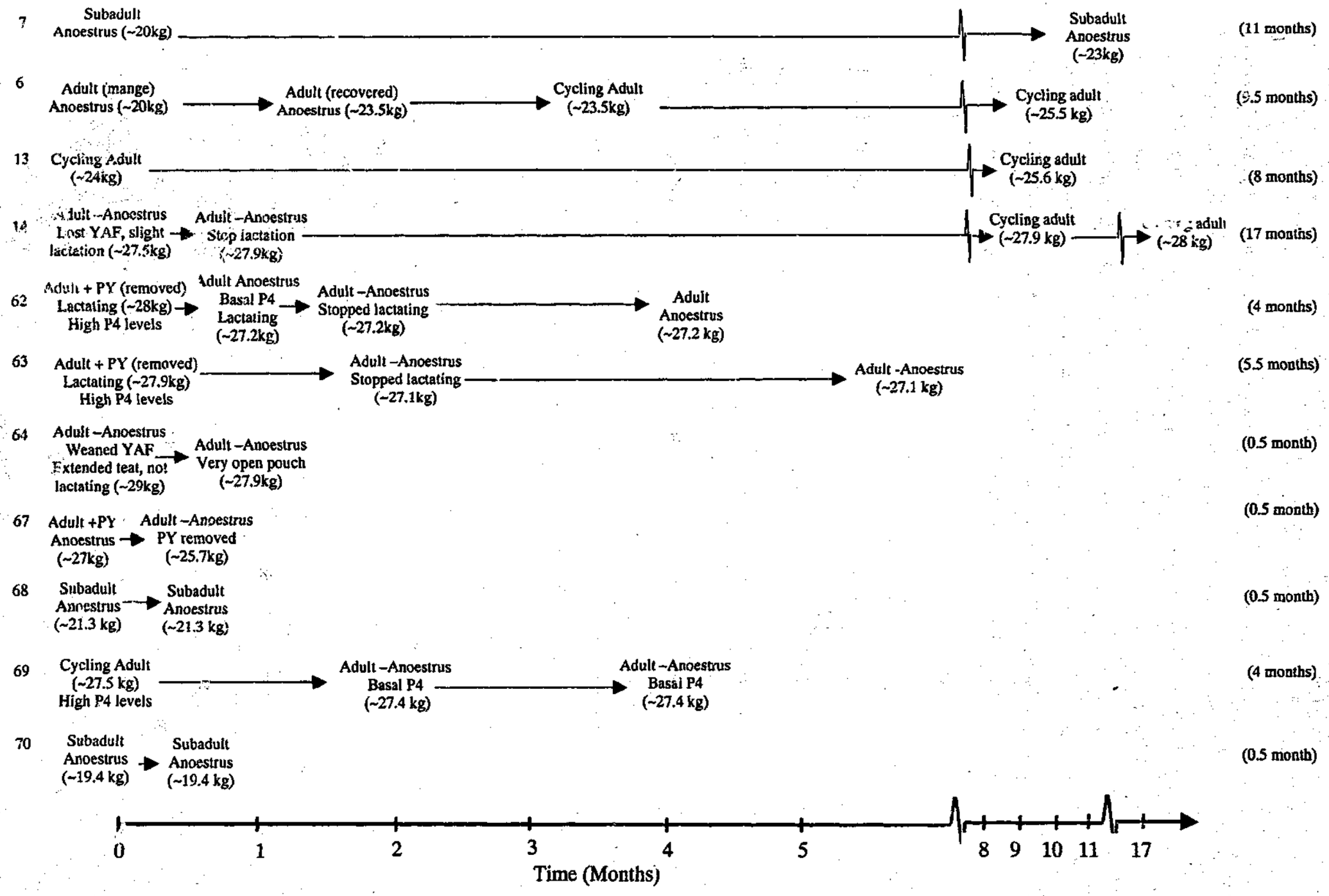
Table 3.7. Months of the year during which oestrous cycle activity was observed. Duration indicates the time which oestrous cyclic activity was observed without intervals between the first observation and the final observation when the study ended ([a]or a wombat entered anoestrous).

Wombat	Oestrous cycle activity		
	First observation	Final observation	Duration (months)
6	November 2000	May 2001	6.5
13	July 2000	March 2001	9
14	July 2001	February 2002	7.5
69*	August 2001	September 2001[a]	1

* suspected cyclic activity, as high progesterone concentrations during this period.

Two anoestrous subadults (wombats 68 and 70) were examined for 2 - 3weeks. One subadult (wombat 7) remained anoestrous for 11 months until the study ended. Three wombats were examined with pouch young. Upon removal of the pouch-young from two of the wombats (wombats 62 and 63) progesterone concentrations increased steeply, similar to that of cycling wombats during the luteal phase, and then returned to basal levels, when the wombats entered anoestrous for 3 - 4months until the study ended. The pouch young was not removed from the other animal (wombat 67), that was in anoestrous during the 2 weeks that she was examined. Another wombat (wombat 64) was captured (without young) and examined for 2 weeks, was anoestrous having a non-lactating extended teat and open scaly pouch, which suggested that she had recently weaned a young-at-foot.

Figure 3.9. The change in reproductive status and body weight of the 11 Common Wombats examined (6,7,13,14,62,63,64,67,68,69,70). (The duration of examination period is shown on the right side of the figure.)


7
Subadult Anoestrus (~20kg)
Subadult Anoestrus (~23kg)
(11 months)
6
Adult (mange) Anoestrus (~20kg)
Adult (recovered) Anoestrus (~23.5kg)
Cycling Adult (~23.5kg)
Cycling adult (~25.5 kg)
(9.5 months)
13
Cycling Adult (~24kg)
Cycling adult (~25.6 kg)
(8 months)
14
Adult –Anoestrus Lost YAF, slight lactation (~27.5kg)
Adult –Anoestrus Stop lactation (~27.9kg)
Cycling adult (~27.9 kg)
adult (~28 kg)
(17 months)
62
Adult + PY (removed) Lactating (~28kg) High P4 levels
Adult Anoestrus Basal P4 Lactating (~27.2kg)
Adult –Anoestrus Stopped lactating (~27.2kg)
Adult Anoestrus (~27.2 kg)
(4 months)
63
Adult + PY (removed) Lactating (~27.9kg) High P4 levels
Adult –Anoestrus Stopped lactating (~27.1kg)
Adult -Anoestrus (~27.1 kg)
(5.5 months)
64
Adult –Anoestrus Weaned YAF Extended teat, not lactating (~29kg)
Adult –Anoestrus Very open pouch (~27.9kg)
(0.5 month)
67
Adult +PY Anoestrus (~27kg)
Adult –Anoestrus PY removed (~25.7kg)
(0.5 month)
68
Subadult Anoestrus (~21.3 kg)
Subadult Anoestrus (~21.3 kg)
(0.5 month)
69
Cycling Adult (~27.5 kg) High P4 levels
Adult –Anoestrus Basal P4 (~27.4 kg)
Adult –Anoestrus Basal P4 (~27.4 kg)
(4 months)
70
Subadult Anoestrus (~19.4 kg)
Subadult Anoestrus (~19.4 kg)
(0.5 month)
0
1
2
3
4
5
8
9
10
11
17
Time (Months)

3.3.11 Comparative lengths of the oestrous cycle

No significant difference (P>0.1) was found between the lengths of the oestrous cycles determined by progesterone (n=9) or the oestrous cycles determined by estradiol concentrations (n=3) or by vaginal cytology (n=12). As progesterone concentrations directly reflect the formation and regression of the corpus luteum and more cycles were assayed for progesterone than for estradiol the length of the oestrous cycle it was considered to be most accurately determined by progesterone concentrations. The length of the oestrous cycle was therefore considered to be ~47.2 days.

3.4 Discussion

3.4.1 Progesterone Profile

Analysis of progesterone concentrations in 10 anoestrous wombats indicates that Common Wombat has a similar basal progesterone concentration to the Koala (*Phascolarctos cinereus*) (Table 3.8). Using the same DELFIA assay, Johnston *et al* (2000) found that the koala has a basal progesterone concentration of 1.2 ± 0.9 (SEM) nmol/L. Progesterone concentrations have now been monitored in the peripheral circulation of 8 marsupials during the oestrous cycle. Basal progesterone concentrations in the Common Wombat are similar to that of the Koala and Virginia Opossum, although all other marsupial species studied so far have slightly lower basal levels (Table 3.8).

Progesterone concentrations were also found to peak at a similar concentration to the Koala (Table 3.8). However peak progesterone concentrations, in the 6 other marsupials studied, have been found to be at least half to a third of that observed in the Common Wombat and Koala. The lowest peak progesterone levels have been observed in the Tammar Wallaby (Hinds and Tyndale-Biscoe 1982b; Shaw and Renfree 1984) and the Quokka (Cake *et al* 1980) this study found concentrations to be 10 - 17 times higher in the Common Wombat. Differences in assays may be responsible for this apparent difference between species. Values given here are

relative since the progesterone assay has not yet been fully validated for wombat plasma.

Table 3.8. Basal and peak progesterone concentrations in the peripheral circulation of 8 marsupial species. Conversion: 1ng/ml = 3.18 nmol/L progesterone.

Species	Basal P4 during anoestrous or follicular phase	Peak P4 during luteal phase	Reference
Common Wombat	2.9±0.8 nmol/L (<6.9nmol/L)	87±5.3 nmol/L	This study
Koala	1.2±0.9 nmol/L[φ] (<6.36nmol/L)	95.4nmol/L	Johnston et al (2000)
Tammar Wallaby	<0.6nmol/L	~1.43-5 nmol/L	Hinds&Tyndale-Biscoe (1982b) Shaw and Renfree (1984)
Common Brushtail Possum	0.61-1.05nmol/L*	21-29 nmol/L	Curlewis et al (1985)
Virginia Opossum	~ <6.36nmol/L[τ]	43.2±4.3 nmol/L	Harder and Flemming (1981)
Eastern Quoll	<3.18nmol/L	14.3-47.7nmol/L	Hinds (1989)
Kowari	1.1±0.7 nmol/L	31.5-36.6nmol/L	Fletcher (1989)
Quokka	1.9nmol/L	7.9nmol/L	Cake et al (1980)

* determined in lactating and ovariectomized possums
[φ] determined during non-mated cycles
[τ] approximate determined from graphed results

Significant variation was observed in the peak concentration of progesterone. This may be an artefact of non-duplicate assays, or of the sampling technique. Alternatively, cycles during which peak progesterone levels differ, may be related to the growth of the corpus luteum. Shorey and Hughes (1973) demonstrated that the progesterone concentrations in the utero-ovarian vein of the Common Brushtail Possum positively correlated with the development of the luteal cells after ovulation. This was also supported by findings of Pilton and Sharman (1962). Peak progesterone concentrations are positively correlated to the sum of the maximum diameter of the corpora lutea on both ovaries in the Virginia Opossum (Harder and Flemming 1981).

The period of basal progesterone levels, between two phases of high progesterone levels was considered to be the follicular phase of the oestrous cycle. The periods of basal progesterone did not vary significantly between individuals, although the length of the follicular phase varied more than the luteal phase. The factors effecting this variation have not been determined but variation in the follicular phase of cows was dependent on the size of the follicles at the beginning of proestrus (Sirois and Fortune 1988). A significantly longer phase was observed in heifers that had a small pre-ovulatory follicle at proestrus, than those with larger follicles.

The period of high progesterone represents the luteal phase of the oestrous cycle. Progesterone concentrations peaked on day ~16 of the mean 28 day luteal phase of the Common Wombat, so that the increasing progesterone concentrations rose for approximately 60% of the luteal phase. The progesterone peak is likely to occur ~ 17 - 18 days after oestrus. Koalas peak ~ 28 days after oestrus (mating) (Johnston *et al* 2000) and increasing progesterone concentrations rise for ~ 70% of the luteal phase, after which the concentration declines steeply. Peak progesterone levels were observed in the Common Brushtail Possum around day 13, after oestrus (Curlewis *et al* 1985), with progesterone concentrations rising for ~ 70% of the luteal phase.

3.4.2 Estradiol Profiles

This study found much lower concentrations of estradiol than previously reported in the other marsupial species (Table 3.9). Even the highest (elevated) estradiol concentrations of the Common Wombat are below the basal estradiol concentrations identified in most other marsupials, except possibly the Common Brushtail Possum.

Curlewis *et al* (1985) had difficulty detecting estradiol levels during the oestrous cycle of the Common Brushtail Possum, using an extraction RIA. Estradiol levels were only detectable during oestrous, and concentrations were highly variable (between <3.6pg/ml and 26pg/ml). Chromatography indicated that estradiol was the major estrogen in Common Brushtail Possums, and its difficulty to detect by RIA was attributed to sampling technique and the sensitivity of the assay. This indicates

that the Common Brushtail Possum has a very brief estrogen peak that may be similar to that observed in the Tammar. An increase from basal estradiol levels in the Tammar Wallaby only lasts for approximately 24 hours (Harder *et al* 1985). Should the estradiol peak in wombats last for a similar period of time, then the sampling technique used during this study, would not have accurately detected it, as samples were collected at intervals of up to 4 days.

Table 3.9. Basal and peak estradiol concentrations in 5 marsupial species, during anoestrous or the oestrous cycle. Conversion: 1pg/ml = 3.67pmol/L estradiol. Data is given as the approximate value or the mean ± SEM.

Species	Basal E2 during anoestrous or luteal phase	Peak E2 during follicular phase	Reference
Common Wombat	0.42 ± 0.05 pg/ml	2.11 ± 0.2 pg/ml[c]	This study
Koala	<15pg/ml[b]	33.9 ± 1.8 pg/ml	Johnston et al (2000)
Tammar Wallaby	8.3± 1.2 pg/ml[d] <10 pg/ml	~20 to 32 pg/ml[a]	Flint&Renfree (1982) Shaw& Renfree (1984) Harder et al (1985)
Common Brushtail Possum	Not detected (<3.6pg/ml)	14.5 ± 5.7 pg/ml	Curlewis et al (1985)
Virginia Opossum	9 ± 1 pg/ml[e]	23 ± 2 pg/ml	Harder&Flemming (1981)

[a] higher estradiol levels observed in Tammar's that display mating behaviour (Harder et al 1985)
[b] estimate from figures
[c] elevated estradiol concentrations above basal
[d] following pouch young removal
[e] lowest point of the cycle

However, as even lower estradiol concentrations (<0.87pg/ml) were observed in the subadult and anoestrous adult wombats, the elevated estradiol concentrations observed in cycling wombats may actually reflect follicular growth. Although confirmation is required, using a greater sample of both anoestrous and cycling wombats and further assessment of the extraction RIA.

In contrast to the estradiol spike in the Common Brushtail Possum and the Tammar Wallaby, the peak estradiol concentrations in the Koala were found to be elevated

for ~ 10 days, with a broad peak ~ 5 days prior to oestrous behaviour (Johnston *et al* 2000). This allowed detection of high estradiol concentrations without such intensive monitoring over a 24 h period. As the Common Wombat is considered to be more closely related to the Koala (Kirsch 1977) it may also have elevated estradiol concentrations over a similar extended period. Despite the considerable variation in the peak estradiol levels of the Koala (Johnston *et al* 2000) estradiol concentrations were, 16 fold greater than the elevated concentrations observed in the Common Wombat, suggesting that the two species might have dissimilar estradiol profiles.

Estradiol has previously been determined to be the major oestrogen in all marsupials examined, including the Tammar Wallaby (Shaw and Renfree 1984) and the Koala (Handesyde *et al* 1990). It is therefore highly likely that the same is true for the Common Wombat, although it still needs to be examined. If estradiol is the most predominant oestrogen compound then we may not be able to exclude the observed rise in estradiol concentrations, prior to the increase in progesterone, as indicative of the actual oestrogen peak. It is possible that as in some eutherian species that either estradiol concentrations are very low, or that another oestrogen is predominant (Desta 1988; Hodges 1998; Lima *et al* 2001).

Peters (1977) examined oestrogen concentrations in the urine of Common Wombats but had difficulty detecting the low concentrations excreted. When oestrogen compounds in the urine of the Common Wombat in Tasmania where measured in urine collected over 24hours, oestrogen was undetectable (< 10μg/24hours) during most of the cycle. However a maximum of 20μg/24 hours was detected at around time of oestrus together with high concentrations of superficial type epithelial cells in the vaginal smears. When a single dose of 0.25mg of exogenous estradiol was administered up to 30μg/24hours over the 3 days following injection. However without additional knowledge of the amount of urine secreted per 24 hours, or determination of the faecal oestrogens, it is difficult to suggest the rate of excretion, or the major excretory pathway of oestrogens.

In this study, although the plasma estradiol concentrations determined using the extraction RIA were low, the general trend suggests that estradiol levels did increase during the follicular phase as determined by progesterone concentrations and coincided with changes in vaginal cytology. Following a period of basal estradiol levels, a small rise in concentrations was observed during the follicular phase, prior to the increase in progesterone concentrations that indicated the luteal phase had started. Estradiol concentrations remained basal during the luteal phase, except in one wombat in which a second small rise in estradiol concentrations was observed towards the middle of the phase. Ovulation may have occurred between the return of basal estradiol concentrations and the subsequent increase from basal progesterone concentrations.

3.4.3 Vaginal Cytology

Inserting a vaginal swab into the urogenital sinus evoked avoidance behaviour (as wombats wiggled their pelvis) even whilst under anaesthesia. As all oestrous cycles examined during this study involved the collection of vaginal swabs we can not exclude the possibility of induced ovulation, during this study. However in another study we investigated the oestrous cycle by faecal steroid analysis (Paris *et al* 2002) and found a similar cycle length and a change in progesterone metabolites in wombats from which vaginal swabs were not taken. Therefore, unlike the Koala (Johnston *et al* 2000), the Common Wombat does not appear to require the physical act of mating, or presence of a male to induce the luteal phase.

Unlike the previous study of vaginal cytology in the Tammar Wallaby by Poole *et al* (1992), mathematical models were not required to interpret the change in vaginal cytology of the Common Wombat. An obvious shift in the proportion of epithelial cell types was observed during the oestrous cycle. Following collection of a series of anoestrous smears, which then formed a database of the basal vaginal cytology, simple analysis of the percentage of epithelial cell types, gave an indication of the stage of the oestrous cycle. However, as indicated by Poole *et al* (1992), consecutive samples were required to determine cyclic activity, and the stage of the oestrous cycle. A high percentage of superficial type epithelial cells, normally indicative of the follicular phase (and oestrus), were found in one anoestrous wombat. Although these smears were rare (≤0.7% of smears) in non-cycling

wombats, care should be taken to use other characteristics to support the determination of reproductive status, or oestrous cycle activity.

Poole *et al* (1992) also noted that one of the sources of variation in the examination of vaginal cytology is the preparation of the smears. Viscosity of the vaginal secretions collected on the swab, the pressure applied to the swab during smearing on the slide (and probably while swabbing the vagina), and their resultant distribution on the slide can all effect the absolute numbers of cells. Repetitive counts of vaginal smears during this study indicated that although there was variation in the percentage of cells counted, the overall trend could still be ascertained.

Peters (1977) found that a high percentage of superficial epithelial cells were present at the time of the follicular phase of the Common Wombats. An increase in superficial type cells coincides with oestrus in the other 13 marsupial species in which vaginal cytology has been examined (Poole et al 1992, Rose and Jones 1996), and this observation is supported in the Common Wombat by Peters (1977). Similar observations were found during this study.

Peters (1997) considered that the vaginal smears of anoestrous females were distinguishable from smears containing similar proportions of cell types collected during the oestrous cycle, by the clumping of cells in the later. This study did not find such a distinction. Although like Peters (1977), no difference was found in the vaginal smears collected from lactating and non-lactating anoestrous adult. Difficulty was found in distinguishing subadult smears from adult anoestrous smears, except by the presence of high percentages of leucocytes, which occurred in approximately 40% of adult smears.

Although the general pattern of vaginal cytology change described by Peters (1977) matches that found during this study, differences in the length of the oestrous cycle have been found. This difference is not attributed to the different stains used between the two studies. When the Shorr's stain used by Peters (1977) was compared to the simpler DiffQuick stain the same pattern of change was confirmed, indicating that the same length of the oestrous cycle would be determined with

either stain. DiffQuick was however, found to more reliably stain the nuclear and cytoplasmic structures of the cells and as it is quicker and simpler to perform, it was the preferred stain. Unlike Shorr's stain, its relative ease would make it suitable for use in the field.

3.4.4 Pouch and Urogenital Opening Morphology

Pouch condition of the Common Wombat did not reliably indicate stage of the oestrous cycle but did provide a rough indication of the reproductive status, as supported by Moritz *et al* (1998). In summary, the length of the teat, lactation of the teat, depth of the pouch, opening of the pouch, scale, the degree of cleanliness, and moisture (secretion), all provided clues to the wombat's reproductive status, especially when used in combination with peripheral progesterone concentrations and vaginal cytology.

The presence of a long elongated teat distinguishes the pouch of wombats with pouch young or young-at-foot, if lactating. In the absence of lactation it suggests that an animal has recently weaned a young at foot, or lost a pouch young. Wide, open and deep pouches are indicative of wombats that have had or have a young-at-foot depending on the presence of lactation. Adults with small pouch young had a tight pouch opening and a lactating teat. The degree of scale found in the pouch is considered to provide an indication of the time since weaning young-at-foot, or pouch young loss, although a larger sample of wombats may be required to provide an accurate estimate.

The pouch of the Common Wombats is first visible in 14-day-old neonates (Green and Rainbird 1987) and develops as a clean and shallow depression in the abdomen during the growth of pouch young and young-at-foot. Weaned young-at-foot that are subsequently referred to as subadults have a dry, mostly clean, shallow pouch, with short teats.

Subadults also have a dry, clean pouch. The interior of the pouch of the subadult is continuous with the abdominal surface at the anterior end. Cycling adults had a deeper pouch than the subadults that was closed with a definite rim, and not

continuous at any point with the abdominal surface. The pouch of cycling adults varied in moistness and cleanliness.

Peters and Rose (1979) found that changes in the pouch of adult Common Wombats in Tasmania, did indicate the stage of the cycle. However these changes were not considered to be as clear cut in the Common Wombats captured in Victoria. In another study, Sharman and Calaby (1964), found, similarly to Peters and Rose (1979), that during the follicular phase of the Red Kangaroo, the pouch was clean but then became more dirty during the luteal phase. Similar observations were also made in other marsupials including the Koala (Johnston *et al* 2000) and Eastern Quoll (Hill and O'Donoghue 1913).

In general the urogenital opening of cycling wombats protruded and engorged only towards the end of the follicular phase, and then became inverted during the luteal phase. Mucous is sometimes observed externally, when the urogenital opening protrudes. Such changes were generally not observed in anoestrous animals. Peters and Rose observed similar changes in cycling Common Wombats in Tasmania, and noted, as we found, that these changes did not occur in all wombats or all cycles. Similar changes were observed in the Eastern Quoll (Hill and O'Donoghue 1913). Conflicting observations have been in the Koala by Brown (1987) suggesting the changes did occur, although Johnston *et al* (2000) reported that these changes were unreliable.

3.4.5 Body temperature

When Peters (1977) intensively studied the changes in body temperature of the Common Wombat, via surgically implanted radiotelemeters, and significant fluctuations occurred both within a 24 hour period and during oestrous cycles. Although the specific body temperature was not reported by Peters (1977) or Peters and Rose (1979), their graphs indicate, that basal body temperature varied between 33 and 36°C with an approximate average of 34.5°C. A similar mean rectal temperature (34.8°C) was found in this study, although greater variation was found in the range of rectal body temperatures (32.1 – 37.6°C). In the current study, no

differences were found in the body temperature between cycling and anoestrous wombats.

Peters (1977) found that maximum body temperatures of the wombat occurred at night, and were associated with increased activity, and a decrease in temperature was observed to coincide with rest periods after eating. Body temperature during the day, was generally found to be lower, when activity was minimal. Some of the variation in the body temperature in our study may be attributed to differences in time of monitoring (usually between 7am and 10am). Additional variation maybe associated with sampling body temperature from the rectum, as in our study, as opposed to internal abdominal sampling, as undertaken by Peters (1977).

Ambient temperatures may have affected the body temperature of wombats, although it was not examined during this study, as the wombats were not housed under conditions of controlled temperature. Body temperatures were measured in wombats kept at a constant temperature (22 ± 1°C) in the study by Peters (1997) and this is likely to be a reason for the lower variation in body temperatures that they observed.

Body temperature was not found to be an indicator of the stage of oestrous during this study, although Peters and Rose (1979) did find a change in the diurnal rhythm when the temperature was monitored hourly under constant environmental conditions. Daily maximum body temperatures occurred late at night (from about 10pm) during the 10 days following oestrous, and earlier in the morning (from about 4am) at other times of the cycle. The change was associated with earlier nocturnal activity noted in wombats during the days following oestrous.

3.4.6 Body weight

This study has found that adult cycling wombats have a minimum body weight of 23.5kg. A subadult wombat's body weight had increased from 18kg to 23.7kg over 11 months and at this body weight, she was considered to be close to the period of her first cycle, although time did not permit observation of the reproductive transition. Such a transition was observed in an adult female recovering from mange, and she did not begin cycling until she had a body weight of 23.5kg. These

findings were consistent with other studies in New South Wales and Victoria. Suggesting that adult Common Wombats have a minimum body weight of ~ 23 - 24kg, that subadults are found to weigh less and the mean body weight of all wombats is ~26kg and a maximum weight of ~35kg (Presidente 1982; McIlroy 1973; McIlroy 1990; Triggs 1996).

Moritz *et al* (1998) examined Common Wombats shot in Tasmanian and found eight females in the luteal phase of the oestrous cycle with body weights of 16.8 to 23.3kg. This lower body weight observed in Common Wombats in Tasmania is also supported by another study by Green and Rainbird (1987) suggesting an average body weight of 19.5 kg, which is considerably less than that of the Common Wombats in Victoria.

3.4.7 Change in reproductive status over oestrous cycle

This study has found that in two adult Common Wombats progesterone concentrations begin to increase shortly after removal of pouch young, 4 and 6 months old; although it is not know if they immediately entered oestrus. Results did suggest that the following a period of high progesterone, the 2 wombats returned to basal progesterone concentrations and remained basal for 3 - 4.5 months, when this study ended. Pilton and Sharman (1962) found that Common Brushtail Possums return to oestrous ~8 days after pouch young removal. Similarly, McNab (1986) found that Matschiei Tree Kangaroos to return to oestrus 7.3days after loosing a pouch young. A complex hormonal milieu and a blastocyst in diapause inhibits the return of oestrous until 26 - 27days after pouch young removal in the Tammar Wallaby (Tyndale-Biscoe and Hawkins 1977; Merchant 1979).

Green and Rainbird (1987) found that in Tasmania, when lactation ends after weaning, the adult Common Wombat enters a phase of anoestrous. This finding was supported in this study when one captive animal that had lost her young-at-foot, and a second animal that had recently weaned a young-at-foot were anoestrous for up to 9 months after lactation ended.

3.4.8 Duration of the Oestrous Cycle

The length of the oestrous cycle of the Common Wombat was highly variable, with a duration between 35 and 60 days. The length of the oestrous cycle of the Koala varies between 47 and 53 days. Cycle lengths vary between 22 – 32 days in the Common Brushtail Possum (mean length of 25.7 days; Pilton and Sharman 1962). Cycle lengths vary in many species including eutherians such as cows (Sirois and Fortune 1988), and elephants (Brannian *et al* 1988). Some variation has been linked to differences in the development of the pre-ovulatory follicle (Sirois and Fortune 1988). The variation observed in the length of the oestrous cycle in the Common Wombat is reflected in the both length of the follicular and luteal phases although most variation was found in the length of the follicular phase. Some of the variation observed in this study is likely to be a result of the sampling technique. Non-invasive faecal steroid studies have supported the cycle length determined (Paris *et al* 2002), or other related captive imposed stress has affected the cycle. It is unlikely that the captive diet would have effected the cycle as wombats were maintained on grass as they predominantly feed on naturally. Further non-invasive studies of free-ranging wombats would support this result.

The duration of the oestrous cycle as determined by this study (~47 days) is longer than that determined for most other marsupials (Tyndale-Biscoe and Renfree 1987). A similar oestrous cycle length of 49.5 ± 1.0 days is reported for the Koala. The only marsupial species examined with longer oestrous cycles are the Kowari (*Dasyuroides byrnei*), Matschie's Tree-kangaroo (*Dendrolargus matschiei*) and also potentially the Timboyok (*Dendrolargus goodfellowi buergersi*) which have lengths 60, 63 and (estimated) 54 days respectively (McNab 1986; Fletcher 1989; Flannery *et al* 1996). By comparison of progesterone and estradiol concentrations in the peripheral circulation, the Koala was determined to have an oestrous cycle of approximately 49.5 days, however ovulation required mating stimulus (Johnston *et al* 2000).

Much shorter oestrous cycles have been determined for other marsupial species (Table 1.1; Tyndale-Biscoe and Renfree 1987). In all marsupials with the exception of a few macropods, the duration of gestation is similar to the length of the luteal

phase (Lyne *et al* 1959; Harder and Flemming 1981;Tyndale-Biscoe and Renfree 1987; Hinds *et al* 1996). Similarly observations of the in the Common Wombat suggest a gestation of ~30 days (Green and Rainbird 1987) and as this study found that the luteal phase is ~28 days which closely fits the general marsupial pattern.

Oestrous cycle activity was observed in wombats in all months of the year (Table 3.7) during this study. Indicating that the Common Wombat in Victoria is polyoestrous capable of breeding all year round. This is further supported by observations of breeding activity during field studies in Victoria.

In Victoria, Nicholson (1963) found that wombats are born between late March and June in the Mt Buller area. Presidente (1982) found that in central Gippsland, Victoria, pouch young born between June - July and December. Our group has found that births occur throughout the year in the Myrtleford area of Victoria, although most births occurred between December and February (unpublished observations). Similar results have also been found in New South Wales, with most births occurring between December and March (McIlroy 1973). McIlroy (1990) suggests that breeding dates vary along a north-south axis with breeding occurring later in the southern areas of the range.

In contrast to the Common Wombats in Victoria, in Tasmania captive cycling Common Wombats went into anoestrus from August to at least December (when the study ended) Peters and Rose (1979). Other field studies in Tasmania by Green and Rainbird (1987) and Moritz *et al* (1998) found that young were produced throughout the year with a peak in births occurring in spring and early summer (October – January).

3.5 Conclusions

This study indicates that the Common Wombat in Victoria has a cycle length of ~ 47days and is polyoestrous. Common Wombats are likely to be monovular as determined by captive and field observations of births of single young (*unpublished observations*; McIlroy 1973; Presidente 1982, Green and Rainbird 1987; Triggs

1996; Boer 1998; Moritz *et al* 1998). The luteal phase occupies ~ 60% of the oestrous cycle. The estimated gestation length of ~30 days (Green and Rainbird 1987) coincides with the length of luteal phase and would represent ~60% of the cycle. The Common Wombat appears to belong to the Type 1/ Group 1 reproductive pattern proposed by Tyndale-Biscoe and Renfree (1987). These findings are similar to those of the Koala, which has a gestation period of 34.8 ± 0.3 days (mean ± SEM) and, an (mated) oestrous cycle length of 49.5 days (Johnston *et al* 2000). Unlike the Koala the Common Wombat is not known to be an induced ovulator.

Pouch condition in combination with body weight permits the rough determination of reproductive status to be subadult, cycling adult, adult with pouch young or young-at-foot or anoestrous adult. The stage of the oestrous cycle can be successfully determined by changes in peripheral progesterone concentration and vaginal cytology examined over consecutive samples. The peak progesterone concentrations were higher than all other marsupials examined so far, except for the Koala that has similar peak concentrations (Johnston *et al* 2000). Estradiol concentrations were very low, in comparison with other marsupials, and further studies should be undertaken to investigate the predominant oestrogen. Progesterone and estradiol concentrations should not be considered as absolute values, but rather indicative of the general trend and further samples should be examined

The lower body weights of cycling animals and shorter oestrous cycles observed in Tasmanian Common Wombats suggest possible inherent differences may occur in wombats from the two states. This variation may be due to differences in environmental factors including climate and nutrition or geographical isolation as the Australian mainland and Tasmanian populations have been separated for 9,000 - 10,000 years.

Chapter 4

Response of the Common Wombat to ovarian stimulation by exogenous hormones

4.1 Introduction

This study investigates the administration of exogenous, porcine derived, FSH (pFSH) and LH (pLH), to induce follicular growth and oocyte maturation of the Common Wombat. Porcine FSH and LH have been previously successfully used to induce ovulation in other marsupial species (Molinia *et al* 1998b, Molinia *et al* 2000). This study investigates the same treatment regime, as that successfully used in previous studies, as no other data is available for the Common Wombat. Recruitment of follicles and the production of oocytes are compared during the follicular phase and anoestrous periods in response to this superovulation regimen. Oocytes are a basic requirement for the assisted reproduction and manipulation of fertility in this and related wombat species.

4.2 Materials and Methods

4.2.1 Animal Use

Eleven wild caught, female Common Wombats were used in this study (Section 2.1).

4.2.2 Experimental Design

The effect of exogenous administration of pFSH plus pLH was examined in eleven Common Wombats divided into three groups on the basis of their reproductive status at the time of treatment (Table 4.1).

A post-lactation anoestrous adult (wombat 14) was examined by laparoscopy before she started to cycle, 13 months prior to treatment. Ultrasound and laparoscopy were performed prior to and during superovulation treatment as per Table 4.2.

Eight 6mg injections of pFSH were administered at 12 hour intervals over a period of 4 days, so that the first pFSH injection was given at 9am on day 1 and the last pFSH

injection was given at 9pm on day 4. One 4mg pLH injection was given at 9am on day 5, 12 hours after the last pFSH injection (Table 4.2). The wombat's reproductive status was monitored during the period of superovulation on days 1, 3, 5 and 6 (Chapter 5). On these days exogenous hormones were administered whilst the wombats were under anaesthesia immediately following collection of a blood sample and vaginal smear.

Table 4.1. The approximate age, reproductive status, and progesterone (P_4) concentration of wombats at the time of superovulation treatment.

Wombat	n	Age group	Reproductive status	P_4 concentration
6 (a), 13 (a)	2	Adult	Cycling (early follicular)	On return to basal
6 (b), 13 (b), 14	3	Adult	Cycling (early follicular)	On return to basal
7, 68, 70	3	Subadult	Anoestrous	Basal
62, 63, 67	3	Adult	Anoestrous (PY removed)	Basal
64, 69	2	Adult	Anoestrous	Basal

Basal progesterone levels were 2.9nmol/L ± 0.8 (range: 0.5 to 6.9nmol/L) (Section 3.3.1).
Two adult wombats were superovulated twice;
(a) 1st superovulation
(b) 2nd superovulation

Table 4.2. Timing of procedures during superovulation of wombats. 1 FSH injection was given twice a day over 4days, followed by an LH injection on day 5 and oocyte recovery 24hrs later on day 6.

Day	-2	-1	0	1	2	3	4	5	6
Wombat				2xFSH	2xFSH	2xFSH	2xFSH	1xLH	Oocyte recovery
6 (a)		B	B		B		B		B, U, Lc, Lm
6 (b)			B	B		B		B, U	B, Lm
7			B	B		B		B, U	B, Lm
13 (a)	B,U,Lp					B, U			B, U, Lc
13 (b)		B	B		B		B		B, Lm
14			B	B		B		B	B, Lm
62			B	B		B		B	B, U, Lm
63			B	B		B		B	B, Lm
64			B	B		B		B	B, Lm
67			B	B		B		B, U	B, Lm
68			B	B		B		B	B, Lm
69			B	B		B		B	B, U, Lm
70			B	B		B		B	B, Lm

(a) = 1st superovulation, (b) = 2nd superovulation
B = blood collection, U = ultrasound, Lc = laparoscopy, Lm = laparotomy

In addition, the reproductive status of two Common Wombats was monitored after superovulation. The wombats were monitored every two or three days following attempted follicular aspiration.

Treatment with pFSH/pLH was repeated in two wombats (wombat 6 and 13), to observe the ovarian response to a repeat treatment. The reproductive condition of each wombat was monitored prior to the first superovulation attempt (Section 2.2; 2.4). Superovulation was first attempted at the return of basal progesterone concentrations, in the peripheral circulation, at the start of a follicular phase. The ovaries were examined *in situ* and oocyte recovery was attempted by laparoscopy (Section 2.4.5). The reproductive condition of each wombat was monitored (Section 2.2) between the two attempts of superovulation and pFSH/pLH was administered following the second luteal phase after the first superovulation attempt. Ovaries were harvested via laparotomy, and then and follicles were aspirated *ex situ* (Section 2.4.6; 2.4.8).

Similarly the ovaries, of all other wombats, were examined and harvested by laparotomy (Section 2.4.6). Follicle aspiration was undertaken *ex situ* between 1 - 3 hrs after the removal of the ovaries. The wombats were euthanised immediately after ovariectomy (Section 2.4.6).

4.2.3 Ovary Examination

Ovarian structures were assessed and measured. Turgid, fluid-filled transparent structures were considered to be healthy follicles, turgid blood filled structures, were considered to be corpora haemorrhagica. Pale pink to pale yellow large solid structures, which were highly vascularized, were considered to be corpora lutea and smaller solid structures that varied in color from dark cream to brown were considered to be corpora albicantia.

4.2.4 Statistics

Results are given as the mean ± SEM (Range). Chi square analysis was used to compare the differences between the number of oocytes collected between wombats, the number of oocytes collected between ovaries of each wombat, and the number of

MII oocytes collected between ovaries of each wombat. One-way ANOVA was used to compare the difference in the number of oocytes collected between cycling adults and the anoestrous adults following removal of pouch young.

4.3 Results

4.3.1 Ovarian appearance prior to and during superovulation

The ovaries were most easily visualised *in situ* when the laparoscope was inserted into an incision in the abdomen via flank rather than the midline. The bursa surrounding the ovaries had to be partially removed to allow visualisation.

Laparoscopy was first attempted on an anoestrous adult wombat (wombat 14, 13 months prior to start of oestrous cycle activity) post-lactation, with basal progesterone concentrations. Laparoscopy indicated that the ovaries were flat with a finely granular surface.

Laparoscopy of one cycling wombat (wombat 13) prior to the first superovulation attempt, indicated that at the end of the luteal phase several small (up to 2mm) follicles occurred on the right ovary, although no follicles were found on the left ovary. These follicles were not detected by ultrasound. When laparoscopy was attempted on day 6, to recover oocytes, one large follicle ~ 5mm in diameter and 3 - 4 smaller opaque structures (~ 4mm) on the right ovary and a corpora luteum ~ 2.1mm and 3 - 4 smaller opaque structures (~ 3 - 4mm) on the left ovary.

An ultrasound examination of 3 wombats (wombat 6(b), 67 and 7) on day 5 (time of LH injection) identified numerous follicles on each ovary, and in particular 6 – 10 large 6-7mm follicles were found on the ovaries of wombat 6(b) (on day 5, 2nd superovulation attempt). These observations were confirmed on 6 by laparotomy, finding that the each ovary had numerous large antral follicles (Table 4.1). Following the initial experiences and development of the ultrasound technique, the ovaries could be found more easily. Ultrasound was most successful when the wombat was in a

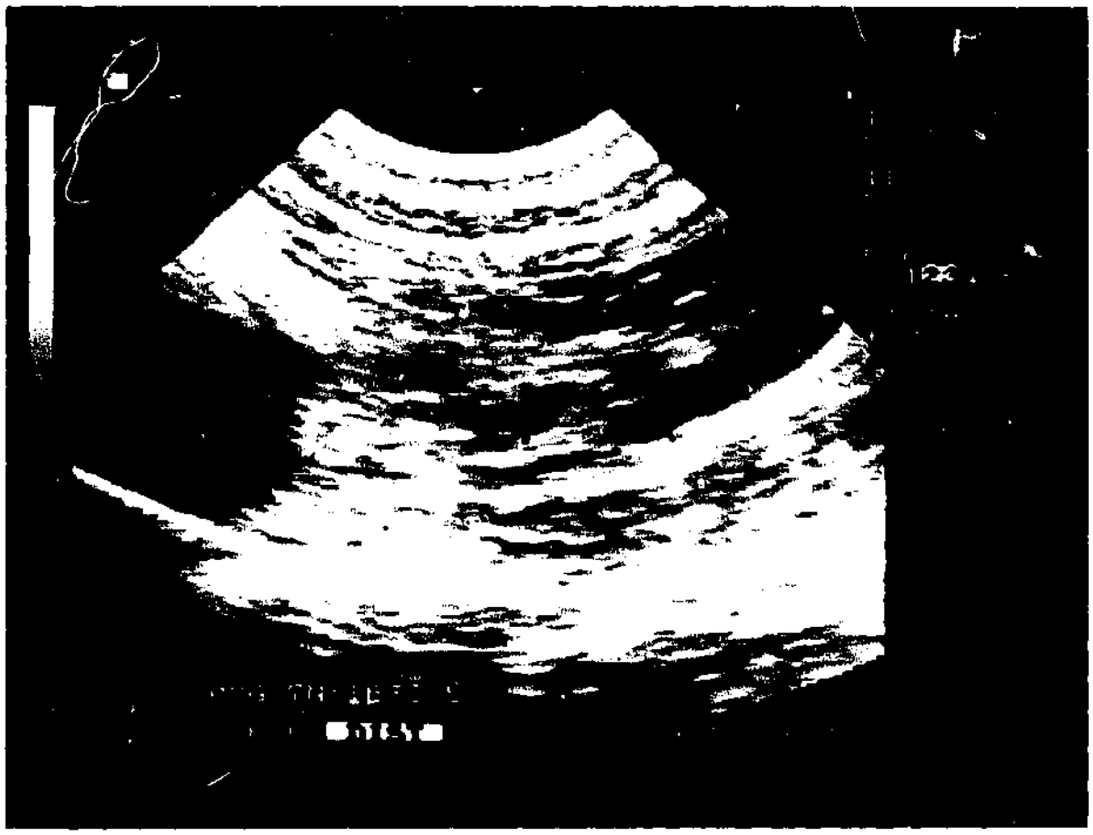


Figure 4.1. Ultrasound image of left ovary of Wombat 69 on day 6 following a five-day treatment period of pFSH/pLH administration. Several antral follicles are visible, two measuring ~ 4mm and ~ 3.5mm are marked: + - + and x – x respectively denote the follicle diameters.

dorsally recumbent position, and the trans-abdominal probe was used on the animal's flank. Ultrasound examination of 4 wombats (wombats 6a, 13a, 62, 69) on day 6 (time of oocyte recovery) indicated that on some large follicles of ~ 3 - 4mm could be identified (Figure 4.1). This was later confirmed by laparoscopy or laparotomy, however not all follicles were reliably detected using ultrasound.

4.3.2 Ovarian appearance 24h after LH administration

Results are shown in Table 4.3. The ovaries of wombats that did respond to the exogenous hormones were 28.6mm ± 0.7 (range: 22 to 36mm) long and 19.8mm ± 0.9 (10 to 30mm) wide, and approximately 10 to 15mm deep. Antral follicles protruded from the ovarian surface, and as large numbers of these antral follicles occurred (Table 4.3), the ovaries resembled a bunch of grapes in appearance (Figure 4.2). Large antral follicles, >1mm in diameter, were found on all ovaries responding to stimulation (Table 4.3). A mean of 21.1 ± 3.0 (range: 8 to 39 follicles) large antral follicles were observed per wombat (n=12) when the ovaries were examined *ex situ*. There was a difference between the number of follicles counted at the first and the second superovulation in the two animals treated twice (4 and 1 large antral follicles (>1mm) compared with 16 and 11) (Table 4.3).

The anoestrous wombat (wombat 68) that failed to respond to the treatment had much, flatter, and narrower ovaries than all other wombats. At autopsy, the pericardium was adherent to the chest wall and a large abscess was present in the left lung. In all other wombats the internal organs were normal.

Antral follicles were between 1mm and 15mm in diameter. When harvested, the ovaries of all wombats could be examined in detail *ex situ*. Excluding the subadult that did not respond, all wombats had ovaries with a wide range of follicular sizes (Figure 4.3). The population of antral follicles (>1mm) examined from all wombats, formed an approximate normal distribution, with a mean follicular size of 5mm (Figure 4.4). Of the total number of follicles found, 40.1% were 4 - 5mm in diameter and 75.2% were ≥ 4mm in diameter. The largest antral follicle found was 15mm in diameter, although only one was found at this size.

One subadult (wombat 70) had 15 large (>1mm) follicles on the left ovary, and 5 large (>1mm) follicles on the right ovary. A relationship was not identified for this difference in ovarian response. All other wombats had a similar number of follicles on each ovary, although slightly more follicles were found on the left ovaries (Table 4.3). This was reflected in the total number of follicles (>1mm) of all wombats, finding that 132 follicles were found on the left ovaries and 110 follicles were found on the right ovaries.

An adult anoestrous wombat (wombat 63), and, a subadult wombat (wombat 7) had the greatest numbers of follicles. Both had 39 antral follicles greater than 1mm in diameter and of these 53.8% and 74.7% were greater than 4mm in diameter, respectively.

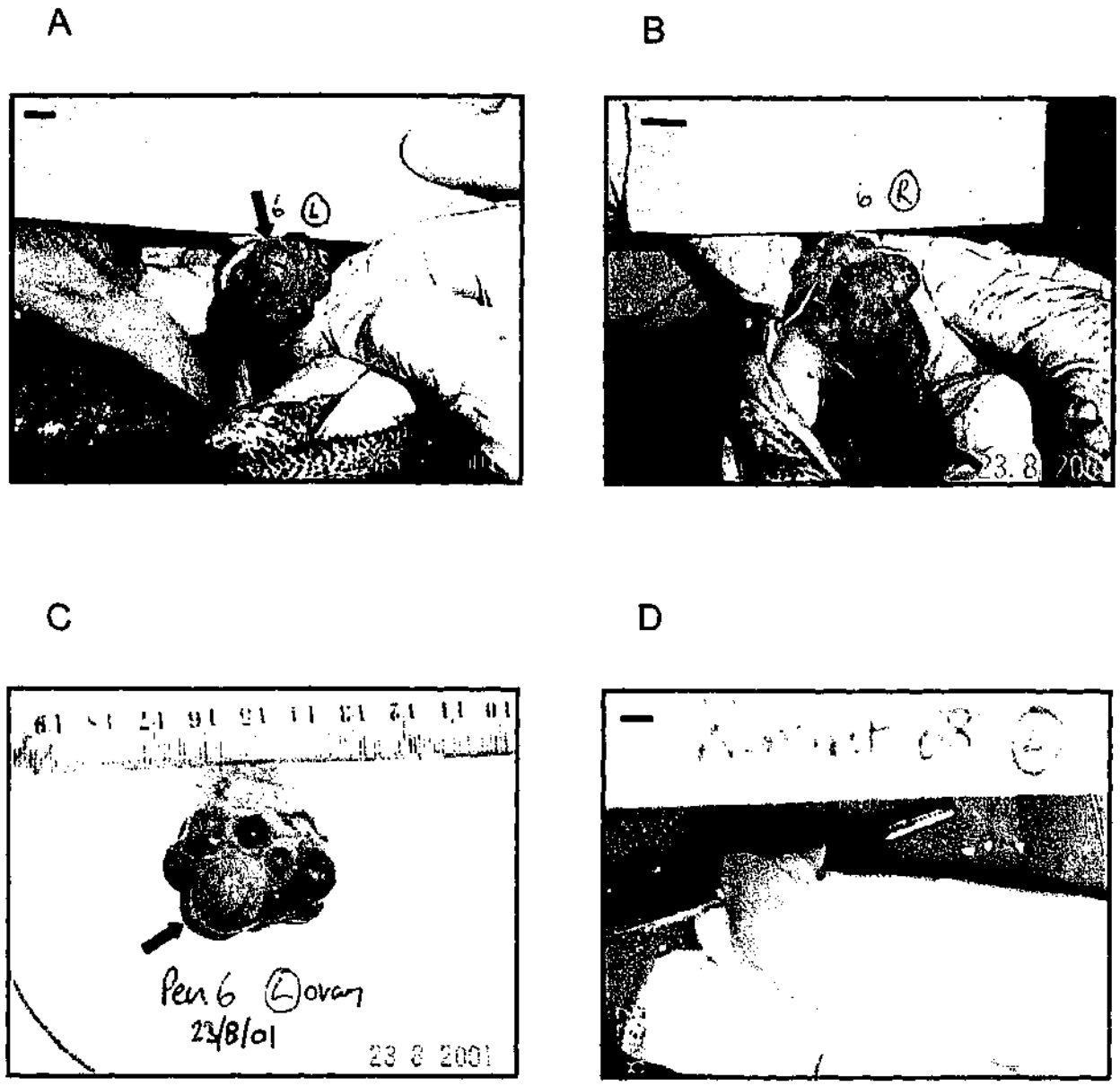
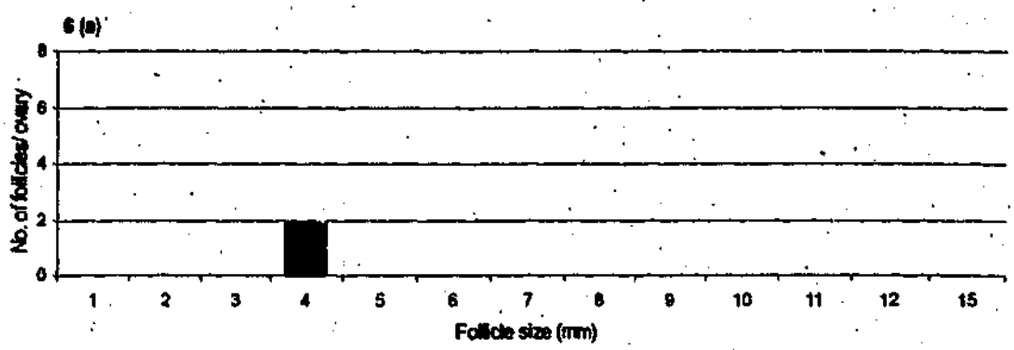


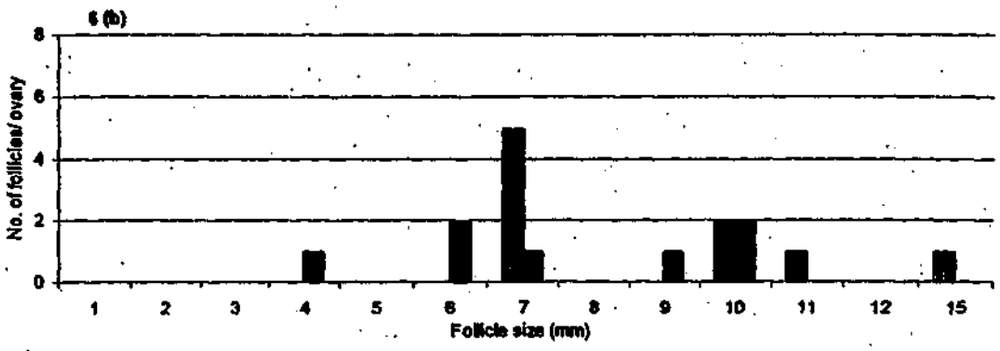
Figure 4.2. Ovaries after pFSH and pLH treatment. A: Left ovary of wombat 6 *in situ*, black arrow shows a corpus luteum surrounded by several large follicles (Scale bar represents 500μm). B: Right ovary of wombat 6 *in situ*, lacking a corpus luteum also containing numerous large antral follicles (Scale bar represents 500μm). C: Left ovary of wombat 6 *ex situ*, black arrow indicates corpus luteum (Scale bar represents 600μm). D: Left ovary of wombat 68 *in situ*, flat in appearance and lacking antral follicles (Scale bar represents 700μm).

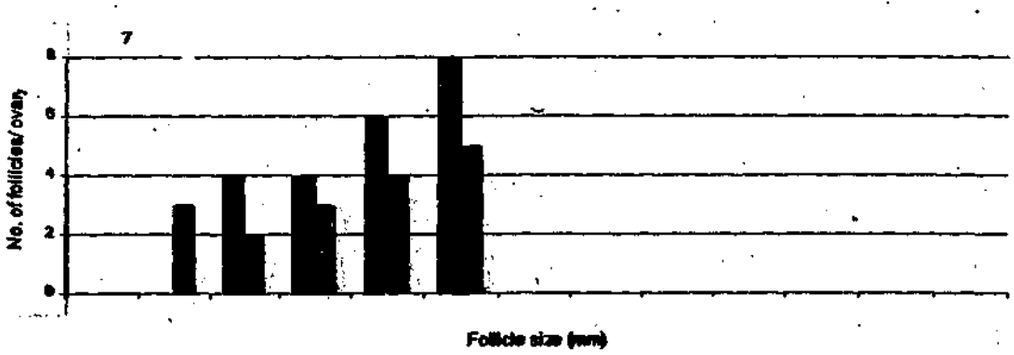
Table 4.3. Number of large antral follicles (>1mm) per ovary and per wombats; and percentage of these follicles >4mm, per wombat, and per ovary. Difficulty in the in situ examination of wombat 6(a) and 13(a) may have reduced their follicular counts.

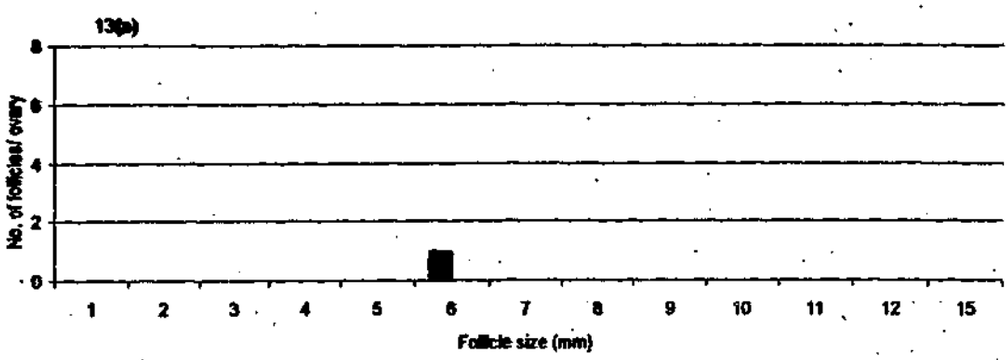
Wombat	Ovary	# follicles/ ovary	# follicles >4mm/ ovary	% follicles >4mm/ ovary	Total # follicles/ wombat	Total # follicles >4mm/ wombat	% follicles >4mm/ wombat
Adult cycling wombats - First superovulation and attempted in situ recovery							
6 (a)	L	2	2	100%	4	4	100%
	R	2	2	100%			
13 (a)	L	-	-	-	1	1	100%
	R	1	1	100%			
Adult cycling wombats – ex situ recovery							
6 (b)	L	9	9	100%	16	16	100%
	R	7	7	100%			
13 (b)	L	4	3	75%	11	10	90.9%
	R	7	7	100%			
14	L	10	9	90%	19	14	73.6%
	R	9	5	55.5%			
Subadults – ex situ recovery							
7	L	22	18	81.8%	39	30	74.7%
	R	17	12	70.6%			
68	L	0	-	-	0	-	-
	R	0	-	-			
70	L	15	6	40%	20	11	55%
	R	5	5	100%			
Adult anoestrous – pouch young removed							
62	L	6	5	83.3%	14	11	78.6%
	R	8	6	75%			
63	L	19	10	52.6%	39	21	53.8%
	R	20	11	55%			
67	L	12	12	100%	21	20	95.2%
	R	9	8	88.9%			
Adult anoestrous – recently weaned young at foot or unexplained anoestrous							
64	L	11	7	63.6%	21	12	52.8%
	R	10	5	50%			
69	L	13	7	53.8%	24	18	79.1%
	R	11	11	100%			

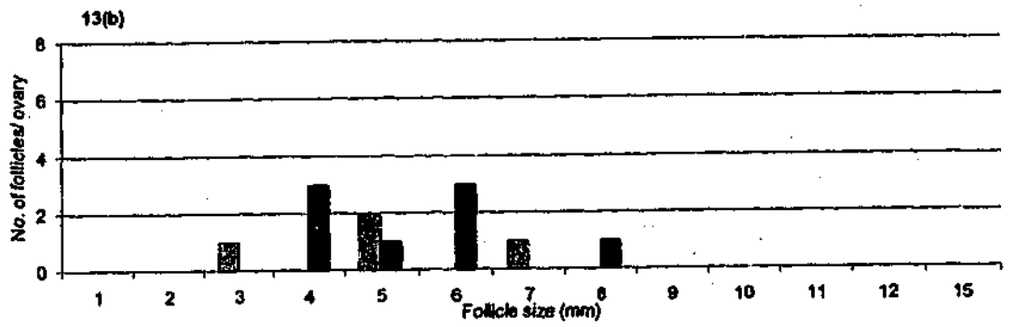
Figure 4.3. Antral follicles measured on the ovaries of pFSH/pLH primed Common Wombats. 6a, 6b, 7, 13a, 13b, 14, 62, 63, 64, 67, 68, 69, 70 (shown on the following 3 pages) depict the follicles measured on the left and right ovary of each of the wombats examined on day 6, 24 h after the last pLH injection. The grey columns represent follicles on the left ovaries and the black columns represent follicles on the right ovaries.

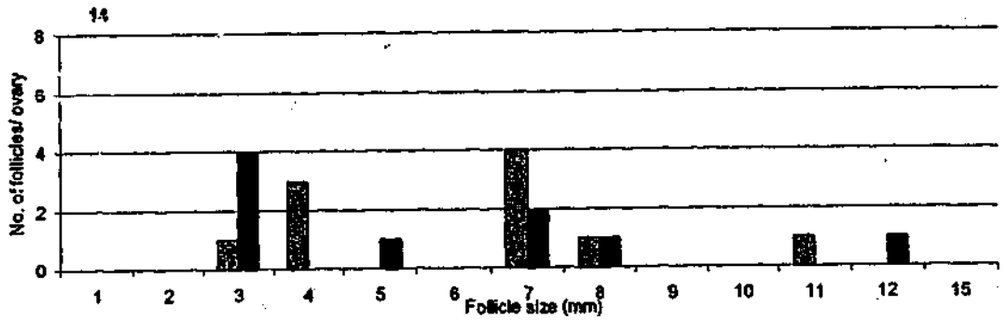

6 (a)
No. of follicles/ ovary
0
2
4
6
8
1
2
3
4
5
6
7
8
9
10
11
12
15
Follicle size (mm)

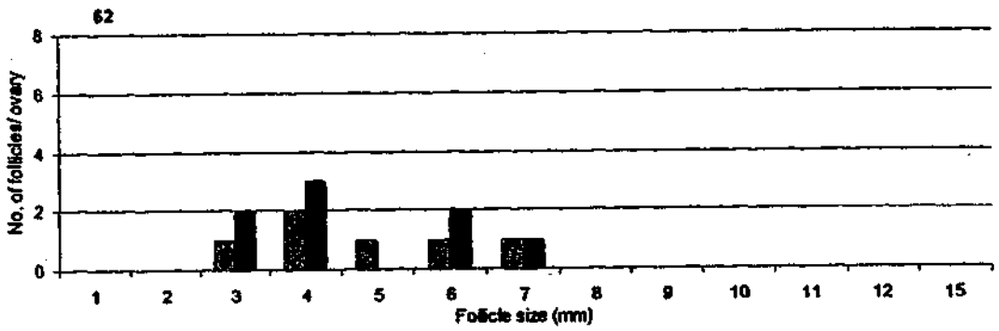

6 (b)
No. of follicles/ ovary
0
2
4
6
8
1
2
3
4
5
6
7
8
9
10
11
12
15
Follicle size (mm)

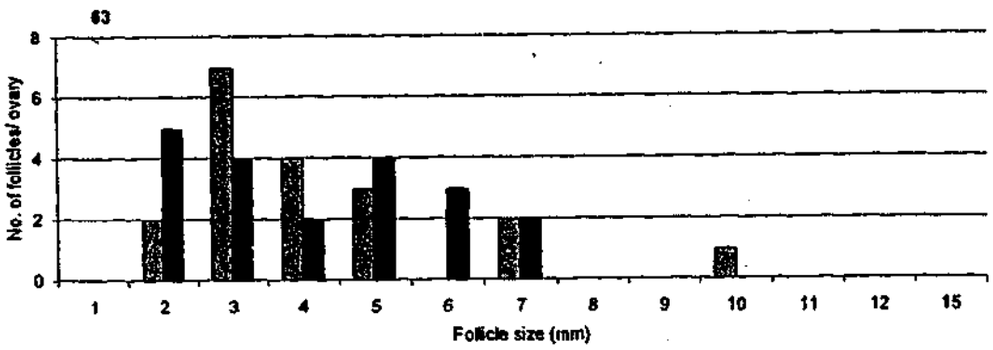

7
No. of follicles/ ovary
0
2
4
6
8
Follicle size (mm)

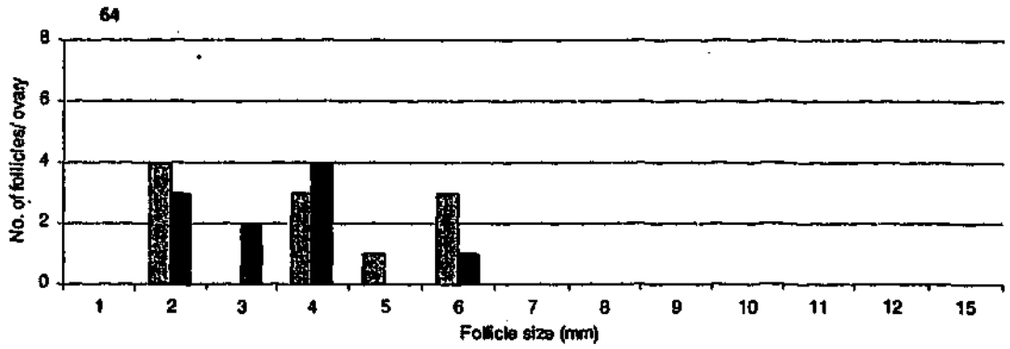

13(a)
No. of follicles/ ovary
0
2
4
6
8
1
2
3
4
5
6
7
8
9
10
11
12
15
Follicle size (mm)

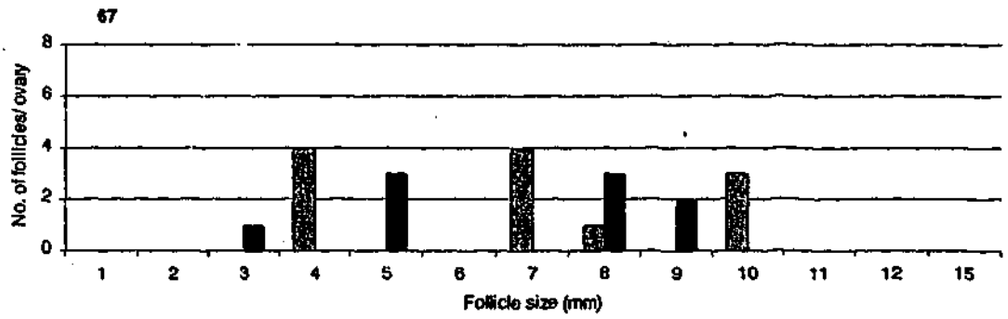

13(b)
No. of follicles/ ovary
0
2
4
6
8
1
2
3
4
5
6
7
8
9
10
11
12
15
Follicle size (mm)

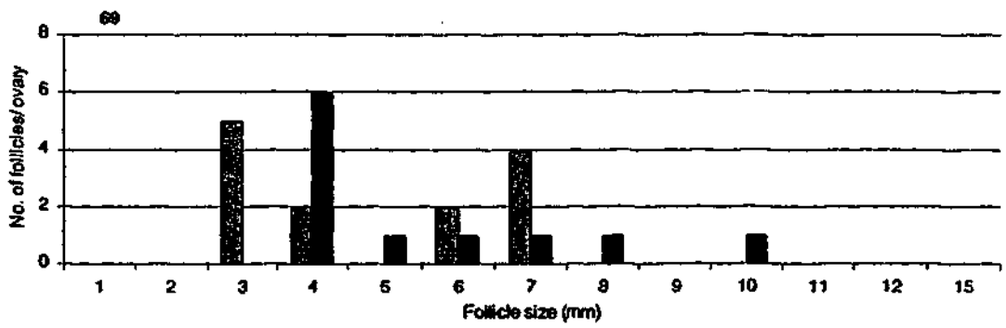

14
No. of follicles/ ovary
0
2
4
6
8
1
2
3
4
5
6
7
8
9
10
11
12
15
Follicle size (mm)

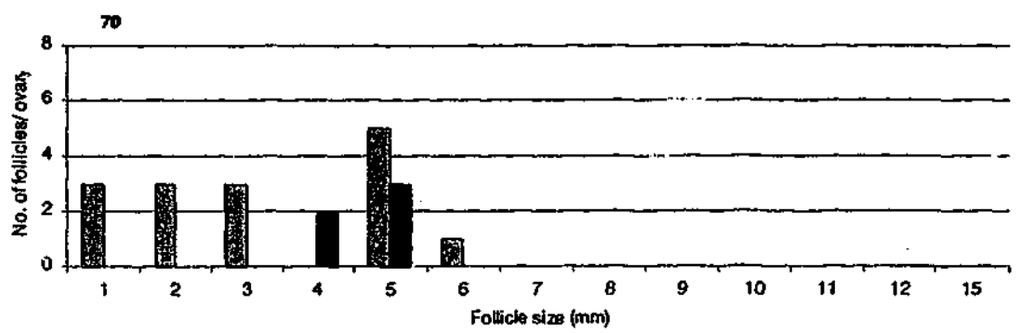

62
No. of follicles/ ovary
0
2
4
6
8
1
2
3
4
5
6
7
8
9
10
11
12
15
Follicle size (mm)


63
No. of follicles/ ovary
0
2
4
6
8
1
2
3
4
5
6
7
8
9
10
11
12
15
Follicle size (mm)


64
No. of follicles/ ovary
0
2
4
6
8
1
2
3
4
5
6
7
8
9
10
11
12
15
Follicle size (mm)


67
No. of follicles/ ovary
0
2
4
6
8
1
2
3
4
5
6
7
8
9
10
11
12
15
Follicle size (mm)


69
No. of follicles/ ovary
0
2
4
6
8
1
2
3
4
5
6
7
8
9
10
11
12
15
Follicle size (mm)


70
No. of follicles/ ovary
0
2
4
6
8
1
2
3
4
5
6
7
8
9
10
11
12
15
Follicle size (mm)

A corpora luteum was found on the left ovaries of cycling wombats. Each cycling wombat also had 1-4 smaller corpus albicans on each ovary. Corpus haemorrhagica were also found on the ovaries of all wombats (Table 4.7), excluding wombat 68 (that did not respond). A small number of corpus hacmorrhagica were also seen on the ovaries of wombats 13 and 6, by laparoscopy, but the technique precluded an accurate count. A mean of 5.6 ± 1.2 (range: 1 to 14) corpus haemorrhagica were found per wombat.

The greatest numbers of blood filled structures (n=14) were found on the ovaries of wombat 64 (Table 4.7). Corpus haemorrhagica varied between 2 and 8mm in diameter, with a mean size of 4.2 ± 0.2 mm.

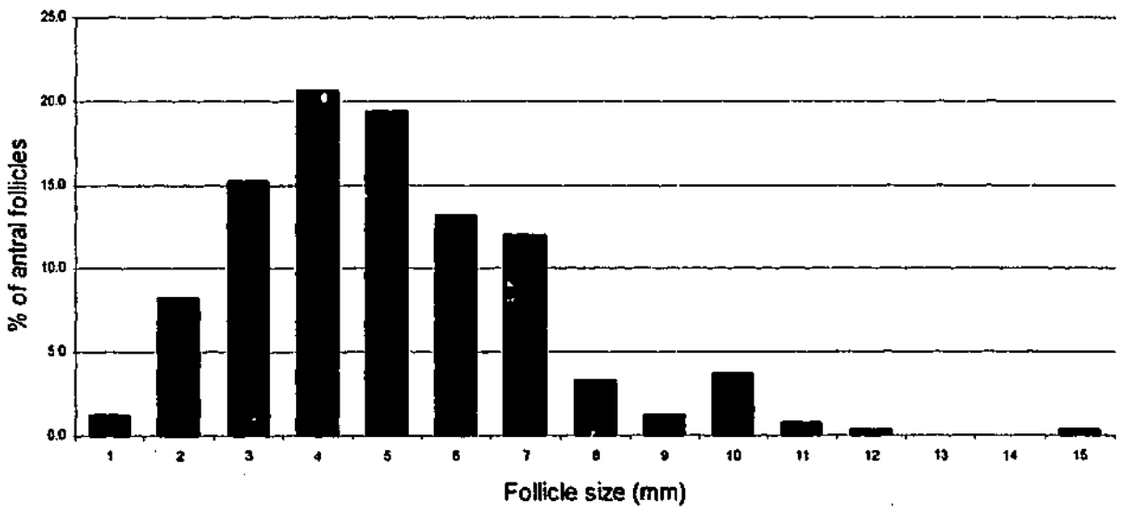


Figure 4.4. Percentage of total follicles of pFSH/pLH stimulated Common Wombats (n=11) measured *ex situ* following ovarian harvest. Follicles were measured from each ovary of Wombats 6b, 7, 13b, 14, 62, 63, 64, 67, 68, 69, 70.

4.3.3 Follicular aspiration and Repeatability of Superovulation

Follicular aspiration via laparoscopy was found to be difficult and unreliable. Following the first superovulation attempt no oocytes were aspirated from wombat 13 or 6, despite the presence of large follicles on the ovaries of both wombats. In an attempt to recover some oocytes, laparotomy was performed on wombat 6, however only two immature oocytes were collected after aspiration via the midline incision.

On both occasions, the manipulation of the ovaries *in situ* required the ovaries to be uncovered from their bursa. The bursa held the ovaries to the dorsal surface of the abdominal wall in the left inguinal region of the abdomen. Manipulation of the ovaries by laparoscopy caused the rupture of the vasculature within the surrounding connective tissue and bursa. As a consequence, scar tissue formed around the ovaries, causing them to become tightly adhered to the abdominal wall. When the wombats (wombat 6 and 13) were examined following the second superovulation, surgical removal of the ovaries was made more difficult by a layer of scar tissue covering each ovary.

Follicular aspiration was, significantly more successful following the removal of ovaries than *in situ* aspiration, and 6 and 19 oocytes were recovered from the two adult cycling wombats after the second superovulation attempt.

4.3.4 Recovered Oocytes

Following superovulation, removal of the ovaries and aspiration of the follicles *ex situ*, a mean of 22.2 ± 5.9 (range: 3 - 68 oocytes) oocytes were recovered per wombat (Table 4.4). The number of oocytes recovered, significantly varied between wombats (α = 0.05; df = 12; χ^2 = 194.73). Aside from the two superovulation attempts that required *in situ* oocyte recovery, the least number of oocytes (n=3) were recovered from wombat 68, who had flat ovaries and no large antral follicles equal to or greater than 1mm in diameter. The greatest numbers of oocytes were recovered from an adult anoestrous wombat (wombat 63) and a subadult wombat (wombat 7), with 68 and 48 oocytes recovered respectively.

More oocytes were collected from the ovaries of wombats than could be accounted for by the number of large antral follicles (>4mm). These additional oocytes were aspirated from smaller follicles and corpora haemorrhagica. Many small follicles (≤1mm), were found on the ovaries in addition to the large antral follicles (>1mm). When the corpora haemorrhagica were aspirated, several contained oocytes. However the numbers of oocytes recovered from the corpora haemorrhagica or small follicles were not delineated from those collected from healthy transparent follicles.

The number of oocytes recovered varied between the left and the right ovary, of a wombat (Table 4.5). More oocytes were collected from the left ovaries of wombats than from right ovaries and an equal number oocytes were collected from 25% of wombats. For most wombats, numbers of oocytes collected between ovaries were not significantly different ($P>0.05$). However for one wombat (wombat 62), significantly more oocytes were collected from the left ovary (Table 4.4) ($\alpha=0.05$; $df=1$; $\chi^2=4$). In total, 141 oocytes were collected from the left ovaries and 105 oocytes were collected from the right ovaries.

Table 4.4. Number of oocytes and percentage of MII oocytes recovered per wombat, and per ovary. ("-" indicates no oocytes were collected).

Wombat	Ovary	# oocytes/ ovary	# MII oocytes/ ovary	% MII/ ovary	Total # oocytes	Total # MII oocytes	%MII per wombat
Adult cycling wombats - First superovulation and attempted in situ recovery							
6 (a)	L	1	-	-	2	-	-
	R	1	-	-			
13 (a)	L	-	-	-	-	-	-
	R	-	-	-			
Adult cycling wombats – ex situ recovery							
6 (b)	L	7	1	14.3%	19	2	10.5%
	R	12	1	8.3%			
13 (b)	L	3	3	100%	6	5	83.3%
	R	3	2	66.7%			
14	L	14	3	21.4%	21	3	14.3%
	R	7	-	-			
Subadults – ex situ recovery							
7	L	30	-	-	48	-	-
	R	18	-	-			
68	L	3	-	-	3	-	-
	R	-	-	-			
70	L	5	-	-	8	-	-
	R	3	-	-			
Adult anoestrous – pouch young removed							
62	L	12	7	58.3%	16	9	56.2%
	R	4	2	50%			
63	L	35	6	17.1%	68	11	16.2%
	R	33	5	15.1%			
67	L	12	2	16.6%	26	3	11.5%
	R	14	1	7.1%			
Adult anoestrous – recently weaned young at foot or unexplained anoestrous							
64	L	6	-	-	8	-	-
	R	2	-	-			
69	L	13	-	-	21	-	-
	R	8	-	-			

Table 4.5. Distribution of total oocytes collected from the ovaries of wombats during the 13 superovulation attempts.

	No. of wombats with recovered oocytes	Wombats with more on L ovary (%)	Wombats with equal no. on both ovaries (%)	Wombats with more on R ovary (%)
Total oocytes	12	66.7	25	8.3
MII oocytes	6	83.3	16.7	-

Of the 246 oocytes aspirated from follicles following superovulation 13.4% were identified as MII oocytes, defined by the presence of a polar body. The number of MII oocytes recovered varied both within individual wombats and between wombats (Table 4.4). MII oocytes were only recovered from cycling adult wombats and adult anoestrous wombats following removal of pouch young. No MII oocytes were collected from 3 subadults, and 2 anoestrous adult wombats (not found with pouch young) despite the presence of large follicles (Table 4.4).

More MII oocytes were collected from the left ovary, of the six wombats from which MII oocytes were recovered (Table 4.5). Despite this trend, there was no significantly difference in the number of MII oocytes collected between the left and right ovary of each wombat (α=0.5; df=1; χ^2<3.84).

A corpus luteum was found of the left ovary of all 3 cycling adult wombats, from which MII oocytes were collected. As the difference in the number MII oocytes found on the ovaries of these wombats was not found to be significantly different ($P<0.05$) the corpora lutea were not considered to effect the production of MII oocytes.

The mean number of MII oocytes recovered per wombat (n=6) was 5.5 ± 1.5 (range: 2 to 11). The greatest number of MII oocytes were collected from wombats 62 and 63 which had pouch young removed 2 weeks to 5 months prior to superovulation (Table 4.6). However less MII oocytes were collected from another wombat when the pouch young was removed on the day of the first FSH injection.

Table 4.6. Time of removal of pouch young and the number of MII oocytes recovered.

Wombat	Age of PY	Sex of PY	Time* of removal of PY	No. of MII oocytes recovered
62	6 months	F	2 weeks	11
63	6 months	F	5 months	9
67	4 months	M	0 days	3

* time prior to first FSH injection

Fewer MII oocytes were collected from adult cycling wombats than anoestrous wombats following pouch young removal. A mean of 3.3 MII oocytes ± 0.9 (range: 2 - 5) were collected from adult cycling wombats. In comparison a mean of 7.6 MII oocytes ± 2.4 (range: 3 - 11) were collected from anoestrous wombats following pouch young removal. This difference was not significant (P=0.166). A significant difference would have been found if the "wombats following pouch young removed" group had not included wombat 67, whose pouch young was removed on the day of 1st FSH injection.

Large follicles (>4mm) were not directly associated with the stage of maturation of oocytes. Neither were other follicular sizes associated with the successful recovery of MII oocytes.

All oocytes without a polar body were, transferred to maturation media and ICSI was performed on MII oocytes (Chapter 6).

4.3.5 Timing of ovarian and oocyte recovery

Variation in the time of recovery of the ovaries did not appear to influence the maturation state of follicles, as defined by follicular size (Table 4.7). Ovulation points were identified in two wombats (wombat 67 and 13(b)) 25.5 to 26hrs after the LH injection. Ovulation was induced when two large follicles, approximately 10-12mm in diameter, were being surgically removed, 25 and 27hrs after LH administration. No other signs of ovulation were observed in any other wombat, despite numerous large pre-ovulatory follicles, and similar ovary recovery times (Table 4.7).

Earlier collection of the ovaries may have permitted the recovery of additional MII oocytes that had potentially ovulated from the 2 wombats with ovulation points, and in which ovulation was observed. For one wombat (wombat 67) the number of MII oocytes recovered may have been as high as 6 (double that observed). However, without recovery of these ovulated oocytes, their development state cannot be assumed.

Time of collection did not influence the number of corpora per wombat. Corpora haemorrhagica were found on the ovaries of 10 of the 11 superovulated wombats whose ovaries were examined *ex situ* (Table 4.7). Although not counted, oocytes were sometimes aspirated from these blood filled structures, indicating that this was not necessarily an indication that ovulation had occurred.

Table 4.7. Time of ovarian harvest after LH injection, comparing numbers of ovulation points, follicles that ruptured at time of harvest and the numbers of oocytes recovered per ovary of each wombat examined.

Wombat	Ovary	Time of harvest	# ovulation points/ ovary	# ruptured follicles at harvest / ovary	# corpora haemorrhagica	# follicles >4mm/ ovary	# oocytes/ ovary	# MII oocytes/ ovary
Adult cycling wombats - First superovulation and attempted in situ recovery								
6 (a)	L	25h	-	-	-	2	1	-
	R		-	-	-	2	1	-
13 (a)	L	25h	-	-	-	-	-	-
	R		-	-	-	1	-	-
Adult cycling wombats – ex situ recovery								
6 (b)	L	27h	-	-	5	9	7	1
	R		-	-	-	7	12	1
13 (b)	L	25.5h	1	-	2	3	3	3
	R		-	-	2	7	3	2
14	L	26h	-	1	1	9	14	3
	R		-	-	-	5	7	-
Subadults – ex situ recovery								
7	L	26.5h	-	-	6	18	30	-
	R		-	-	5	12	18	-
68	L	26.5h	-	-	-	-	3	-
	R		-	-	-	-	-	-
70	L	27h	-	-	3	6	5	-
	R		-	-	-	5	3	-
Adult anoestrous – pouch young removed								
62	L	26h	-	-	2	5	12	7
	R		-	-	2	6	4	2
63	L	25.5h	-	-	2	10	35	6
	R		-	-	3	11	33	5
67	L	26h	2	1	2	12	12	2
	R		-		2	8	14	1
Adult anoestrous – recently weaned young at foot or unexplained anoestrous								
64	L	26h	-	-	10	7	6	-
	R		-	-	4	5	2	-
69	L	25.5h	-	-	3	7	13	-
	R		-	-	2	11	8	-

4.4 Discussion

4.4.1 Ovarian appearance prior to and during superovulation treatment

Sham injected controls could not be used in this study as numbers of wombats were restricted by practical and ethical considerations. An attempt was therefore made to examine ovaries before superovulation treatment. Exploratory surgery and ultrasound were used. Although neither technique had been previously developed for the Common Wombat, results suggest that both laparoscopy and ultrasound could become useful monitoring techniques for this species.

Laparotomy is currently the best and most reliable method to examine the ovaries *in situ*. The ovaries were difficult to examine thoroughly by laparoscopy and scar tissue formed following damage to the vasculature and soft tissue surrounding the ovary. Laparoscopy was successfully used to examine reproductive change in the Common Brushtail Possum (Crawford *et al* 1997) and the Tammar Wallaby (Rodger *et al* 1993). In the Common Brushtail Possum, the pre-ovulatory follicle develops an accumulated blood supply at the apical pole. Similar reliable surface markers do not appear to occur in the Common Wombat or Tammar Wallaby. Crawford *et al* (1997) found that this accumulation of blood is visible on the dominant follicle in the Common Brushtail Possum for approximately 5 days prior to ovulation. This was not observed during this study, or during the examination of culled material by our group (unpublished) or Moritz *et al* (1998).

The ovaries were visible during trans-abdominal ultrasound and large follicles could be visualised. Ultrasound has been used for a number of divergent wildlife species, including the African Elephant (*Loxodonta africana*) and Hare (*Lepus* species) and provides a less invasive method of examination (Hildebrandt *et al* 2000). Application to wildlife often requires the adaptation and modification of probes, especially for trans-rectal or trans-vaginal examination. Currently available probes only permit trans-abdominal ultrasound in the Common Wombat, however the position of ovaries in the abdomen is considered favourable for this technique.

Examination of the ovaries by laparoscopy and ultrasound technique has indicated that in cycling wombats that a range of follicle sizes less than 1 – 2 mm in diameter was visible prior to hormonal stimulation. Similar observations were made during the treatment period in anoestrous and cycling adults. Small follicles less than 2mm could not be reliably observed by ultrasound, using a 5 or 7.5 MHz probe.

The developmental stage of follicles present on the ovary varies with reproductive status. Ovarian morphology and histology of culled wombats has been examined by our group (unpublished) and was similar to observations made by Moritz *et al* (1998). Small preantral follicles are present at all reproductive stages (Moritz *et al* 1998). Pouch young ovarian tissue appears flat and consists of predominantly small preantral follicles, although histological analysis has found early antral follicles (M. Cleary *pers comm*). Subadult ovaries also contain predominantly small pre-antral follicles (<124μm), and some large antral follicles (≥2mm) can be identified on the external surface.

Moritz *et al* (1998), found that cycling adults in pro-oestrous had one or two large antral follicles (≥2mm), although no pre-ovulatory follicles were found. Larger follicles (~4mm) were reported during the mid- and late-luteal periods of cycling wombats (Moritz *et al* 1998). These follicles were considered pre-ovulatory as histologically, they appeared the same as the pre-ovulatory follicles classified in the mouse by Pederson and Peters (1969). The pre-ovulatory period has not yet been examined in the Common Wombat, and therefore the size of the pre-ovulatory follicle immediately prior to ovulation is not known. Moritz *et al* (1998) found that the pre-ovulatory follicle develops on the ovary contralateral to the previous corpus luteum. And in the early-luteal phase, the newly forming corpus luteum has been measured at ~8mm. Observations of the ovaries during the pro-oestrous and early luteal phases, led Moritz *et al* (1998) to conclude that the Common Wombat is monovular.

Lactating anoestrous Common Wombats have a range of follicles including some antral follicles up to ~ 2mm in diameter. One pre-ovulatory follicle 4 to 4.5mm was found on the ovaries of a lactating wombat with a 5 – 7 week old pouch young (Moritz *et al* 1998). Wombats in post-lactation, had flat ovaries, with predominately

small follicles (Moritz *et al* 1998). In other marsupials this ovarian appearance is considered to be a sign of 'true' anoestrous (Tyndale-Biscoe and Renfree 1987). These observations indicate that the follicular development is on going in all reproductive states of the Common Wombat. Although, the final stages of follicular growth require gonadotrophin stimulation (Tyndale-Biscoe and Renfree 1987).

4.4.2 Ovarian appearance 24h after LH administration

Porcine FSH/LH treatment induced the recruitment of multiple follicles in 10 of the 11 wombats. Moritz *et al* (1998) reported that several ~ 2mm follicles could be found in culled un-stimulated wombats in varying reproductive states, and a maximum of one large pre-ovulatory follicle ~ 4 - 4.5mm was identified. In contrast, the number of large antral follicles after pFSH/pLH treatment exceeded this natural follicular development. Up to 30 large antral follicles ≥ 4mm, were found on the ovaries of stimulated wombats, 24 - 27 h after pFSH/pLH administration. The number of large antral follicles > 4mm varied between wombats, and did not appear to be related to reproductive status prior to stimulation.

One subadult female did not appear to respond to stimulation, as only very small follicles (<1mm) were found, and the ovaries appeared flat with a finely granular surface. Pathological findings (Section 4.3.2) were not manifest as clinical illness. But they may have affected the response to pFSH and pLH in this animal. Interestingly the wombat in which the greatest number of large follicles (≥ 4mm) was observed was also a subadult.

Another study found, significantly more follicles grow in response to FSH treatment compared to sham injected and cyclic Common Brushtail Possums (Mcleod *et al* 1999). Approximately twice the number of large antral follicles (≥ 2.5 mm) were observed in anoestrous possums that were stimulated with ovine FSH (oFSH) treatment, when compared to follicles of cycling adults (Mcleod *et al* 1999). After 3 days of oFSH injections at 12 h intervals, 10 ± 2.2 follicles ≥ 2.5 mm were present.

Varying follicular sizes have been found in other marsupials in response to exogenous gonadotrophin treatment. Roger *et al* (1992) reported follicular growth of between

0.67 mm in the Grey Short-tailed Opossum and up to 5mm in the Tammar Wallaby. The hormonally stimulation growth of the follicles in the Tammar Wallaby was slightly larger than the natural pre-ovulatory follicles diameter of ~ 4.2mm (Tyndale-Biscoe and Rodger 1978).

Pre-ovulatory follicular sizes varies between species, and in both eutherians and marsupials final pre-ovulatory size has been found to be positively correlated with adult body size (Parkes 1932; Linton-Moore *et al* 1976; Tyndale-Biscoe and Renfree 1987). Pre-ovulatory follicle sizes in the Eastern Grey Kangaroo (*Macropus giganteus*) has been reported at 6mm in diameter and an adult body weight of ~22kg (Clark and Poole 1967). As adult Common Wombats in Victoria have minimum bodyweight of 23.5kg (Section 3.3.6) we would expect the pre-ovulatory follicle size to be at least 6mm. Following superovulation, follicles were found to be up to 15mm in diameter, although most were 2 - 7mm in diameter. It is consistent then, that larger antral follicles should be found in the Common Wombats in Victoria, when compared to the Common Wombats in Tasmania which weigh less (minimum of 15.4kg), and have a pre-ovulatory follicle size of ~ 4mm (Green and Rainbird 1987; Moritz *et al* 1998).

The actual maximum size of a pre-ovulatory follicle, cannot be determined unless it is actually measured immediately prior to ovulation. This is because there is an increase in pre-ovulatory follicle size associated with the pLH surge (Hilier 2001). Amongst the few species in which this period has been monitored closely, follicles were observed to increase from 5mm - 13mm in the cow (Sirois and Fortune 1988), and ~ 19 - 25mm in the human (Trounson *et al* 2001). The two follicles that were observed to ovulate, in this study, did so at approximately 10-12mm in diameter, and this is more likely to be the pre-ovulatory follicular size of Common Wombats in Victoria.

In the Common Brushtail Possum the natural pre-ovulatory follicular size does not have to be attained prior to ovulation when artificially stimulated. Molinia *et al* (1998b) observed that Common Brushtail Possum follicles were capable of ovulation at a diameter of 2mm in response to LH or GnRH stimulation in spite of the natural pre-ovulatory diameter to of ~5mm (Crawford *et al* 1997).

Zoletil and isoflurothane, were used to anaesthetise wombats during this study. Previous studies in the Common Brushtail Possum suggest that the use of halothane anaesthesia was unlikely to have effected follicle development (Crawford *et al* 1997). Anaesthesia is considered to inhibit ovulation in some other species, such as the domestic cat (Howard *et al* 1992). There was no evidence that anaesthesia effected ovulation during this study, although as oocytes were aspirated from the follicles, inhibition of ovulation may have only helped collection.

No significant difference was found between the number of follicles on each ovary for most wombats in response to the pFSH/pLH treatment. A significant difference was only found in one subadult wombat (wombat 70), and a relationship was not identified. Similarly, Molinia *et al* (1998b) reported bilateral stimulation of the ovaries of Tammar Wallabies in response to the same pFSH/pLH treatment regimen. In contrast, when a different regimen of PMSG was administered, more follicles were produced on the contralateral ovary, to the one bearing a corpus luteum following PMSG administration to Tammar Wallabies (Rodger *et al* 1993; Molinia *et al* 1998b). A corpora luteum was found on one ovary of each of the cycling wombats, and it did not appear to have an affect.

Luteinized cysts are commonly associated with superovulation treatment in many species including sheep (Gilbert *et al* 1990) and Common Brushtail Possums (Mcleod 1999) and are recognised as a sign of over stimulation (Rodger and Mate 1988; Hinds *et al* 1996). Several small corpora lutea were found on the ovaries of hormonally stimulated Common Wombats, although histological analysis is required to confirm their structure. Smith and Godfrey (1970) found that luteinized follicles of Fat-tailed Dunnarts (*Sminthopsis crassicaudata*) were difficult to distinguish morphologically from corpus lutea until examined by histology and entrapped oocytes were found.

4.4.3 Recovered oocytes

Following treatment, immature oocytes were collected from all wombats (n=11). In most wombats, many more immature oocytes were collected than mature oocytes. The superovulation treatment induced maturation of multiple MII oocytes in 6 wombats. The mean number of MII oocytes (5.5 ± 1.5 oocytes) collected from

stimulated wombats exceeded the natural ovulation rate (1 MII oocyte) of the cycling Common Wombat. The superovulation treatment was therefore considered successful in this species.

Unlike most other studies in marsupials, oocytes in this study have been collected from aspirated follicles. Other studies have investigated the number of oocytes induced to ovulate and recovered them from the reproductive tract (Rodger and Mate 1988, Molinia *et al* 1998b, Hickford *et al* 2001). Recovery of oocytes from the reproductive tract invariably results in some losses (Glazier and Molinia 1988; Molinia *et al* 1998b). Losses also occur when aspirating follicles. Two oocytes were potentially lost in this study, as follicles ruptured at the time of surgical removal of the ovary. Another 3 oocytes were potentially lost as follicles had ovulated prior to surgical recovery, defined by ovulation points, and they were not flushed from the reproductive tracts. In Common Wombats, the ovulation points can be recognised at least until the early formation of the corpora luteum, and have been described, as a protrusion of the thecal layer (Moritz *et al* 1998).

Comparable numbers of mature MII oocytes (2 to 11/wombat) were collected from wombats in this study, as have been previously collected from other monovular marsupials. A mean of 3.9, 6.1 and 13.1 oocytes were collected from the tracts of superovulated Common Brushtail Possums treated with various doses of PMSG and GnRH (Roger and Mate 1988; Glazier and Molinia 1998). Glazier and Molinia (1998) later recovered a mean of 9.5 oocytes from 6 Common Brushtail Possums in response to PMSG/pLH administration. Approximately 60% of oocytes recovered from the reproductive tract of FSH/LH treated possums were considered to be mature (Molinia *et al* 2000), but when collected from both the reproductive tract and pre-ovulatory follicles, only 11% were mature MII oocytes (Molinia *et al* 2001). Similarly we collected ~13% MII oocytes (33/246 oocytes) from follicles of pFSH/pLH treated wombats. In another study, in the Common Brushtail Possum, 56% of oocytes were considered to be MII following PMSG/pLH treatment and recovery from the preovulatory follicles (Mate *et al* 2000). A higher percentage (80%) of oocytes were considered mature (MII) following PMSG/pLH treatment when recovered from the

reproductive tract, although less ovulations occurred, than pFSH/pLH primed possums (Molinia *et al* 2000).

Early studies in the Tammar Wallaby induced the ovulation of a mean 2.2 oocytes (in 5 of 9 wallabies) in response to a PMSG/anti-PMS/GnRH regimen (Renfree *et al* 1988). Modification of the regimen, and the use of pLH instead of GnRH increased this ovulation rate and after mating a mean of 3.5 blastocysts were recovered (from 2 of 4 wallabies; Renfree *et al* 1988). The induced ovulation rate has been further increased in recent studies on the Tammar Wallaby due to improvements in the superovulation protocol. In response to the same pFSH/pLH regimen as used in this study, between 7 - 13 oocytes could be recovered from the reproductive tract (Molinia *et al* 1998b), although examination of the ovaries suggested that 3 - 25 oocytes had ovulated.

Reproductive status prior to treatment seems to be an important factor on the MII oocyte yield following pFSH/pLH stimulation of the Common Wombat. MII oocytes were only collected from cycling adults and adults following pouch young removal. The time of pouch young removal did not appear to affect the outcome, although the data may suggest that more MII oocytes were produced in animals following a longer interval of pouch young removal and hormonal induction. Data also suggest, but not significantly, that more MII oocytes can be collected from adults following pouch young removal than from cycling adults during the follicular phase. Further studies are required to investigate significant differences.

Similarly, superovulation was found to be successful in the Tammar Wallaby when stimulated during the follicular phase with various regimens using PMSG (Renfree *et al* 1988). The pFSH/pLH stimulation regimen was more successful than previous PMSG regimens, and induced ovulation in both cycling and non-cycling Tammar Wallabies (Molinia et al 1998b). Dose has been found to effect the ovulatory response of Stripe-faced Dunnarts in different stages of the cycle when stimulated with PMSG (Hickford et al 2001). Another study suggested that PMSG/GnRH treatment was able induce superovulation in all reproductive states, including juveniles, but excluding pre-ovulatory Common Brushtail Possums (Rodger and Mate 1988). Glazier (1998) found more specifically that even though most Common Brushtail Possums will

respond to PMSG administration, the number of large follicles that develop depends on the season. Common Brushtail Possums are seasonal breeders and responded better during the autumn breeding season than during other seasons, particularly during summer. The effect of season was not investigated in this study. As Common Wombats have a peak breeding (Green and Rainbird 1987; McIlroy 1990) similar to the Common Brushtail Possum (Pilton and Sharman 1962; Gemmell and Serina 1995), season may have an effect. However, it remains evident that reproductive status does influence artificially induced oocyte maturation.

In addition to species, age, reproductive status and season, factors effecting superovulation response of marsupials are likely to be as variable as has been found in the cow (Kafi *et al* 1997) and factors are likely to include environment, nutrition, health and individual differences.

4.4.4 Timing of ovarian and oocyte recovery

Results may indicate that, the 5 day pFSH/pLH stimulation regimen, used in this study was too long, although further investigation of the hormonal milieu and histology of stimulated ovaries is required.

Three different signs of ovulation were observed on day 6, that had occurred prior to the time of ovarian harvest. The rupture of two oocytes at surgical removal of the ovaries was difficult to avoid, suggesting that some follicles may have been capable of ovulation 25 - 27 h after pLH. Alternatively, oocytes maybe recovered from the reproductive tract as in other marsupial studies (Roger and Mate 1988, Molinia et al 1998a; Glazier and Molinia 1998). Ovulation points were also observed in two wombats suggesting that oocyte collection may have already occurred prior to ovarian harvest. Ovulation points are visible in the newly form corpus haemorrhagican of the Common Brushtail Possum and at formation of the corpus luteum, however they are less conspicuous in other marsupials, (Crawford *et al* 1997; Tyndale-Biscoe and Renfree 1987) including as we have found, the Common Wombat. Both of these observations indicated that oocyte aspiration might have been more successful if undertaken several hours earlier. Although, this may compromise the maturation stage of the recovered oocytes, which have been observed to continue to develop after follicular antrum formation in other marsupials (Roger *et al* 1992a).

An additional, but unconfirmed sign of inappropriately timed ovary collection and oocyte recovery, was the presence of large numbers of corpora heamorrhagica. As some oocytes were observed to be aspirated from several corpora haemorrhagica, they were not all considered to be a sign that ovulation had occurred. Following natural ovulation in the bandicoot species Lyne and Hollis (1979) found that as the follicle wall is largely retained, blood from burst vessels fills the follicle, forming a corpus haemorrhagican. Within 24 hours luteal cells infiltrate the blood filled antrum and by 4 to 6 days the ovulated follicle has become a fully formed corpora luteum. These observations suggest that corpus haemorrhagica may have formed, in the hormonally stimulated wombats, ~ 24 h prior to harvest. As blood clotting had not yet occurred in some, these corpus haemorrhagica may have formed more recently. Should, the majority of corpus haemorrhagica have formed following ovulation, it would indicate that the hormonal regimen may need to be reduced to encompass a shorter stimulation period and earlier oocyte recovery.

Multiple corpora haemorrhagica are often identified in hormonally stimulated ovaries, however this has mostly been reported in eutherian species. Corpora haemorrhagica formed in 79% of ewes stimulated with GnRH (Keisler and Keisler 1989). In another study, the numbers of corpora haemorrhagica were greater on the ovaries of wild felids (particularly cheetahs and tigers) if small to large antral follicles were present prior to stimulation (Phillips *et al* 1982). Certainly observations of the ovaries of culled wombats (unpublished) and observations prior to hormone stimulation indicate that small antral follicles are present on the ovaries of most wombats in all reproductive states. It is therefore likely that small to large follicles were present in all wombats prior to the first pFSH injection during this study. The greatest number of corpora haemorrhagica occurred on the ovaries of an anoestrous female in post-lactation. Moritz *et al* (1998) found that the ovaries of females in this state, are relatively inactive (consisting of mostly small pre-antral follicles) compared to wombats in other reproductive states.

Should, the majority of corpora haemorrhagica have formed following ovulation, then it would suggest that artificial induction of ovulation does not require administration

of pLH. Instead some ovulation may have occurred in response to the repeated pFSH injections. In some other marsupial species, such as the Fat-tailed and Stripe-faced Dunnart, Common Brushtail Possum, ovulation can be induced in response to a single stimulation of PMSG (Rodger *et al* 1992b; Hickford *et al* 2001; Harding 1969). Harding (1969) however observed that although Common Brushtail Possums did respond to a single treatment of PMSG, many of the ruptured follicles retained the oocytes. Most marsupials require both hormonal administration (PMSG or FSH) to recruit follicles and (GnRH, hCG or LH) to induce ovulation (Rodger and Mate 1988; Rodger *et al* 1992b, Hinds *et al* 1996).

4.4.5 Repeatability of SO

Repeated superovulation in the Common Wombat is limited by the difficulties associated with ovary examination and oocyte recovery *in situ*. This has proved difficult so far in this species. Previous studies marsupials report the collection of oocytes from harvested reproductive tracts and ovaries, of euthanaised animals (Renfree 1988; Rodger and Mate 1988; Molinia *et al* 1998b; Hickford *et al* 2001). A working procedure for repeated superovulation has not yet been reported for a marsupial, however it is currently being examined in the Tammar Wallaby (G.M. Magarey *pers comm*). Methods for the recovery of oocytes *in situ* from hormonally stimulated ovaries are well developed in several eutherian species including humans and sheep (Dr J Leeton *pers comm*; Torres and Sevellec 1987).

In this study, MII oocytes were only recovered from two cycling wombats following the second superovulation treatment and aspiration of the follicles *ex situ*. When the ovaries of another cycling wombat were aspirated *ex situ*, MII oocytes were recovered following a single pFSH/pLH treatment. This implies that repeated superovulation is possible in the Common Wombat, so long as the technique of *in situ* recovery is improved. The number of times a wombat can be superovulated remains to be identified, and will include factors such as the interval between superovulation treatments and possibly the animal's immune response to the exogenous hormones.

Repeated superovulation in other species, such as sheep, suggest that the reduced ovulatory rate occurs depending on the number of times it is repeated and the interval between each treatment regimen (Al-Kamali *et al* 1985; Torres and Sevellec 1987;

Forcada *et al* 2000). If extended intervals are allowed between treatments then no differences were found in up to three consecutive treatments (Boland and Gordon 1982).

4.5 Conclusion

The response to superovulation treatment has not been previously reported in the Common Wombat. The pFSH/pLH protocol, previously used in the Tammar Wallaby (Molinia *et al* 1998b), successfully induced development of multiple large antral follicles (≥4mm) and the maturation of multiple MII oocytes in the Common Wombat. Cycling adults (n=3) and adults (n=3), following pouch young removal, responded better than subadults (n=3) and anoestrous adults (n=2). The mean number of MII oocytes (5.5 oocytes) collected from stimulated wombats (n=6) exceeded the natural ovulation rate of the monovular cycling Common Wombat. These results represent an important step for attaining mature oocytes for manipulation of reproduction in wombat species.

The health of the follicles found on the stimulated ovaries needs to be assessed by histological analysis which would also provide a more detailed study of the proportion and types of follicles based on cell layers and cell types (Moritz *et al* 1998). Further information could be obtained by examining the histology of ovaries collected from pFSH/pLH wombats.

This method of superovulation is not optimal, and dose, duration of administration and the type of exogenous hormones should be examined, and comparisons made with the effect on the hormonal milieu. Further studies of repeated superovulation in this species should attempt to develop techniques that are less damaging to the ovaries and surrounding tissue on inspection *in situ*. The use of ultrasound is one possible method, however this technique still requires further development. The viability of the resultant oocytes still needs to be examined.

Chapter 5

Hormonal profiles and vaginal cytology during and following superovulation treatment

5.1 Introduction

Hormonal and vaginal changes normally occur during the natural oestrous cycle of the Common Wombat (Chapter 3) and similar changes may occur in response to hormonal stimulation. This study compares the hormonal, vaginal, pouch and urogenital opening changes in artificially stimulated Common Wombats to those observed during the natural oestrous cycle. This information will help define the effect of the superovulation regimen, and its potential to support the growth of fertilised oocytes.

5.2 Materials and Methods

5.2.1 Animal Use

Superovulation was investigated in 11 Common Wombats listed in Table 5.1.

Table 5.1. Body weight, age group and reproductive status of Common Wombats at the time of superovulation treatment.

Wombat	Body Weight (kg)	Age group	Reproductive Status
6	25.5	Adult	Cycling
7	23	Subadult	Anoestrous
13	25.6	Adult	Cycling
14	28	Adult	Cycling
62	27.2	Adult	Anoestrous (RPY)
63	27.1	Adult	Anoestrous (RPY)
64	27.9	Adult	Anoestrous
67	25.7	Adult	Anoestrous (RPY)
68	21.3	Subadult	Anoestrous
69	27.4	Adult	Anoestrous
70	19.4	Subadult	Anoestrous

RPY = pouch young removed before treatment
Subadult defined as <23kg, Adult defined as >23kg

5.2.2 Experimental Design

Plasma and vaginal smears were collected at regular intervals during the induction of superovulation of eleven Common Wombats. Two of the wombats were superovulated twice, and plasma and vaginal smears were collected following the first superovulation and during the second superovulation attempt. Changes in pouch and urogenital sinus condition, body temperature and body weight were all monitored at the times of plasma and vaginal smear collection (Section 2.2). Plasma was assayed for progesterone and estradiol concentration (Section 2.3).

5.2.3 Monitoring of Reproductive Status

Plasma samples, vaginal smears, body weight, body temperature and observations of changes in pouch and urogenital sinus condition were gathered 2 to 3 times per week as described in Sections 2.3.

5.2.4 Timing of Superovulation and Collection of Samples

The reproductive status, of all wombats, was monitored for a minimum of 12 days prior to superovulation (Table 4.2). Cycling wombats were monitored for at least 3 complete cycles prior to the 1st superovulation and for one complete cycle prior to the 2nd superovulation.

Porcine FSH/LH treatments are summarised in Table 4.2 Eight 6mg injections of pFSH were administered at 12 h intervals over a period of 4 days. The first pFSH injection was given on day 1. A 4mg pLH injection was given on day 5 and the ovaries and oocytes were collected on day 6.

In cycling adult wombats, the previously monitored oestrous cycles were used to predict the day of return to basal progesterone concentrations, at the end of the last normal luteal phase monitored. The 1st pFSH injection of the superovulation regimen was administered to cycling wombats as the progesterone concentrations were returning to the basal levels (>7.6 nmol/L) at the beginning of the next follicular phase. The 1st pFSH injection was administered to the remaining 8 wombats as listed in Table 4.2 pFSH/pLH administration was repeated in two wombats (wombat 6 and 13), with an interval of one natural cycle.

5.2.5 Data Analysis and definitions

Progesterone and estradiol concentrations were determined from peripheral plasma samples (Sections 2.3). Elevated estradiol levels were determined by calculating the mean ± SEM (range: minimum to maximum) of the maximum concentrations observed for each wombat during the pFSH/pLH treatment period. Stimulated cycles were defined as those during which a wombat received pFSH/pLH administration at the beginning of that cycle. Day 1 was the first day of pFSH administration. The natural cycle that immediately followed the stimulated cycle was termed the subsequent cycle. During these cycles, the reproductive status of the wombats was monitored at intervals of 1 - 4 days. The average lengths of the stimulated and subsequent cycles for each wombat were determined by calculating the time interval midpoint between observed changes.

5.3 Results

5.3.1 Estradiol Profiles

Estradiol concentrations in the peripheral circulation were generally found to increase after day 1 (1st pFSH injection), peak between days 3 and 5, and then decrease by day 6 (ovary/oocyte recovery). This general pattern was observed in 6 of the 11 wombats (Table 5.2), and potentially 7 wombats although some of the samples of this last wombat (wombat 6(b)) were considered to be inaccurate (poor duplicate result).

On day 1, estradiol concentrations were found to be basal (< 0.86pg/ml; Section 3.3.2) for 7 of the 13 superovulation attempts (Table 5.2). Estradiol concentrations were not determined for 2 wombats on day 1 as shown in Table 5.2. Two of the cycling wombats (wombat 6(a) and 13(b)) and 2 of the anoestrous adult wombats that had their pouch young removed (wombats 63 and 67) had basal estradiol concentrations on day 1. MII oocytes were collected from these 4 wombats. The estradiol concentration on day 1 did not appear to have affected the MII oocyte yield, as MII oocytes were also collected from 2 wombats (wombat 14 and 62) that had basal estradiol concentrations.

By day 3, the estradiol concentrations, in 10 of the 13 superovulation attempts, had increased above basal levels (Table 5.2). The estradiol concentrations of 5 of these wombats at day 3, were found to be greater than the elevated estradiol levels (2.11pg/ml ± 0.2 (range: 1.18 to 3.67pg/ml)) observed during the normal oestrous cycle. MII oocytes were collected from all wombats with estradiol concentrations equal to or greater than 4.66pg/ml on day 3. It is likely that as very high estradiol concentrations were observed on day 5 of wombat 6(b), that the estradiol was also high on day 3. MII oocytes were recovered from wombat 6(b). It is likely that multiple MII oocytes would have been produced by wombats 13(a) and 6(a), which had estradiol concentrations at or above natural elevated levels (2.11pg/ml) by day 3 or 4. However no MII oocytes were collected from these animals. This may have been due to insufficiencies in the *in situ* aspiration technique used. MII oocytes were collected following the 1st superovulation treatment of the other cycling wombat (wombat 14), although higher estradiol concentrations were observed, compared to the 1st superovulation attempt of the other two cycling wombats (wombats 13(a) and 6(a)).

By day 5, most wombats (7/13) had high estradiol concentrations (Table 5.2). Basal estradiol concentrations were observed in 3 wombats (wombat 64, 68 and 70). The estradiol concentrations of 6 wombats (n=11), at day 5, were found to be greater than the elevated estradiol levels observed during the normal oestrous cycle (Section 3.3.2).

By day 6, estradiol concentrations had decreased in most wombats (4/7) that had high levels at day 5 (Table 5.2). In these wombats, estradiol was still greater than the elevated concentrations observed during the natural cycle. Estradiol concentrations had increased further by day 6 in one wombat (wombat 7) and remained constant in the other wombat (wombat 69).

Although estradiol concentrations of consecutive plasma samples have not been determined for all superovulation attempts of the cycling wombats, the general pattern was consistent in two superovulation attempts of cycling wombats (wombat 14 and 13(a)), when consecutive samples had been assayed. The intermittent results from the

other superovulation attempts of cycling wombats (wombat 13(b), 6(a), 6(b)) also support this general pattern.

Estradiol concentrations of 2 subadults (wombat 68 and 70) and one anoestrous adult (wombat 64) remained below basal levels during the entire superovulation period. In contrast, the estradiol levels of the third subadult (wombat 7), had increased above basal concentrations by day 3, and then continued to increase up until, and including, day 6.

Estradiol concentrations during superovulation (days 2 - 5) were found to peak at a mean 11.23pg/ml ± 2.13 (range: 3.64 to 20.13pg/ml; Figure 5.1). This was approximately 5 times greater than the mean peak estradiol concentrations (2.11pg/ml ± 0.22) determined in 3 natural oestrous cycles (Section 3.3.2).

Table 5.2. Change in estradiol concentration compared to the number of MII oocytes recovered. Estradiol samples were collected at day 1 (1st FSH injection), day 3 (5th pFSH injection), day 5 (pLH injection) and day 6 (time of ovary collection).

Wombat	Estradiol Concentration (pg/ml)							No. of MII oocytes
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	
6 (a)	1.75		2.11		*		3.84	φ
6 (b)		*		*		11.64	*	2
7		< 0.23		1.15		2.76	5.22	-
13 (a)				4.97			*	φ
13 (b)	1.44		13.35		12.59		2.07	5
14		0.71		6.45		18.30	2.87	3
62		0.32		5.15		8.32	1.95	9
63		1.07		4.66		20.13	9.37	11
64		0.85		< 0.23		< 0.23	*	-
67		1.66		4.81		10.64	3.59	3
68		< 0.23		< 0.23		< 0.23	< 0.23	-
69		0.49		1.56		3.64	3.61	-
70		< 0.23		< 0.23		0.44	0.68	-

(a) 1st superovulation attempt; (b) 2nd superovulation attempt
φ failed to recover oocytes using *in situ* technique
* samples with poor duplicate results

A general trend suggested that unless estradiol concentrations were ≥ 2.11 or 4.66 pg/ml by day 3, or ≥ 8.32pg/ml by day 5, then MII oocytes could not be recovered from the ovaries on day 6, 25 to 27hrs after pLH (Table 5.2).

In the 6 wombats that produced multiple MII oocytes, estradiol concentrations had increased by day 3. Estradiol concentrations then continued to increase until day 5, during 5 of the 6 superovulation attempts that yielded MII oocytes (wombats 6(b), 14, 62, 63, 67) and remained constant during the other superovulation attempt (wombat 13(b)). By day 6, the time of pLH injection, a considerable decrease in the concentration of estradiol had occurred in all of the responding wombats, however, the estradiol concentrations remained higher than basal levels.

5.3.2 Progesterone Profiles

Progesterone concentrations were at basal levels both before and during superovulation treatment in all anoestrous wombats (subadults 7, 68, 70; adults 62, 63, 64, 67, 69) (Table 5.3). Different progesterone profiles were observed in the cycling adults (Table 5.3).

Treatment of the cycling wombats was designed to start on the first day of basal progesterone concentrations at the end of the previous cycle. This point had to be estimated (based on previously examined cycles (Chapter 3)). For two superovulation attempts this estimate was correct (wombat 13(a) and 14) (Table 5.3). For the other three, progesterone concentrations had not reduced to basal levels by day 1 (wombat 13(b), 6(a), 6(b)). The progesterone concentration at day 1 did not appear to affect the number of MII oocytes produced (Table 5.3). By day 3, all wombats had basal progesterone concentrations. Generally the progesterone concentrations of adult cycling wombats remained basal during the administration of exogenous hormones, until day 6 (time of ovary/oocyte recovery) when they increased above basal levels (Table 5.3).

Blood samples were not collected from one wombat (wombat 13(a)) on days 1 or 5. The previous samples collected on day –2 and 3, were at basal progesterone concentrations. It is likely that the samples on day 1 and 5 were also basal (Table 5.3).

On day 5 (time of the LH injection), progesterone concentrations were basal in all but one cycling wombat (wombat 6(a)) which had a slightly higher progesterone concentration. On day 6, progesterone concentrations had increased above basal levels, in all but one cycling wombat (wombat 14) that had basal progesterone concentrations throughout the superovulation treatment period in that wombat.

Table 5.3. Change in progesterone concentration and the number of MII oocytes recovered. Progesterone samples were collected at day 1 (time of 1st FSH injection), day 3 (5th FSH injection), day 5 (LH injection) and day 6 (time of oocyte recovery).

Wombat	Progesterone Concentration (nmol/L)							No. of MII oocytes
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	
6 (a)	8.6		7.2		9.7		15.9	φ
6 (b)		8.1		4.9		6.8	12.7	2
7		3.1		3.8		5.4	6.1	-
13 (a)		< 7.6*		3.3		< 7.6*	8.9	φ
13 (b)	13.4		6.3		7.4		22.5	5
14		3.6		3.2		2.9	5.6	3
62		4.1		3.5		3.8	3.8	9
63		3.9		3.4		4.1	3.2	11
64		3.8		4.9		9	8.1	-
67		3.9		4.0		4.8	6.8	3
68		4.0		2.9		4.0	6.8	-
69		4		2.9		3	3.9	-
70		3.5		3.1		3.9	4.6	-

(a) 1st superovulation attempt; (b) 2nd superovulation attempt
φ failed to recover oocytes due to poor technique
* although a sample was not taken, estimated concentration to be basal (<7.6nmol/L).

No definitive relationship occurred between the progesterone concentration at any sample point and the number of MII oocytes produced in response to treatment (Table 5.3). On day 6, MII oocytes could still be recovered from 2 cycling wombats (wombat 13(b) and 6(b)), despite high progesterone concentrations. Low progesterone concentrations were identified in all other wombats (n=4) that yielded MII oocytes.

The progesterone concentrations increased at least 1 to 3 days prior to the decline in estradiol concentrations in 4 superovulation attempts of the cycling wombats (Figure

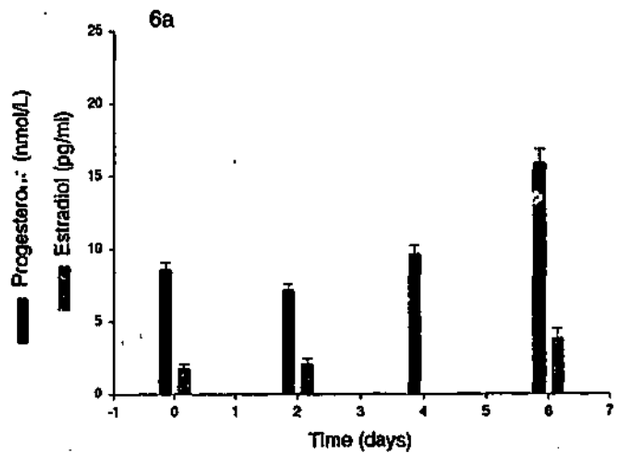
5.1). In all other wombats progesterone concentrations had not increased above basal concentrations before ovarian harvest, and euthanasia.

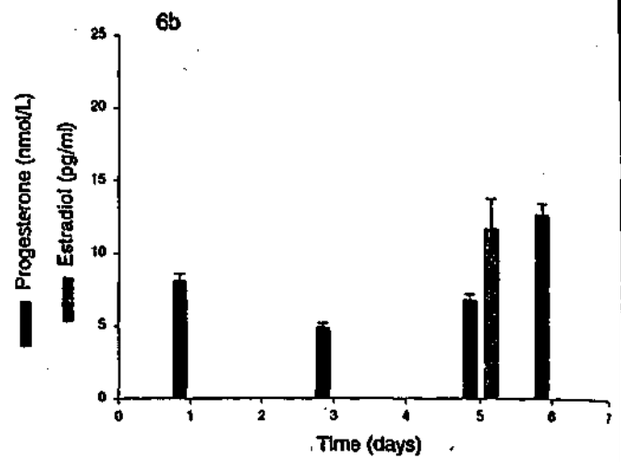
5.3.3 Hormonal changes following superovulation

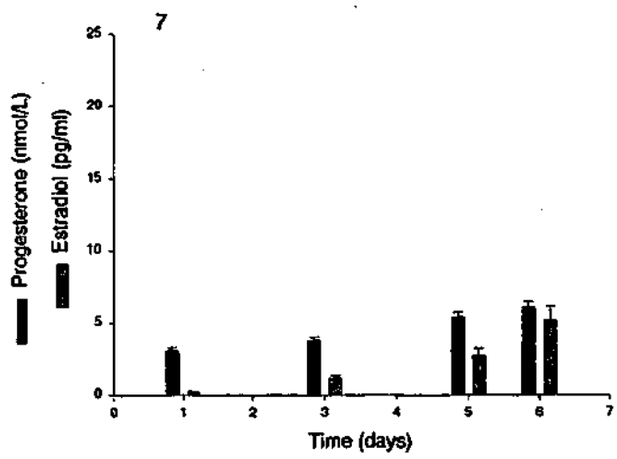
The length of the artificially stimulated cycles, of two cycling females (wombat 6 and 13), were not significantly different (P>0.1) from that determined during the previous natural cycles examined (Figure 5.2). However, the average stimulated cycle lengths for each wombat were both slightly shorter than the mean natural cycle lengths of each wombat. Additionally, the average subsequent cycles of each wombat, following the stimulated cycles, were not significantly different (P>0.1) from the mean natural cycle lengths.

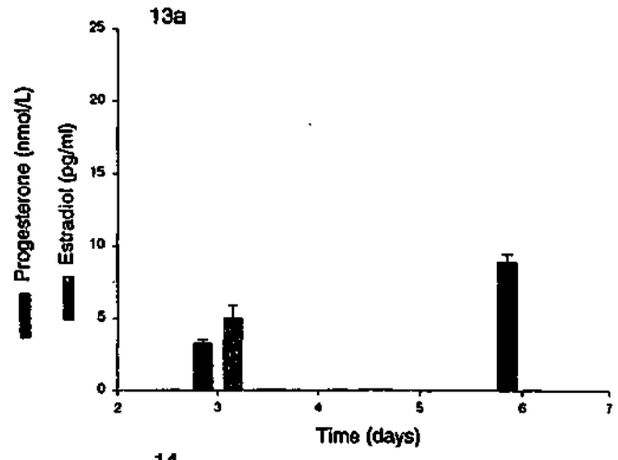
Peak progesterone concentrations, during the stimulated cycle, were not significantly different (P<0.1) from that of natural cycles. However in 4 superovulation attempts progesterone (wombats 6a, 6b, 13a, 13b) concentrations began to rise above basal levels earlier than during natural cycles (Figure 5.2). During the natural cycles (n=7) progesterone concentrations were observed to increase 3 –7.5 days after the return of basal estradiol concentrations (Section 3.3.2). During the stimulated cycles progesterone concentrations were observed to increase 1 –3 days prior to the return of basal estradiol concentrations.

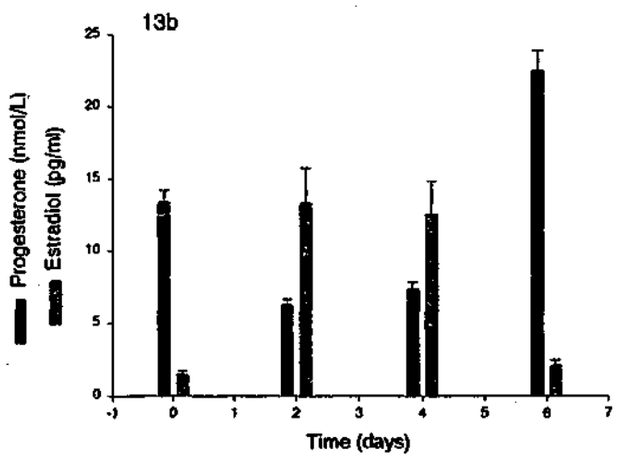
No distinct differences were observed between the hormone profiles of the natural cycle and of the subsequent oestrous cycles, following the stimulated cycle, of wombats (Figure 5.3).

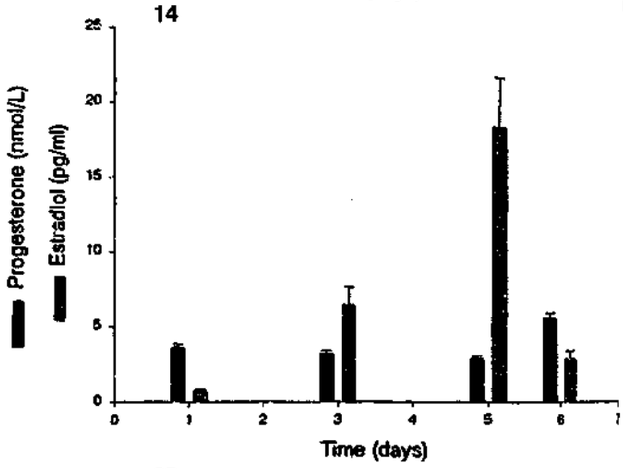

6a
Progesterone (nmol/L)
Estradiol (pg/ml)
Time (days)

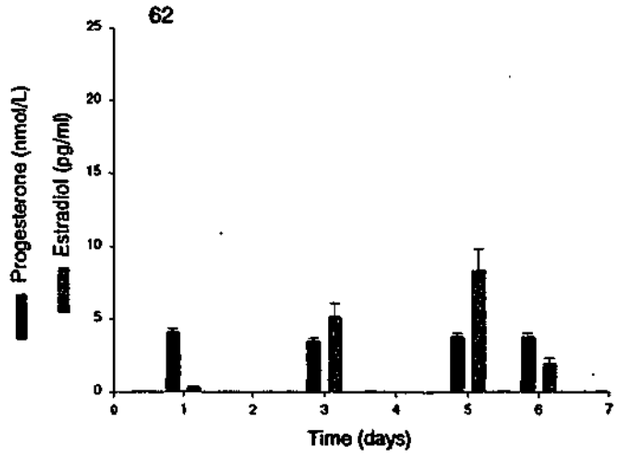

6b
Progesterone (nmol/L)
Estradiol (pg/ml)
Time (days)

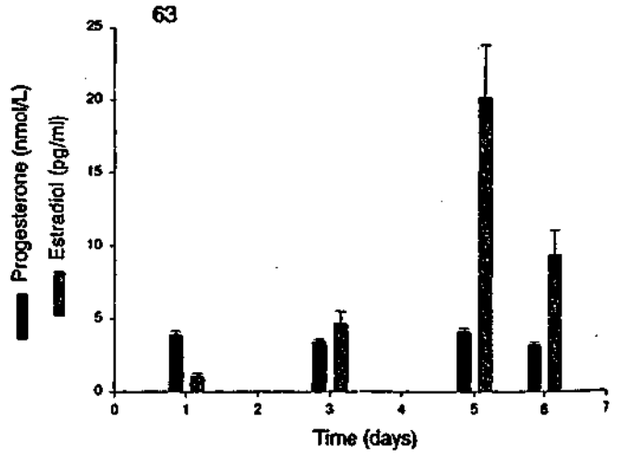

7
Progesterone (nmol/L)
Estradiol (pg/ml)
Time (days)


13a
Progesterone (nmol/L)
Estradiol (pg/ml)
Time (days)


13b
Progesterone (nmol/L)
Estradiol (pg/ml)
Time (days)


14
Progesterone (nmol/L)
Estradiol (pg/ml)
Time (days)


62
Progesterone (nmol/L)
Estradiol (pg/ml)
Time (days)


63
Progesterone (nmol/L)
Estradiol (pg/ml)
Time (days)

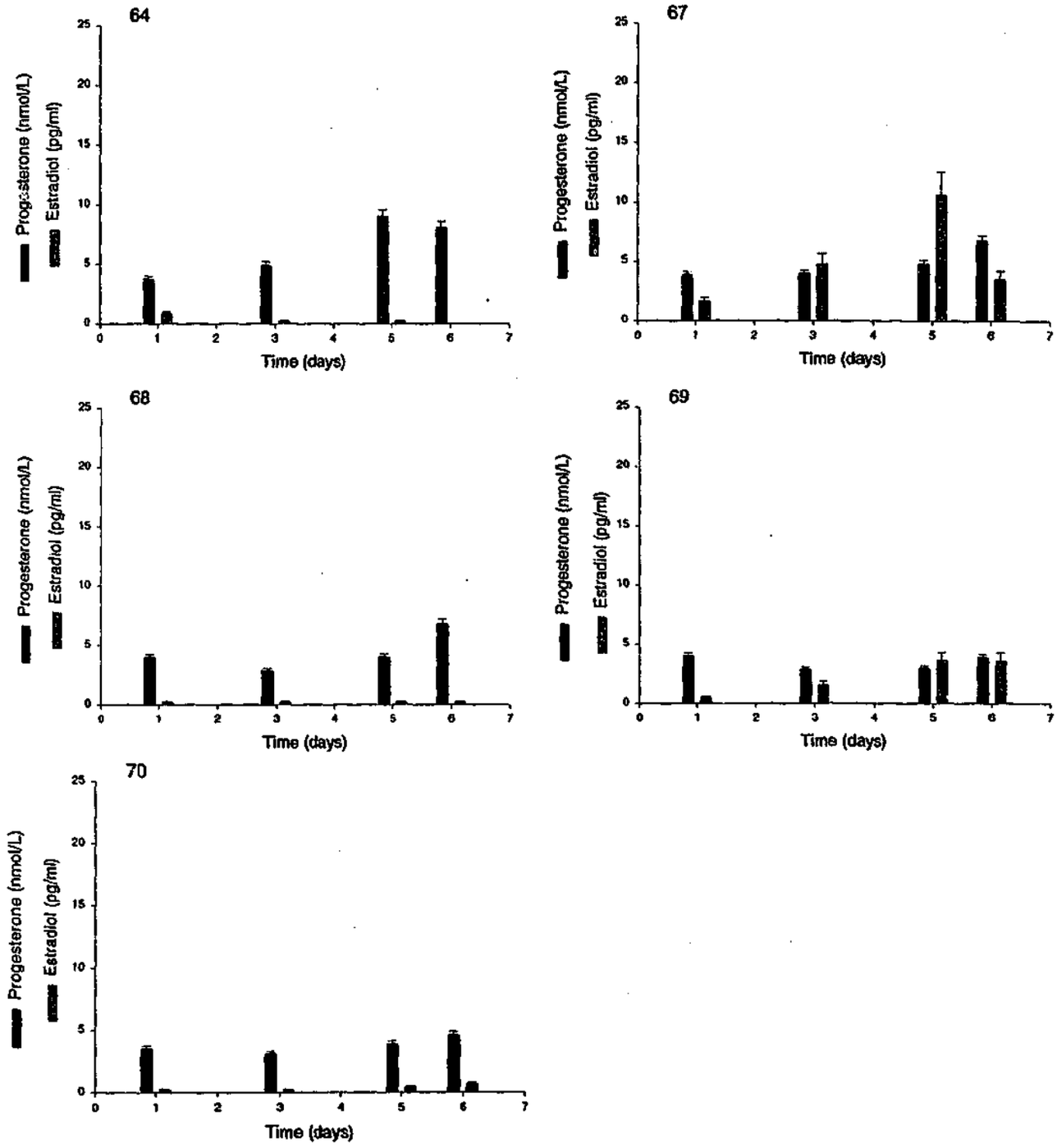


Figure 5.1. Change in progesterone and estradiol concentrations during 13 pFSH/pLH treatments of 11 wombats (wombats 6,7,13,14,62,63,64,67,68,69,70). Wombats 6 and 13 were treated on two separate occasions denoted by 6a, 6b, 13a, and 13b. Day 1 was the first pFSH injection; the treatment regime consisted of 8 x pFSH at 12 h intervals and 1 x pLH injection on day 5.

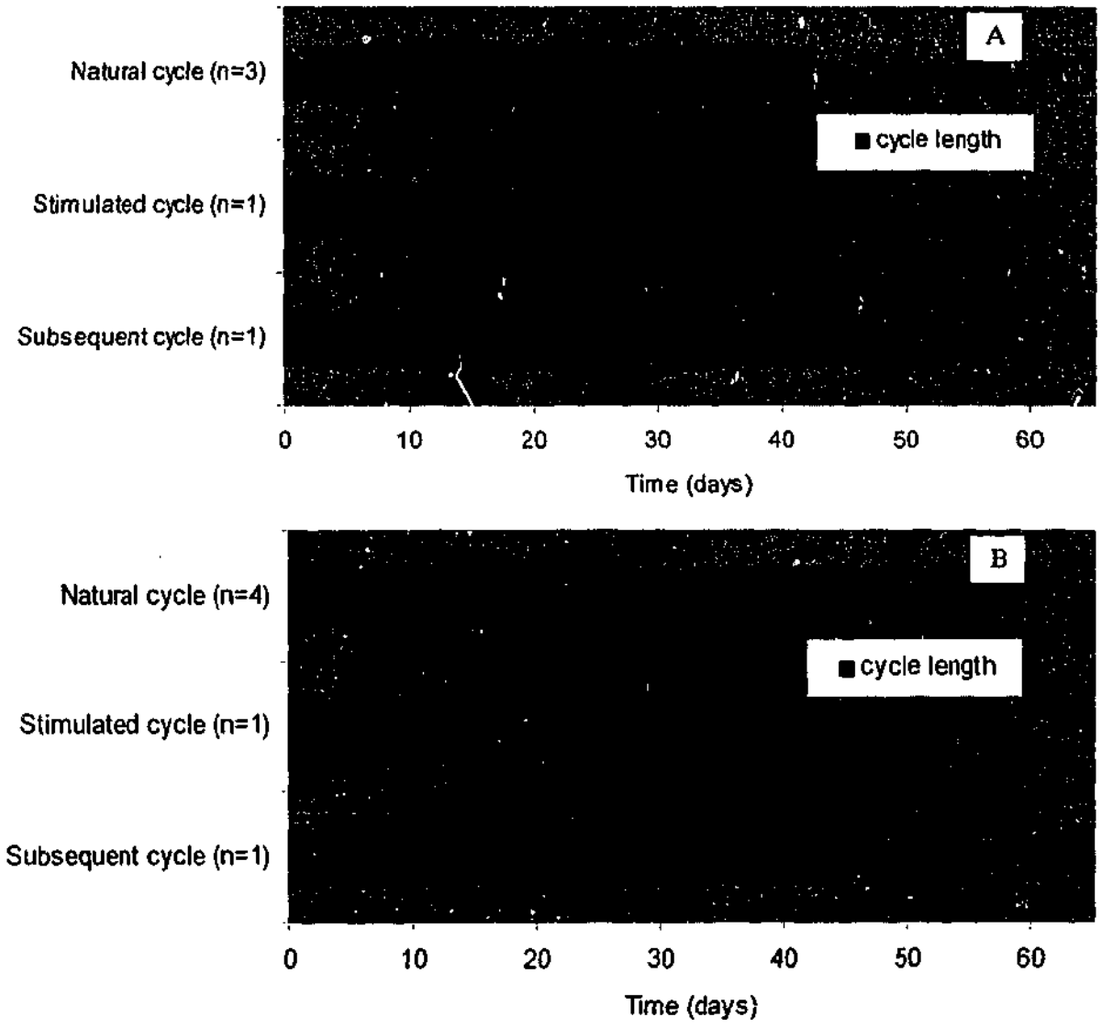


Figure 5.2. Cycle lengths of wombat 6, defined by progesterone concentrations, determined during the natural cycles (n=3 and 4), a stimulated cycle when FSH/LH was administered (n=1) and the subsequent oestrous cycle that immediately followed the stimulated oestrous cycle. A – Wombat 6; B- Wombat 13. Error bars show the range of each cycle length.

5.3.4 Vaginal cytology changes during superovulation

A general pattern was observed in most wombats, with the initially low percentage of superficial type cells increasing on day 3 or 5 to a high percentage of superficial type cells. Variations in this general pattern were observed, particularly in those wombats that did not respond as well to superovulation (those in which MII oocytes were not recovered) (Table 5.4).

One subadult (wombat 68) and one anoestrous adult (wombat 69), that did not produce MII oocytes, had a low percentage of superficial type cells throughout the superovulation period (Table 5.4). The vaginal cytology was typical of an unstimulated subadult or anoestrous adult, even though an crease in estradiol concentrations was observed in the anoestrous adult. Estradiol remained basal in the subadult. These observations were reflected in the follicular growth. Morphological examination of the subadult ovaries showed that ≥1mm follicular growth did not occur, however, 18 antral follicles > 4mm in diameter did develop in the anoestrous adult.

A high percentage of superficial type cells were observed for the entire duration of the first superovulation attempt of one cycling wombat (wombat 6). Numerous large antral follicles were identified in this wombat, although failure to collect MII oocytes is attributed to the *in situ* recovery technique. All other cycling wombats, from which MII oocytes were recovered, had a general pattern of vaginal cytological change typical of naturally cycling wombats in the follicular phase.

A high percentage of superficial type cells towards the end of the superovulation period did not necessarily indicate that an animal was responding well during hormone administration. However, wombats that had high percentage of superficial type cells by day 3 or 5 were more likely to have numerous large follicles and numerous MII oocytes were more likely to be recovered (Table 5.4). One subadult (wombat 70) had a typical low percentage of superficial type cells until day 6 (ovarian harvest) when the percentage of superficial type cells increased. This was typical of a naturally cycling wombat. On day 6, the ovaries of this wombat had 11 antral follicles (> 4mm), although no MII oocytes were recovered.

Elevated estradiol concentrations (>0.86pg/ml) were found to coincide with 88.5% of observations of a high percentage of superficial type cells. However 11.5% of observations of elevated oestradiol concentrations coincided with a low percentage of superficial type cells (Table 5.4).

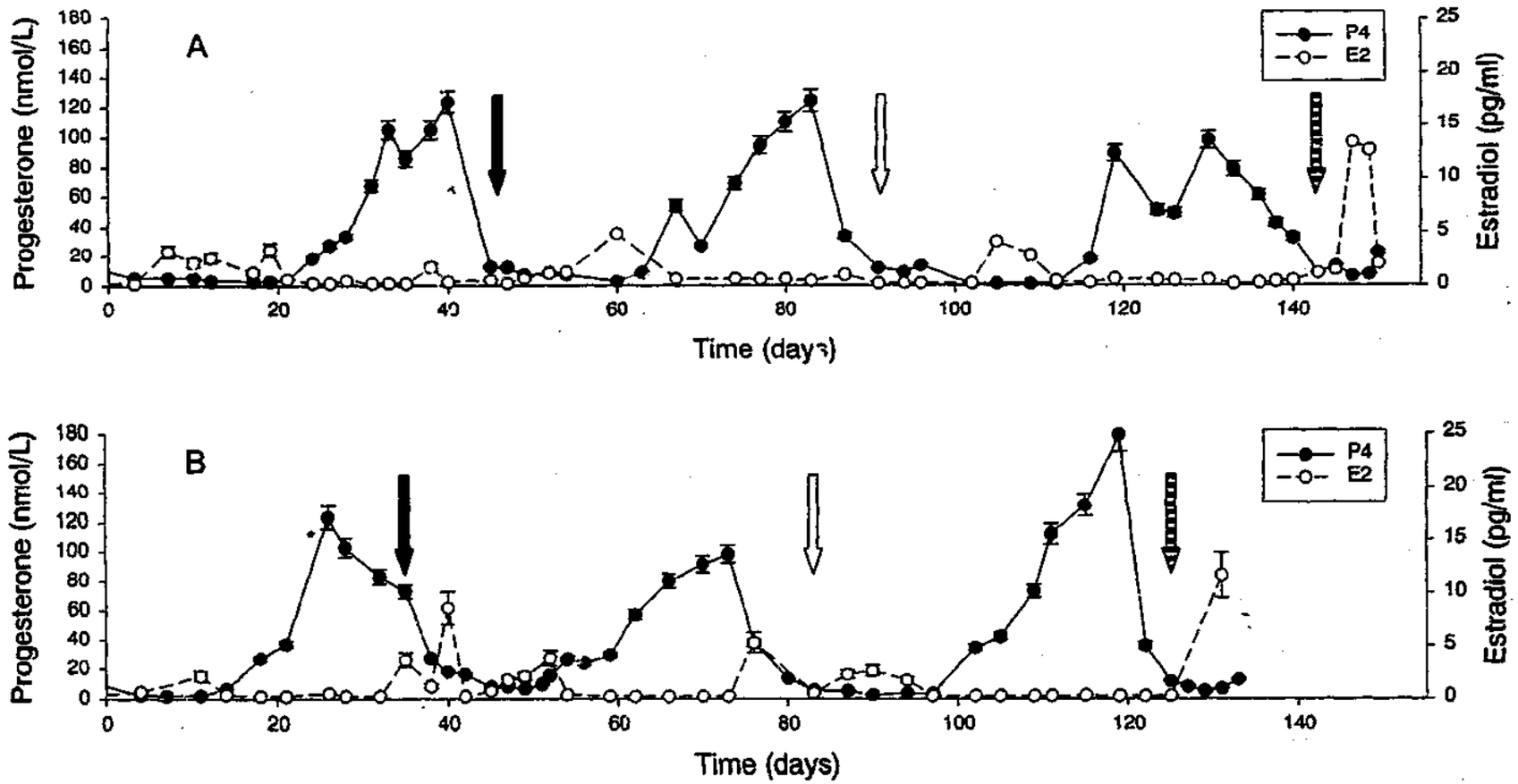


Figure 5.3. Progesterone (P4) and estradiol (E2) profiles of Common Wombats. Natural cycle prior to 1st superovulation treatment, stimulated cycle immediately following superovulation treatment, subsequent cycle (after stimulated cycle), and 2nd superovulation treatment where elevated progesterone (>6.9nmol/L) respectively indicates the luteal phases of each cycle. Day 1 is the firsts day of the follicular phase of natural cycle. The solid black arrows indicate the start of the pFSH/pLH treatment, the open arrow indicates the start of the subsequent cycle and the hatched arrow indicates the start of the 2nd pFSH/pLH treatment. Error bars indicate the within assay variation.

Table 5.4. Change in vaginal cytology during the superovulation treatments in wombats. Vaginal smears samples were collected at day 1 (1st FSH injection), day 3 (5th FSH injection), day 5 (LH injection) and day 6 (time of ovary harvest). Minus (-) = superficial type cells represented ≤ 46% of the epithelial cell population. Plus (+) = superficial type cells represented > 46% of the epithelial cell population.

Wombat	Vaginal Cytology				No. of MII oocytes
	Day 1	Day 3	Day 5	Day 6	
Adult cycling wombats – first superovulation and attempted in situ recovery					
6 (a)	+	+	+	+	φ
13 (a)	*	-	*	+	φ
Adult cycling wombats – ex situ recovery					
6 (b)	-	-	+	+	2
13 (b)	-	+	+	+	5
14	-	+	+	+	3
Subadults – ex situ recovery					
7	-	-	+	+	-
68	-	-	-	-	-
70	-	-	-	+	-
Adult anoestrous – pouch young removed					
62	-	-	+	+	9
63	-	+	+	+	11
67	-	-	+	+	3
Adult anoestrous – recently weaned young-at-foot or unexplained anoestrous					
64	+	-	+	+	-
69	-	-	-	-	-

(a) 1st superovulation attempt; (b) 2nd superovulation attempt
* samples not collected
φ failed to recover oocytes using *in situ* technique

5.3.5 Vaginal changes following superovulation

The vaginal cytology changes of the stimulated cycle and the subsequent cycle followed a general pattern comprising a phase of high superficial type cells (high cytology) followed by a phase of low superficial type cells (basal cytology). This same general pattern of change was observed during the previous natural oestrous cycles of these two wombats in which superovulation was repeated (wombat 6 and 13). FSH/LH stimulation, therefore, did not appear to have an effect on the general

cytological changes that occurred during the cycles that followed superovulation treatment. Wombats with a high percentage of superficial type cells, did not have a decline in the percentage, so long as estradiol concentrations were above basal levels.

The stimulated and subsequent cycle lengths were not significantly different (P>0.05) when compared to the natural oestrous cycle length of each wombat (Section; 3.3.3; Figure 5.4).

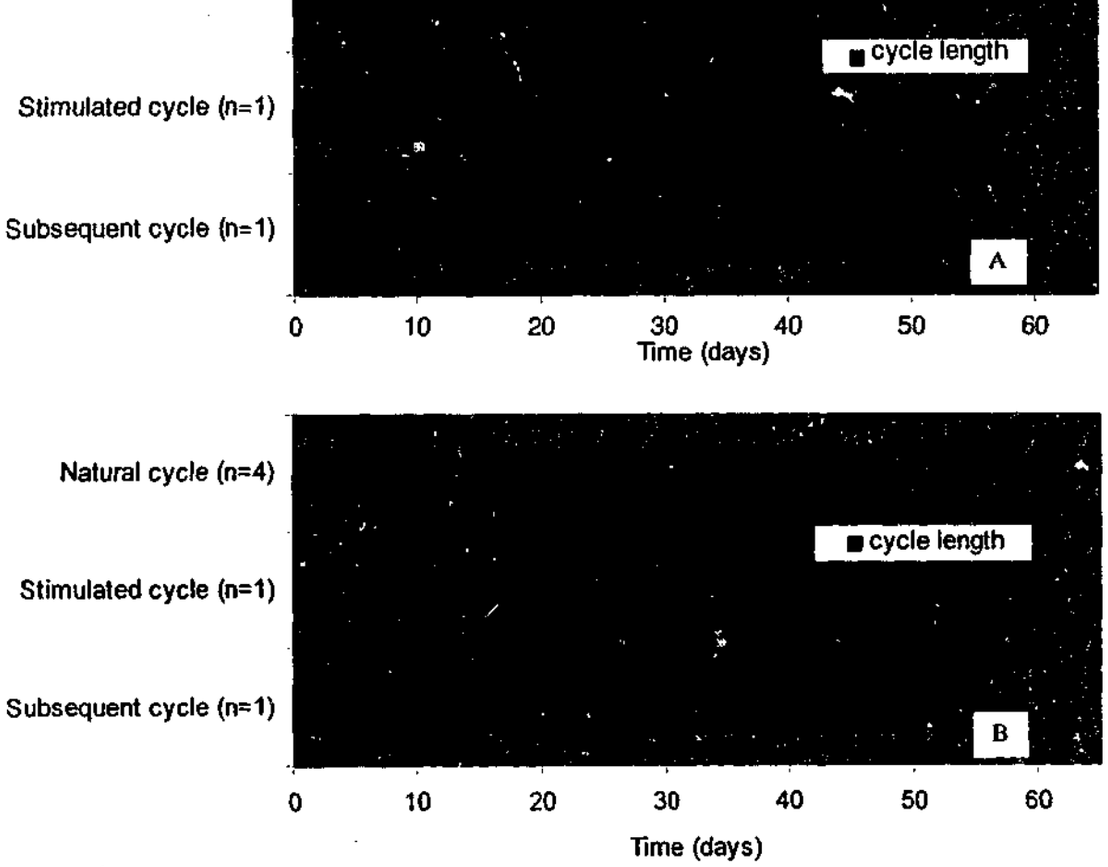


Figure 5.4. Cycle lengths, defined by vaginal cytology, determined during the natural cycles (n=4), a stimulated cycle when FSH/LH was administered (n=1) and the subsequent oestrous cycle (n=1) that followed the stimulated cycle. A – Wombat 6; B- Wombat 13. Error bars show the range of each cycle length.

5.3.6 Morphological changes during and following superovulation treatment

Exogenous hormone administration did not have a significant effect on the pouch condition of cycling adults and most anoestrous adults (Table 5.5). Increased secretion in the pouch of an anoestrous adult, and two subadults (wombat 64, 7 and 70) occurred by day 5 or 6 during the superovulation treatment period. No change in pouch condition was observed in the only subadult wombat (wombat 68), that did not have follicular growth (≥1mm).

The condition of the urogenital opening changed in all subadult, cycling adult and adults following pouch young removal, over the superovulation treatment period (Table 5.6). Generally, the urogenital opening became more protruded by day 3 or 5, than observed in the period monitored prior to hormone stimulation. On day 6 the urogenital opening of all subadults was observed to become less protruded.

This general urogenital opening pattern was observed in all but the two anoestrous adult wombats. In one anoestrous adult (wombat 64), that had recently weaned a young wombat, the urogenital opening remained inverted until day 6 when it became protruded. No MII oocytes were recovered from this animal, however, large follicles were found that contained oocytes that could be successfully matured *in vitro*. No significant change was observed in the urogenital opening of the other anoestrous female (wombat 69). This animal had an abnormally enlarged, but not inflamed, urogenital opening throughout the entire monitoring period (4 months).

The general pattern of change of the urogenital opening resembled the pattern of change during the natural oestrous cycle.

Table 5.5. Pouch changes during 13 superovulation attempts of 11 wombats.

Wombat	Pouch changes during superovulation							
	cleanliness				moistness			
	Day 1	Day 3	Day 5	Day 6	Day 1	Day 3	Day 5	Day 6
Adult cycling wombats – first superovulation and attempted in situ recovery								
6 (a)	+	+	++	+	xxx	xx	xx	xxx
13 (a)	-	+++	-	+++	-	x	-	xx
Adult cycling wombats – ex situ recovery								
6 (b)	+++	++	++	++	xxx	xxx	xxx	xxx
13 (b)	++	+	++	+	xx	xx	x	x
14	+	o	+	+	xxx	xxx	xxx	xx
Subadults – ex situ recovery								
7	o	o	o	+	o	o	o	o
68	o	o	o	o	o	o	o	o
70	o	o	o	o	o	o	x	x
Adult anoestrous – pouch young removed								
62	+	+	+	+	x	x	o	x
63	+	+	+	+	x	x	x	x
67	o	o	o	o	x	x	x	x
Adult anoestrous – recently weaned young-at-foot or unexplained anoestrous								
64	o	o	+	++	xx	xxx	xxx	xxx
69	++	++	++	++	xx	xx	xx	x

(a) 1st superovulation attempt; (b) 2nd superovulation attempt
o = clean, + = mildly dirty, ++ = moderately dirty and +++ = very dirty
o = dry, x = mildly moist, xx = moderately moist and xxx = very moist.

Table 5.6. Urogenital opening changes during 13 superovulation attempts of 11 wombats.

Wombat	Urogenital opening changes during superovulation							
	protrusion				Dry/moist/mucous			
	Day 1	Day 3	Day 5	Day 6	Day 1	Day 3	Day 5	Day 6
Adult cycling wombats – first superovulation and attempted in situ recovery								
6 (a)	o	+	++	++	x	x	x	xx
13 (a)	-	+++	-	+++	x	x	x	x
Adult cycling wombats – ex situ recovery								
6 (b)	oo	++	++	++	x	x	x	x
13 (b)	+	++	+++	+	o	o	o	x
14	o	++	+	+	o	x	x	xx
Subadults – ex situ recovery								
7	o	+	++	+	o	o	o	o
68	o	o	+	++	o	o	o	o
70	o	o	+	o	x	x	o	x
Adult anoestrous – pouch young removed								
62	o	+	++	o	x	x	x	x
63	o	+	++	+	o	x	x	x
67	oo	o	++	+	x	x	o	o
Adult anoestrous – recently weaned young-at-foot or unexplained anoestrous								
64	o	o	o	++	o	x	o	xx
69	E	E	E	E	x	x	x	x

(a) 1st superovulation attempt; (b) 2nd superovulation attempt
E=enlarged, oo =very inverted, o =inverted, + =mildly protruded, ++ =moderately protruded,+++ =very protruded
o =dry,x = moist, xx = mucous

5.4 Discussion

In previous superovulation attempts in marsupials, the main assessment criterion of an animal's response to exogenous hormones has been the number of oocytes that ovulate, and the extent of follicular recruitment (Rodger and Mate 1988; Hinds *et al* 1996; Glazier and Molinia 1998, Hickford *et al* 2001). Previous studies have also considered the effect of superovulation on behaviour, and the ability to fertilize the resultant ovulated oocytes (Renfree *et al* 1988; Rodger *et al* 1992; Rodger *et al* 1993; Molinia *et al* 1998b; Jungnickle *et al* 2000; Jungnickle and Hinds 2000; Hickford *et al* 2001). Many additional questions remain to be answered; including what effect

exogenous hormones have on the animal's hormonal milieu (Mate *et al* 1998) although increasing data is now being published to answer this question (Fletcher 1983; Renfree *et al* 1988; Rodger *et al* 1993; Jungnickel and Hinds 2000). We have found that pFSH/pLH treatment changes the progesterone and estradiol concentrations in the peripheral circulation of the Common Wombat and similarly changes also occur in the vaginal cytology and urogenital opening morphology.

5.4.1 Estradiol profiles

High estradiol concentrations were generally found to indicate follicular maturation and production of MII oocytes. *In vitro* research in Common Brushtail Possums indicates that high concentrations of estradiol are secreted from FSH stimulated follicles (McLeod *et al* 1999). This is also confirmed by *in vivo* studies of the Tammar Wallaby showing that the large antral follicles are the main source of estradiol (Harder *et al* 1985).

MII oocytes were collected from wombats with high estradiol concentrations ≥ 4.66pg/ml on day 3. Furthermore, it is likely that MII oocytes could have been collected from all wombats that had estradiol concentrations, on day 3, at or above the natural elevated estradiol concentrations (~2.11pg/ml) observed during the follicular phase of cycling wombats (Section 3.3.2). MII oocytes were not collected from two wombats with elevated estradiol concentrations on day 3 and this may have been a artefact of the *in situ* recovery technique. Alternatively these wombats may not have produced MII oocytes. Despite this, estradiol concentrations may be used to indicate follicular growth.

Peak estradiol concentrations during the natural oestrous cycle may be later found to be similar to the peak estradiol concentrations (11.23pg/ml) observed during this study. Failure to detect such concentrations during the natural oestrous cycle may therefore have been an artefact of the sampling technique, as samples were collected at intervals of 1 – 4 days (Section 2.2). Sampling during the stimulation period was more intensive, so that blood was collected on days 1, 3, 5 and 6. A more thorough investigation of estradiol concentrations, during the late follicular phase of the natural oestrous cycle is required.

Other studies have suggested that estradiol concentrations during treatment are normally similar to that observed during the natural follicular period of Tammar Wallabies (Rodger *et al* 1993). Significantly higher concentrations of estradiol detected during superovulation treatment were suggested to be indicative of over-stimulation (Renfree *et al* 1988; Jungnickel and Hinds 2000).

Evidence that follicular growth was still occurring at the time of oocyte harvest was found in two wombats (wombat 7 and 69). Estradiol concentrations were apparently still increasing between days 5 and 6 (Table 5.2). Monitoring of estradiol concentrations may therefore be a useful guide to the length of superovulation treatment and adjustments needed for different individuals. Estradiol concentrations may also indicate that we should not attempt to try to collect oocytes from wombats that have low estradiol concentrations. During this study, no MII oocytes were collected from wombats with basal estradiol concentrations. Further studies are therefore required to examine different administration periods, times of MII oocyte development and correlations with estradiol concentrations.

Prediction of the time of ovulation, in response to FSH treatment, would also be more accurately determined by monitoring LH concentrations. Studies have investigated the timing of ovulation in relation to the LH peak for the Tammar Wallaby (Tyndale-Biscoe *et al* 1983; Harder *et al* 1985). However, the timing of ovulation is poorly understood in most marsupials (Hinds *et al* 1996) and as yet no study has investigated LH in Common Wombats. Both LH and FSH circulating concentrations of FSH/LH stimulated Tammar Wallabies were found to be within the normal ranges of those detected during the natural oestrous cycle (Jungnickel and Hinds 2000). A peak in plasma LH concentrations was observed 2 hours after pLH injection, and then slowly returned to basal level by 12-14 hours after injection. Molinia *et al* (1998b) found that ovulation of FSH/LH treated Tammar Wallabies occurs ~36 hours after the LH injection. Further studies are required to investigate estradiol concentrations in the Common Wombat, to verify the RIA, however the general trend suggests that estradiol concentrations increases in response to pFSH/PLH treatment in most wombats. The factors inhibiting response to treatment also need to be examined.

5.4.2 Progesterone profiles

Progesterone concentrations of cycling wombats were found to increase earlier than has previously been observed during the natural oestrous cycle of Common Wombats. An overlap (of 1 – 3 days) was observed so that progesterone increased in superovulation attempts of 4 cycling wombats prior to the time of estradiol decline. In contrast, progesterone concentrations in naturally cycling wombats, increased above basal levels, (3 – 7.5 days) after the return of basal estradiol concentrations at the end of the follicular phase (Section 3.3.1). In all other wombats progesterone concentrations had not increased above basal concentrations before ovarian harvest. In contrast Jungnickel and Hinds (2000) found that the time of the increase in progesterone concentrations of FSH/LH treated Tammar Wallabies (4 - 5 days after ovulation) was similar to observations during the Tammar's natural oestrous cycle.

The differences observed in the progesterone concentrations between adult cycling wombats and adult anoestrous wombats, that had their pouch young removed, suggest that the superovulation protocol was more suited to the latter group. This is because the high progesterone levels at the time of oocyte recovery indicated that luteolysis of follicular cells had begun and progesterone was being produced. In superovulated cows, a similar increase in progesterone concentrations at the time of estradiol decrease has been suggested to indicate initiation of the luteinization process (Kemper Green *et al* 1996). In marsupials, progesterone secretion has been most thoroughly studied in the Tammar Wallaby, and it has been demonstrated that the corpus luteum is clearly the main source of progesterone (Findlay *et al* 1983).

Basal progesterone concentrations during most (61.5%; n=13) superovulation attempts suggested that luteolysis had not yet begun. Of these, corpora haemorrhagica were found in 4 wombats (1 anoestrous adult, 3 subadults) at day 6, suggesting that ovulation may have already occurred. However, the basal progesterone concentrations detected suggested the corpora haemorrhagica had not yet been invaded by luteal cells that had started to secrete progesterone. This may indicate that corpora haemorrhagica had recently formed compared to other wombats that had higher progesterone concentrations.

There were 5 types of structures on the ovaries by day 6: (i) large clear antral follicles that had not ovulated, (ii) large non-ovulated follicles in which the fluid was blood tinged that had ovulated and had a small pinpoint hole in the follicle wall, (iii) corpora haemorrhagica, (iv) corpora lutea and (v) corpora albicantia (Sections 4.3.2; 4.3.5).

Progesterone concentrations did not increase during the treatment period in the adults that had pouch young removed. The higher numbers of MII oocytes collected from these adults suggests that ovulation may have occurred soon after the ovarian harvest if ovaries had been left *in situ*. This is supported by the ovulation of one follicle and the two recent ovulation points observed on day 6, at the time of ovarian harvest. Ovulation was also observed of one follicle from a cycling female wombat (wombat 14), that did not have an increase in progesterone concentrations prior to harvest.

A dichotomy has arisen in this study during 5 superovulation attempts. Progesterone concentrations and the presence of corpora haemorrhagica suggested ovulation had occurred prior to the time of ovary collection. Estradiol concentrations and the presence of large antral follicles suggested ovulation was about to occur at the time of collection.

How do we then suggest what the appropriate collection time should have been? The trouble being that not all corpora haemorrhagica were considered to be a sign of actual ovulation. Oocytes were observed from some of these structures at aspiration, and potentially ovulated oocytes were not recovered from the oviducts. These observations prevented the determination that ovulation had actually occurred. However ovulation prior to collection is strongly indicated by the early rise in progesterone concentrations, which is likely to have been secreted from recently formed luteal cells. On the other hand, MII oocytes could be collected from antral follicles at the time of ovarian harvest, indicating that some follicles had matured towards a preovulatory stage.

The best time for harvest is likely to be when the follicles are just about to rupture. Studies in other marsupials, including the Common Brushtail Possum and the Tammar Wallaby found that that oocyte maturation continues into the period immediately prior to ovulation (Rodger *et al* 1992a). Determination of the peak

estradiol levels associated with pre-ovulatory follicular growth would be one way of defining a likely optimal oocyte harvest time. Conversely, determination of the time of an increase in progesterone concentrations may indicate that some ovulation had already occurred.

The results could suggest that there were two waves of follicular recruitment. The first wave is likely to have induced further development of the most developed follicles, potentially the medium to large follicles, which already existed on the ovaries. Based on the study by Moritz *et al* 1998, medium to large follicles were considered to be >1mm follicles but ≤4mm. It seems that their growth to a pre-ovulatory stage may have been accommodated, within the 4-day period of FSH stimulation or around the time of LH administration. The second wave of follicular growth may have been induced in smaller follicles (≤1mm), that did not reach preovulatory stage until after the LH injection, at around the time of ovarian harvest.

Although follicular waves have not yet been studied in marsupials, the occurrence of follicular waves is well documented in eutherian species. Sirois and Fortune (1988) found that approximately 3 follicular waves occur in cows and that in each wave a single follicle becomes dominant whilst other follicles regress. A similar event is likely to occur in marsupials. Follicular growth is not restricted to the follicular phase of Common Wombat and instead is on going at all reproductive stages (Moritz *et al* 1998), indicating that natural recruitment is likely to occur in waves. Studies in other marsupial species indicates that the final stimulus for follicle development is inhibited by a lack of gonadotrophin stimulus, in most reproductive stages, except during the follicular phase of the oestrous cycle, when the dominant follicle(s) ovulates (Tyndale-Biscoe and Renfree 1987).

Treatment with exogenous FSH/LH has increased the number of follicles developing towards a pre-ovulatory stage, than has been previously documented for unstimulated Common Wombats (Moritz *et al* 1998). In addition, in some wombats, more than one group of follicles has reached this stage. Similar observations have been made in more thoroughly studied species such as the cow by repeated ultrasound (Kaneko *et al* 1992).

MII oocytes were collected from stimulated cycling wombats irrespective of their progesterone concentrations at the initiation of superovulation treatment. This suggests that we may not have needed to wait until progesterone concentrations had returned to basal levels to begin treatment.

Other studies in Marsupials have considered the reproductive status of an animal, rather than the specific differences in endocrine levels at the initiation of stimulation. Those studies have shown the response of an animal is dependent on both reproductive status and the hormones administered (Molinia *et al* 1998b; Hickford *et al* 2001) and additionally the individuals response to treatment (McLeod *et al* 1999). The variation in progesterone concentrations observed throughout the superovulation period, indicates that a number of additional factors, other than the hormonal milieu, are likely to effect an animals response.

5.4.3 Hormonal changes following superovulation

This is the first study, that we are aware of, that has investigated the endocrine response of a marsupial for an extended period following superovulation treatment. Jungnickel and Hinds (2000) did examine the response of Tammar Wallabies for up to 5 days following pFSH/pLH treatment. Although our study was limited to two wombats, the length of the oestrous cycle does not appear to be significantly affected by pFSH/pLH administration. This suggests that at the endocrine level, superovulation treatment is not detrimental to Common Wombats. Particularly as a normal oestrous cycle length was detected subsequent to the stimulated cycle. As for reasons already explained, we cannot conclude if the estradiol concentrations observed during this study are significantly different to the peak estradiol concentrations of a natural cycle. The only definitive difference between the endocrine responses was the overlap of estradiol decrease and progesterone increase observed during the stimulated cycle.

In theory, it seems likely then that superovulation treatment may be repetitively administered to Common Wombats. An interval, of the duration of a luteal phase following pFSH/pLH administration and the subsequent cycle, may be required between superovulation attempts. This is supported by the results, which indicated

that MII oocytes could be collected on the second superovulation attempt, when this interval was allowed. An efficient collection or aspiration technique that does not require the removal of the ovaries still needs to be developed. Repeated superovulation has been successful in many eutherian species including cows (Kafi *et al* 1997) and sheep (Forcada *et al* 2000). In sheep, response to repetitive superovulation treatments is improved by allowing an appropriate interval between treatment periods (Boland and Gordon 1982).

5.4.4 Vaginal changes during and following superovulation

Vaginal cytology indicates the response of the vaginal epithelium to hormonal changes. Not all wombats responded by increased maturation of epithelial cells, in response to elevated estradiol concentrations. Estradiol receptors are found throughout the reproductive tract of the Common Brushtail Possum (Young and McDonald 1982) and the Tammar Wallaby (Renfree and Blanden 2000). It is known in both these species that, when comparing studies of vaginal cytology and estradiol concentrations, changes in vaginal cytology are associated with an increase in estradiol concentrations (Pilton and Sharman 1962; Shaw and Renfree 1984; Curlewis *et al* 1985; Harder *et al* 1985; Poole *et al* 1992). There was a lag time of at least 2 days between the appearance of high estradiol concentrations and the change to a high percentage of superficial epithelial cells.

As variation in the percentage of superficial type cells was observed during the natural oestrous cycle it is not surprising that variation has also been found in the superovulated wombats. The response of the vaginal epithelium did not infer the ovarian response. However a similar pattern of change has been observed during the superovulation treatment period to that observed during a natural follicular phase (Section 3.4.3). Porcine FSH and LH administration therefore appears to have induced "normal" vaginal cytology changes. Hickford *et al* (2001) has also recently reported that changes observed in the urine cytology of Stripe-faced Dunnarts resembles the changes observed in natural oestrous cycles.

Fletcher (1983) reported that the urine cytology also changed in stimulated Kowaris, however this change did not infer that the animals would mate or were capable of producing litters. Similar changes in urine cytology were made whilst studying the

Fat-tailed Dunnart's in response to PMSG/hCG treatment, although ovulated oocytes were retained in the reproductive tract for unusually long periods of time. It is therefore difficult to allude to how the observed changes at the cytological level of the vagina, relate to the changes that may be occurring elsewhere in the reproductive tract or how behaviour may be affected. The reproductive tract has been demonstrated to support fertilisation following mating (Renfree 1988; Jungnickel and Hinds 2000;) or artificial insemination (Molinia *et al* 1998a, Jungnickel *et al* 2000) in other marsupials including the Tammar Wallaby and Common Brushtail Possum. Although so far, only one marsupial, the Fat-tailed Dunnart, has been found to be capable of producing live young following mating (Rodger *et al* 1992b). Further assessment of the normality of the reproductive tract (by histological examination) and behaviour of pFSH/pLH treated wombats is required.

Results suggested that, the vaginal cytology changes observed following pFSH/pLH administration during the stimulated cycle and the subsequent cycle resembled changes observed during the previous natural oestrous cycles examined. Studies in other marsupials have not yet reported vaginal cytology after superovulation treatment.

5.4.5 Morphological changes during and following superovulation treatment

Porcine FSH/LH administration appears to have affected the urogenital opening of most wombats that produced multiple large antral follicles in response to superovulation treatment. Changes were not observed in the urogenital opening of the two anoestrous adults. In the animals that did respond, there was a lag time between the start of the superovulation regimen and the morphological change in the urogenital opening. Changes that resembled the normal cycle were observed by day 3 or 5 of the superovulation regimen. Although some changes in the pouch were observed, that are likely to have been induced by the superovulation regime, most wombats did not differ significantly from the pouch condition observed prior to treatment. Changes in pouch condition that were similar to those observed naturally during pre-parturition, have been reported for the Kowari in response to PMSG/hCG treatment. Normal changes, similar to those observed during the natural oestrous cycle, were also observed in the pouch and urogenital opening during the stimulated and subsequent cycles following the 1st superovulation attempt in two wombats.

5.5 Conclusion

Changes in progesterone and estradiol concentrations, and vaginal cytology all provide an indication of the success of the pFSH/pLH treatment. This study suggests that pFSH/pLH superovulation regimens may need to be tailored for the individual Common Wombat. Monitoring such parameters as progesterone and estradiol concentrations will allow determination of the required duration of treatment. Some ovulation may occur prior to the pLH injection, and may be induced by repetitive pFSH administration. The duration of pFSH stimulation may have insufficient to induce follicular and oocyte maturation in the subadults and some anoestrous adults. A shorter duration of pFSH stimulation may be sufficient to induce follicular growth and oocyte maturation in other wombats, such as the cycling adults in the follicular phase.

Further studies should investigate, circulating pFSH and pLH concentrations and examine ovarian histology to provide further insight into the treatment regimen and the timing of collection. Variation in doses of pFSH and pLH should also be considered and compared with the associated changes of the hormonal milieu.

This study has not investigated behavioural changes, including oestrus. Normal changes were observed in vagina cytology and the urogenital opening in response to pFSH/pLH treatment, and may allude to other normal changes else where in the reproductive tract. Further studies are required to assess the ability of the reproductive tract to support fertilisation of superovulated oocytes.

Chapter 6

In vitro maturation (IVM) and intracytoplasmic sperm injection (ICSI) of oocytes collected following hormonal ovarian stimulation

6.1 Introduction

Many methods of assisted reproduction require a protocol to increase the production of MII oocytes collected from ovaries and to assess the viability of MII oocytes produced following superovulation treatment or *in vitro* maturation (IVM). Immature oocytes and superovulated oocytes were collected after hormonal treatment (Chapter 4). The studies in this chapter investigate IVM of oocytes from pFSH/pLH primed wombats and the fertilisation, culture and development of *in vitro* and *in vivo* derived metaphase II (MII) oocytes following intracytoplasmic sperm injection (ICSI).

6.2 Materials and Methods

6.2.1 Animal Use and Oocyte collection

Oocytes were aspirated from the follicles from 13 superovulation attempts of 11 wild caught female Common Wombats as previously outlined in Sections 2.1.1 and 2.4.

6.2.2 Experimental Design

All oocytes were assessed following superovulation using brightfield microscopy (Section 2.4.9). ICSI was performed on MII oocytes 2 - 4hrs following recovery. All immature oocytes were transferred to TCM 199 maturation media and examined at 6 - 8hr intervals. ICSI was performed on MII oocytes collected from superovulated ovaries and on the resultant MII oocytes after 24hrs IVM. Injected oocytes were examined using brightfield microscopy at 6 - 12hr intervals for signs of degeneration, fertilisation or cleavage after 66 - 114hrs in culture. Oocytes were then stained with DNA Hoechst or DAPI and examined using fluorescence microscopy to determine their nuclear status.

6.2.3 *In vitro* maturation

Oocytes were recovered from follicles aspirated with TCM 199 aspiration media (Section 2.4.8; Appendix 5). After a brief examination, oocytes were transferred to TCM 199 maturation media (Section 2.4.9; Appendix 5) for IVM.

All immature oocytes were maintained in vitro in TCM 199 maturation media (Appendix 5) at 35°C and 5% CO_2 in air. Oocytes were examined under a light microscope at 6 – 8 hr intervals (Section 2.4.9). Cumulus cells were attempted to be removed at 0, 12, 24, 30, and 48 hours after collection by gentle pipetting through a finely pulled pipette to determine maturation status. MII oocytes identified following 24 hours IVM underwent ICSI. All remaining immature oocytes were stained using the DNA-specific dye Hoechst 333432 48 to 96 hours after collection.

ICSI was performed on all MII oocytes in vitro matured for 24hrs. After 48 – 90 hrs IVM immature oocytes and oocytes that had matured to MII during this time were fixed and stained (Sections 6.2.9 and 6.2.10).

6.2.4 Sperm collection and preparation for ICSI

Spermatozoa were collected from the epididymis of six culled male wombats from the Myrtleford area of Victoria and cryopreserved in 0.2M Trehalose and 10% glycerol or 0.3M Trehalose and 8% glycerol, and stored at -196°C by Bickell (2001). Straws were removed from liquid nitrogen, exposed to room temperature for 10 seconds, plunged into a 35°C water bath for 30 seconds then opened into a sperm Talp solution and gently mixed (Appendix 6) as described by Bickell (2001). The sperm solution was then maintained at 35°C until ICSI..

6.2.5 ICSI

Intracytoplasmic sperm injection was performed using an Leica inverted microscope with horfman optics and left and right micromanipulators to operate holding and injection pipettes respectively. Mature oocytes (MII) were placed in a 40µl droplet of TCM 199

aspiration medium (Appendix 5) under embryo-tested oil in a micromanipulation chamber. Prepared wombat spermatozoa were placed in a second 40µl droplet of Hepes buffered TCM medium supplemented with 10% FCS in the micromanipulation chamber. From this second droplet, a motile spermatozoon was then randomly selected and aspirated, tail first, into a glass microinjection pipette with an inner diameter of 5 – 7 µm. The injection pipette was then moved to the droplet containing the oocytes. An oocyte was then picked up by a rounded glass holding pipette. The zona pellucida was then drilled by piezo pulses (using a Piezo-Drill; Burleigh Instruments) allowing the injection pipette to pass through the zona into the perivitelline space and then into the oocyte cytoplasm. The sperm was then gently injected into the ooplasm and the injection pipette withdrawn from the oocyte. This process was repeated for each MII oocyte.

After ICSI, injected oocytes were then transferred to TCM maturation media for culture.

6.2.6 Culture of injected oocytes

After ICSI, injected oocytes were transferred into a 4 well dish containing TCM 199 maturation media (Appendix 5) and placed in an incubator at 35°C and 5%CO_2. Oocytes were then checked at intervals of 6 to 8 h. Injected oocytes from wombats 6(b), 63, 64, 67, and 70 were fixed (Section 6.2.7) and stained with DAPI (Section 6.2.8) 48 –96h after ICSI to examine the nuclear development. All other injected oocytes were fixed (Section 6.2.9) and stained with DNA Hoechst (Section 6.2.10).

6.2.7 Chromosome fixation of injected oocytes for DAPI staining

A sterile hypotonic solution comprising 0.25g of tri-sodium citrate (Sigma) in 30ml of embryo tested tissue culture water (CSL) was made in a 50ml Falcon tube (Becton Dickinson). Bovine serum albumin (BSA) (30mg/ml) was added to the 50ml Falcon tube and the volume increased to 50ml with embryo tested water (CSL). A fixative solution was then made by combining 6ml Glacial Acetic Acid (AnalaR) and 3ml Anhydrous Methanol (AnalaR).

After 42 - 90hrs in culture, injected oocytes were transferred to 300µl of the hypotonic solution in a 4-well dish for 40 minutes at room temperature. Using a glass pipette, each oocyte was individually placed onto a glass slide with a minimum amount of hypotonic solution. Under a light microscope, the hypotonic solution was observed until it evaporated from around the oocyte. Just as the hypotonic solution finally evaporated a single drop of fixative solution was dropped onto the oocyte on the slide. The drop of fixative was observed until it evaporated when another drop of fixative was applied in the same manner. After a number of drops of fixative (usually 3 - 6) were added the oocyte swelled and then gently burst, spreading the ooplasm and nucleus onto the slide. The fixative was allowed to dry, in preparation for DAPI staining.

6.2.8 DAPI Staining

Chromosome fixed injected oocytes (Section 6.2.7) were stained with DAPI (Sigma). A 5µl aliquot of 125ng/ml DAPI in sterile cell culture deionized water (20601; CSL) was dropped onto each oocyte and a cover slip placed on top. The oocytes were then examined under fluorescence microscopy for nuclear staining. The number of pronuclei and nuclei as well as stained polar bodies were examined in injected oocytes and the number of nuclei were counted in cleaving embryos.

6.2.9 Fixation for DNA Hoechst staining

A fixative solution of 3% glutaraldehyde (Electron Microscopy Sciences) in 0.1M cacodylate buffer (Pro Sci Tech) was warmed to 35°C in a water bath. 250µl of the fixative was placed in a 96-well Falcon tissue culture plate (Becton Dickinson) and maintained at 35°C on a warming stage. Immature and mature oocytes were individually transferred with a minimum amount of media into the fixative for 30mins at room temperature and then overnight at 4°C.

6.2.10 DNA Hoechst Staining

Oocytes were removed from fixative and individually transferred, using a glass pipette, into PBS for 5 minutes at room temperature. Oocytes were then transferred into 5µg/ml DNA Hoechst (333432; Sigma) in PBS (Gibco BRL) for 10 minutes. During this time the

oocytes were kept under dark wrapped in foil at room temperature. The oocytes were then washed in PBS and the nuclear status assessed using fluorescence microscopy within 10 minutes after staining. The number of nuclei, pronuclei and polar bodies were counted in injected oocytes.

6.3 Results

6.3.1 IVM of oocytes from pFSH/pLH primed Common Wombats

A total of 246 oocytes were recovered from 13 superovulation attempts in 11 female Common wombats. From these 33 (13.4%) were MII oocytes (Figure 6.1). Eleven oocytes (4.4%) showed signs of degeneration that included a shrunken or irregular ooplasm, a partially or completely absent zona pellucida, and fragmented ooplasm (Figure 6.1). Excluding these degenerating oocytes, a total of 204 immature oocytes that lacked a polar body, or had cumulus cells that could not be removed were recovered. This immature group consisted of 33 cumulus oocyte complexes (COC's), 17 of which had their cumulus cells removed by gentle pipetting prior to IVM. The immature cumulus surrounded (n=16) and naked (n=188) oocytes were then matured *in vitro* (Figure 6.1; Table 6.1).

After 24hrs maturation, 29 (14.2%) of the 204 immature oocytes extruded a polar body. After 30hrs maturation, another 4 (1.9%) immature oocytes had extruded a polar body. Despite maturation for up to 66 and 114hrs no further MII oocytes developed. A total of 33 MII oocytes (16.2%) were produced.

MII oocytes were produced from superovulated wombats in all reproductive stages however, the number of oocytes that progressed to the MII stage, varied between wombats (Table 6.1). The highest number of IVM MII oocytes (11 MII; Table 6.1) was from an adult anoestrous wombat (wombat 63) following pouch young removal. MII oocytes did not develop from the few immature oocytes that were recovered from two immature wombats (wombat 68 and 70) and another adult anoestrous wombat (wombat

62) following pouch young removal. However, when similar numbers of oocytes were collected from an adult anoestrous wombat (wombat 64) that had recently weaned a young wombat, the majority (60%) did extrude a polar body and become MII oocytes.

A greater percentage of MII oocytes developed when matured with cumulus cells attached than without (Table 6.2). 56.3% of the 16 COC's developed to the MII stage after IVM. In contrast, 17.5% of 188 cultured immature naked oocytes developed to MII stage. Only one naked MII oocyte developed when there were no cumulus cells in the maturation media.

Table 6.1. The developmental state of oocytes following follicular aspiration from superovulated wombats.

Wombat	Total No. oocytes/ wombat	No. naked oocytes	No. degenerate oocytes	No. COC's*	No. immature oocytes	# MII oocytes
Adult cycling wombats - First superovulation and attempted in situ recovery						
6 (a)	2	1	1	-	1	-
13 (a)	-	-	-	-	-	-
Adult cycling wombats – ex situ recovery						
6 (b)	19	17	-	2	17	2
13 (b)	6	6	-	-	1	5
14	21	15	-	6	18	3
Subadults – ex situ recovery						
7	48	47	1	-	47	-
68	3	2	-	1	3	-
70	8	6	-	2	8	-
Adult anoestrous – pouch young removed						
62	16	9	2	2	5	9
63	68	52	-	16	57	11
67	26	26	3	-	20	3
Adult anoestrous – newly weaned young at foot or unexplained anoestrous						
64	8	7	1	-	7	-
69	21	13	1	7	20	-

*COC = cumulus oocyte complex

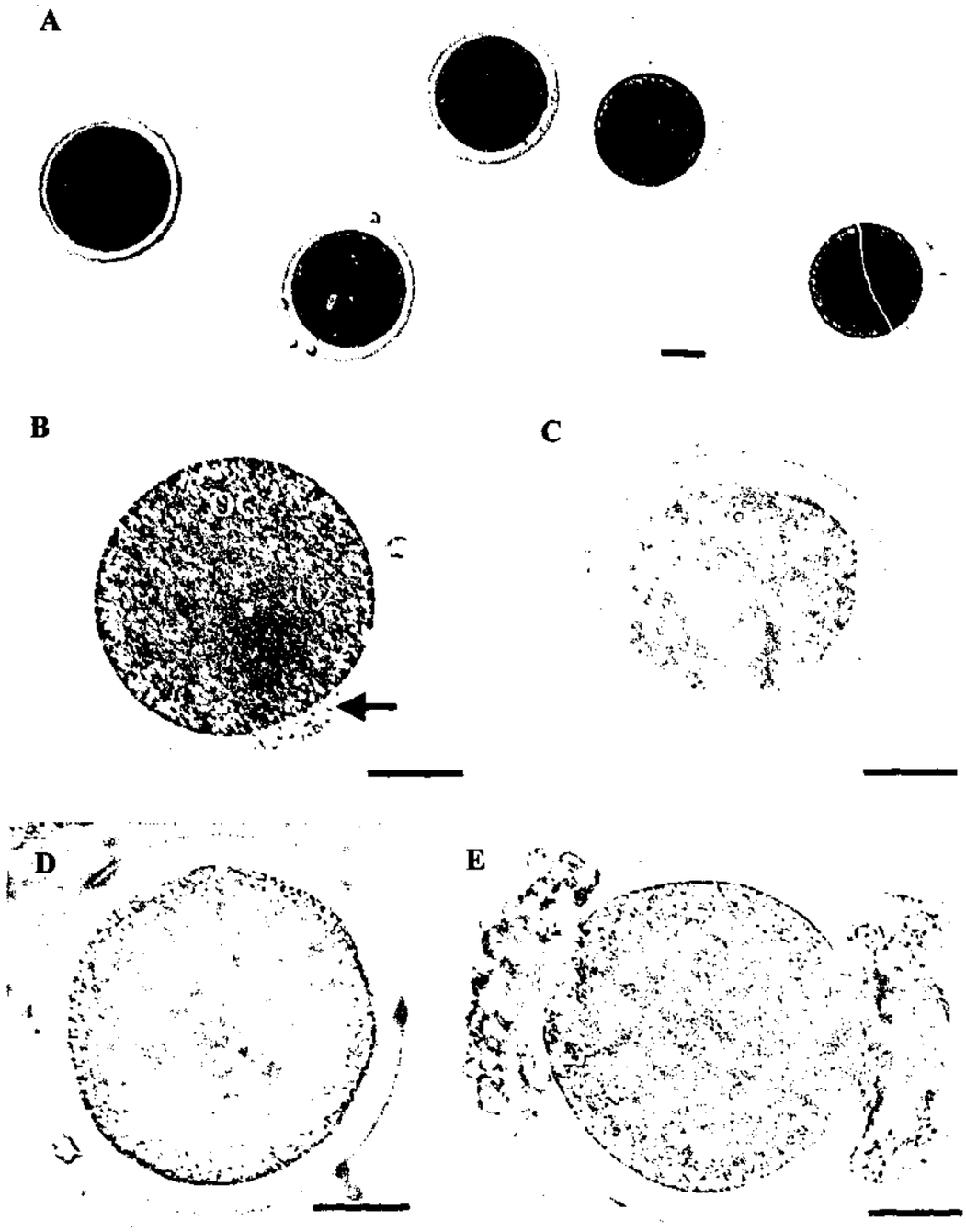


Figure 6.1. Examples of oocytes aspirated from the follicles of pFSH/pLH primed Common Wombats. A: 4 immature oocytes and 1 MII oocyte (MII); B: MII oocyte and the arrow indicates the polar body; C: degenerate oocyte, D: immature oocyte as it lacks a polar body; E: oocyte partially surrounded by attached cumulus cells. ZP denotes zona pellucida, PV denotes perivitelline space, OC denotes the oocyte cytoplasm, CU denotes cumulus cells. Scale bars indicate 50μm.

Table 6.2. The maturation of immature oocytes, with (COC's) or without (naked) cumulus cell attached, after 24 and 30hrs in culture, comparing those that matured to MII status from naked oocytes or COC's.

Wombat	No. naked Immature oocytes in IVM	No. COC's* in IVM	No. MII oocytes after 24hrs	No. MII oocytes after 30hrs	Total No. MII oocytes following IVM	No. MII oocytes developed from COC's
Adult cycling wombats - First superovulation and attempted in situ recovery						
6 (a)	1	-	-	-	-	-
13 (a)	-	-	-	-	-	-
Adult cycling wombats – ex situ recovery						
6 (b)	17	-	1	-	1	-
13 (b)	1	-	-	-	-	-
14	12	6	5		5	2
Subadults – ex situ recovery						
7	47	-	3	-	3	-
68	3	-	-	-	-	-
70	8	-	-	-	-	-
Adult anoestrous – pouch young removed						
62	3	2	-	-	-	0
63	41	16	10	4	14	5
67	20	-	2	-	2	-
Adult anoestrous – newly weaned young at foot or unexplained anoestrous						
64	7	-	3	-	3	-
69	13	7	5 (only 3 successfully injected)	-	5	2

*COC = cumulus oocyte complex

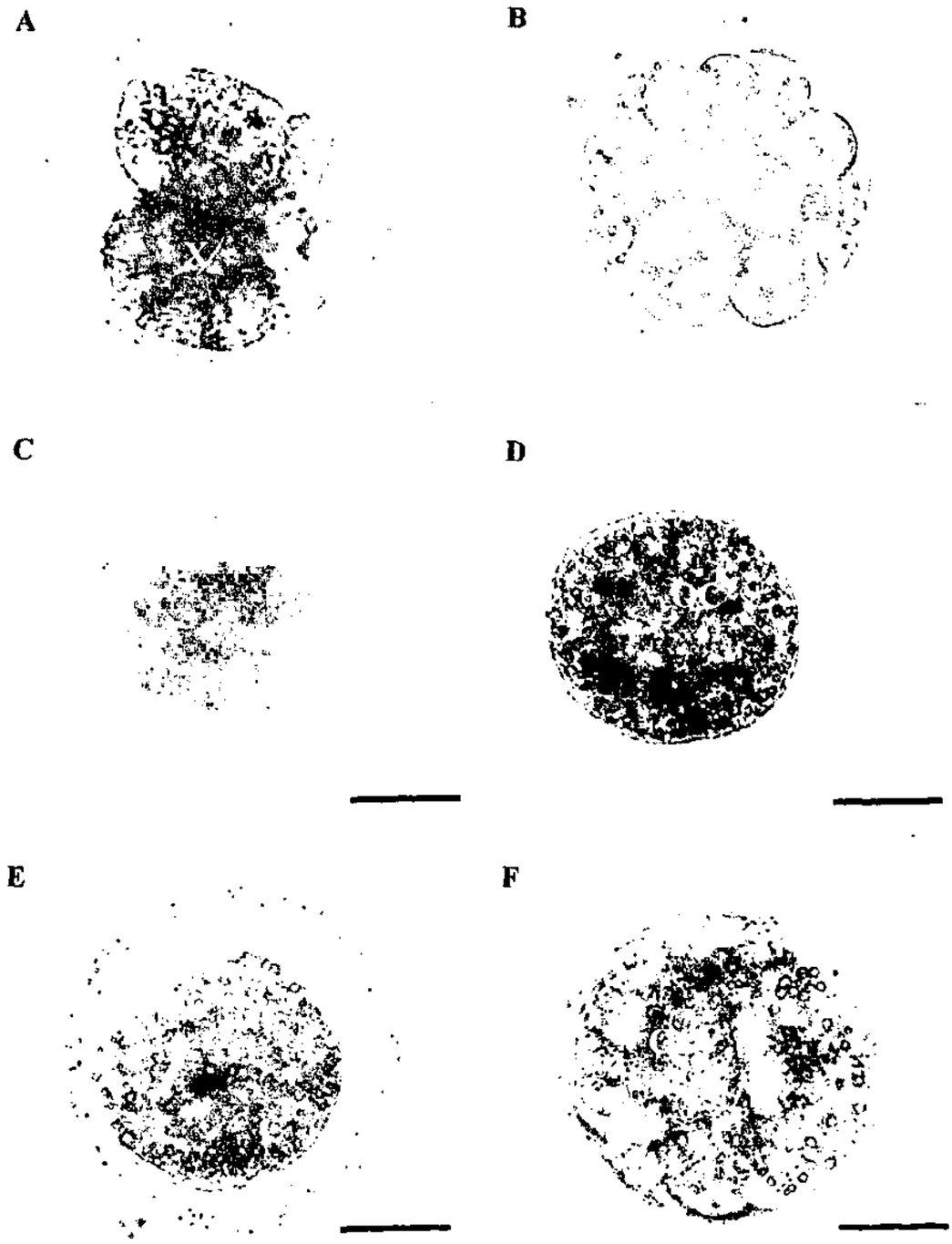


Figure 6.2. Parthenogenetic and degenerating oocytes after 48h in TCM 199 maturation media. A: 2 cell parthenotes. B: 8-16 cell parthenotes and is stained with Hoechst showing that nuclei only occur in two cells. C and E both show show oocytes with irregularly shaped ooplasms and the Ooplasm is also shrunken in E. D: an oocyte with a shrunken ooplasm. F: fragmented oocyte. ZP denotes zona pellucida, PV denotes perivitelline space, OC denote the oocyte cytoplasm, X denotes a cell of the parthenotes. Scale bars = 50μm.

Cellular division, without ICSI, was observed in immature oocytes (1 oocyte at 18 hrs, 6 oocytes at 30hrs and 2 oocytes at 42hrs) in maturation media, from wombats 63, 14 and 70 (respectively). In the absence of sperm injection, or other forms of artificial fertilization this cellular division was considered to be parthenogenetic, and upon staining few nuclei were found (Figure 6.2).

Signs of degeneration of the oocytes included, fragmented ooplasms and the formation of numerous small lipid bodies were identified. Fragmentation and shrinkage of the ooplasm were observed in degenerating oocytes that were kept in culture longer than 24hrs. The formation of lipid bodies was observed in the majority of otherwise normal looking immature oocytes that had been kept in maturation media in excess of 66 to 96hrs (Figure 6.2).

6.3.2 ICSI of MII oocytes collected 24hrs after pFSH/pLH treatment

Following follicular aspiration, a total of 33 oocytes had a polar body and were considered mature MII oocytes of which 30 underwent ICSI. The 3 MII oocytes not injected were found to have an unusual ooplasm that was very pale in appearance and unevenly distributed.

ICSI was successfully performed on 27 MII oocytes (81.8% of 33), which were then transferred to culture media. The first signs of change in injected MII oocytes were detected after 18hrs in culture (Table 6.3), when the ooplasm began to shrink in 3 injected oocytes of two wombats (wombat 14 and 63).

Cellular division was first observed in 3 injected oocytes from one wombat (wombat 13) after 48hrs in culture (Table 6.3). Between 36 and 48 hours in culture, 2 injected oocytes had divided once to form 2 cells, and 1 injected oocyte had divided to form 6 to 8 cells. Development was permitted to proceed until injected oocytes had appeared to have formed approximately 100 cells (Figure 6.3). Three injected oocytes had reached this stage by 72hrs after ICSI and two were then fixed. No change was observed in the 3rd

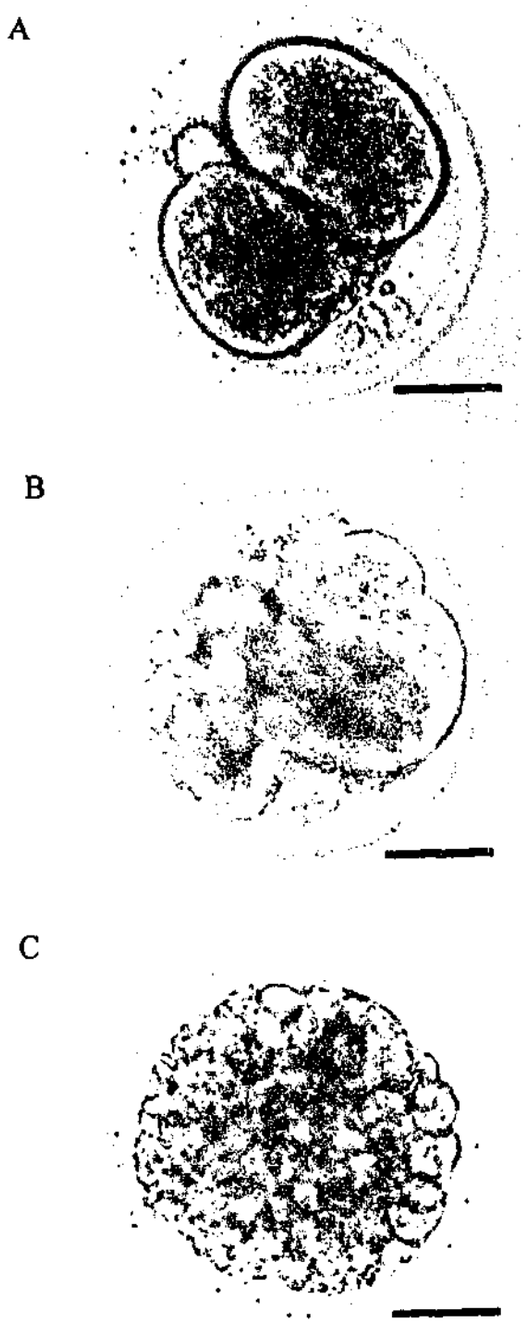


Figure 6.3. Cleavage of Common Wombat oocytes 48 – 65 h after intracytoplasmic sperm injection. A shows cleavage to the 2-cell stage (48 h), B shows cleavage to 16-32 cells or fragmentation, C show cleavage to 100 cells or fragmentation (65 h). ZP denotes the zona pellucida, PV denotes the perivitelline space, X denotes the cytoplasm of one cell. Scale bars = 50μm.

injected oocyte that appeared to have formed 100 cells by 87hrs when it was also fixed (Table 6.3). When the oocytes were stained with DNA Hoechst, some nuclear staining was evident, however it was difficult to ascertain how many cells contained nuclei.

Table 6.3. Developmental appearance of Common Wombat MII oocytes, collected from the 2nd superovulation of wombat 13, following ICSI. Early cleavage was most likely parthenogenetic division and subsequently fragmentation.

Time (hours)	0	12 - 36	48	65	72	87
Developmental Rate	ICSI of 5 MII oocytes	No change	2 x 1 cell 2 x 2 cell 1 x 6-8 cell	1 x 1 cell 3 x ~ 100 cell 1 x fragmented	1 x 1 cell 3 x ~ 100 cell 1 x fragmented	4 x fixed 1 x 100 cell (no change)

Ooplasm fragmentation was observed 65hrs after injection in one injected MII oocyte from wombat 13 collected during the second superovulation attempt and 42hrs after ICSI in 3 injected oocytes from wombat 67 (Figure 6.2). Irregular ooplasm shape was another degenerative change seen in injected oocytes 42hrs after ICSI (Figure 6.2). No evidence of fertilisation was observed in the injected oocytes of other wombats.

6.3.3 ICSI of MII oocytes following IVM

ICSI was performed on 29 MII oocytes following IVM, 22 to 26hrs after collection. Of these 2 MII oocytes (6.9%) had thickened zona that made penetration of the injection pipette more difficult.

The first signs of change following ICSI of the IVM MII oocytes were detected after 66 hrs in culture (Table 6.4). Excluding polar bodies, nuclei were not visible in unstained injected oocytes under light microscopy. When stained with DNA Hoechst or DAPI nuclei were visible under fluorescence microscopy. After staining with DAPI (Figure 6.4), Pronuclei were only observed in 3 of the 27 IVM, injected MII oocytes. This included 1 MII oocyte of wombat 63 after 42hrs and 2 MII oocytes of wombat 64 after 90hrs in culture.

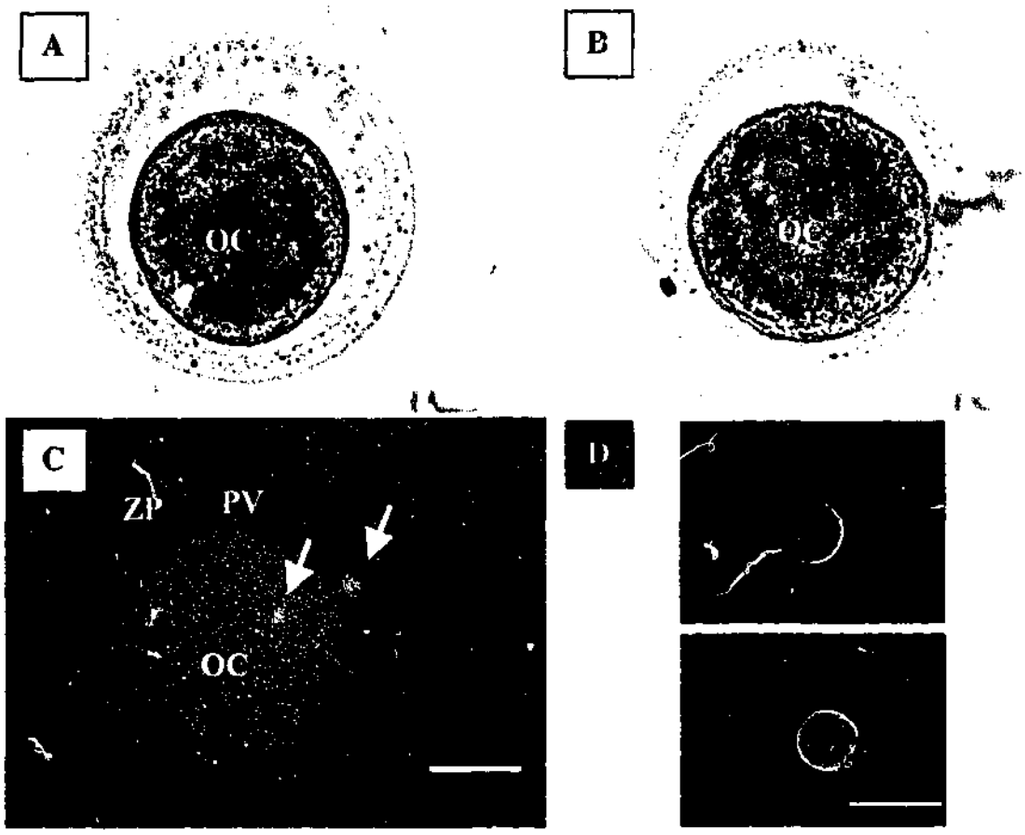


Figure 6.4. Stained injected Common Wombat oocytes 72 – 96 hours after intracytoplasmic sperm injection. A and B respectively show two different injected oocytes 72 and 96 h after ICSI. C shows A after staining with DNA Hoechst and the nucleus and polar body are still visible. D shows B after fixation on a slide and staining with DAPI, and 2 pronuclei are shown stained blue. The arrow in A shows the polar body and the arrows in C show the nucleus and polar body (top right arrow). ZP denotes the zona pellucida, PV denotes the perivitelline space, OC denotes the oocyte cytoplasm. Scale bars = 50μm.

A shrunken ooplasm, recognised as a sign of degeneration, was observed 42hrs after ICSI (66hrs after collection) in 2 injected oocytes of one wombat (wombat 62) and in 2 injected oocytes of another wombat (wombat 7) (Figure 6.4). All other injected MII oocytes appeared normal under light microscopy, although upon staining the nuclei generally appeared fragment.

Table 6.4. Development IVM injected MII oocytes. After 24hrs IVM, ICSI was performed on MII oocytes at time 0hrs.

	Time in culture					
Wombat	**0hrs ICSI**	**42hrs**	**66hrs**	**72hrs**	**78hrs**	**90hrs**
6 (b)	1 MII	nc	nc	1 x NSF		
14	5 MII	5 x NSF				
63	10 MII	{ 1 x 2 PN 9 x NSF				
7	3 MII	nc	nc	3 x NSF		
67	2 MII	nc	nc	2 x NSF		
64	3 MII	nc	nc	nc	nc	{ 2 x 2 PN 1 x NSF
69	3 MII	nc	nc	3 x NSF		

NSF = no sign of fertilisation upon staining, PN = pronuclei, nc = no change observed

6.4 Discussion

6.4.1 IVM of oocytes from pFSH/pLH primed Common Wombats

The range of different oocytes collected by aspiration of follicles after pFSH/pLH treatment reflects the variation in the health and development of follicles. In particular it indicates that 4mm follicles are not pre-ovulatory follicles in the Common Wombat in Victoria, as has been suggested for Common Wombats in Tasmania (Mortiz *et al* 1998). Further studies are required to correlate the morphological appearance of follicles with

histological appearance so as to differentiate between healthy follicles and atretic follicles.

Immature oocytes from all reproductive groups of wombats could be matured in vitro. By 24-30 hrs IVM, the yield of MII oocytes collected after superovulation treatment was doubled. *In vitro* maturation was therefore considered to be successful. By 30hrs 16.1% of oocytes had matured to the MII oocyte stage. The maturation protocol used in this study is not optimal, as higher percentages of immature oocytes have progressed to the MII oocyte stage in other marsupial studies (Mate and Rodger 1993; Mate and Buist 1999). IVM studies in the Tammar Wallaby have found that, nuclear maturation had not occurred by 24hrs, however 20% matured to MII status within 36 hrs after collection from PMSG primed animals (Mate and Rodger 1993). By 48hrs a total of 40% of the immature Tammar oocytes had matured to the MII stage. In another study, Tammar Wallaby MII oocytes were produced between 18 and 42hrs IVM from FSH primed animals (Mate and Buist 1999). By 36 hrs, 20% of immature Tammar oocytes were reported to have matured to the MII stage, and by 42 hrs, a total of 76% had matured.

FSH/LH stimulation prior to IVM, may have assisted maturation of Common Wombat MII oocytes *in vitro*. The major difference between our study and those of the Tammar Wallaby was that, the Common Wombats received a final LH injection to begin to induce ovulation, which was not administered to the wallabies prior to IVM (Mate and Rodger 1993; Mate and Buist 1999). Nuclear maturation of the common wombat oocytes in vitro may have therefore proceeded faster in response to the final LH injection.

In our study no further nuclear maturation occurred after 30hrs IVM, and 83.9% percent remained immature oocytes (although the nuclear stage was not defined). No further nuclear maturation was reported for the Tammar Wallaby after 42hrs in FSH primed wallabies, and 24% did not mature to the MII stage (Mate and Buist 1999). In an earlier study 60% of oocytes did not mature to the MII oocyte stage in PMSG primed Tammar Wallabies after 48hrs (Mate and Rodger 1993). Many immature Common Wombat oocytes instead began to show signs of degeneration, the longer they were left in the

maturation media. Signs of shrinking ooplasms and fragmentation suggest that the media (TCM-199 + 10%FCS + pFSH + pLH) was not suitable. Further studies should investigate modifications the maturation conditions and in particular the media. Factors such as osmolarity, pH, gonadotrophins of the media have been studied in eutherians such as pigs, cows and domestic cats, and are appropriate conditions are imperative for the production mature oocytes and culture of embryos (Yamauchi *et al* 1999; Geshi *et al* 1999; Wolfe and Wildt 1996).

The maturation conditions required, have been found vary in the Tammar Wallaby, in response to the stimulation protocol prior to oocyte collection. FSH primed wallabies required the addition of pLH to the media to induce maturation (Mate and Buist 1999), where as the addition of gonadotrophins (PMSG and/or pFSH/pLH) to the maturation media of PMSG primed wallabies did not improve maturation of oocytes (Mate and Rodger 1993).

MII oocytes were produced more readily, when immature oocytes were matured with cumulus cells attached (56.3%, n=16) than naked oocytes (17.8%, n=188) (Table 6.2). The developmental state of these oocytes was not known prior to IVM. Immature status was assumed because the cumulus cells could not be removed. The presence of polar bodies could not be ascertained because these cells obscured the oocyte. Normal development of MII oocytes and embryos in vitro have been suggested to be improved by co-culture with other cell types, such as granulosa or cumulus, as these cells can provide signals that can not be produced by the media itself (Geshi *et al* 1999). Mate and Buist (1999) found that attached granulosa cells were required to mature oocytes from FSH primed wallabies. In contrast, immature oocytes of PMSG primed wallabies did not require granulosa to mature (Mate and Rodger 1993). As Mate and Buist (1999) have previously pointed out, maturation of eutherian oocytes *in vitro* may be blocked if granulosa are stimulated with FSH, and that LH can override this inhibition. The early removal of cumulus cells from immature oocytes, and the aspiration of oocytes, which occasionally removed cumulus cells may have compromised the percentage of oocytes maturing in this study.

It is clear that the oocytes from FSH/LH primed Common Wombats, matured better with cumulus cells attached however, the need for pFSH and pLH in the maturation media is yet to be determined. FSH and LH might be most beneficial for the early maturation of the germinal vesicle oocytes. In Tammar Wallabies, Mate and Rodger (1993) suggested that pFSH and pLH might play a role in accelerating germinal vesicle break down.

The successful maturation of immature oocytes from one subadult and two anoestrous adults, indicates that with further stimulation these animals may have produced MII oocytes *in vivo*. On the other hand, maturation of immature oocytes from all reproductive groups may be stimulated *in vitro*, irrespective of superovulation. After collection from unstimulated wombats, MII oocytes were recovered following 60 hrs IVM in EMEM (M. Cleary *pers comm* unpublished).

TCM-199 was investigated as it has proved to be a successful maturation media for cow oocytes achieving very high maturation rates (of at least 80%) (Dr A French *pers comm*)". EMEM and DMEM have been previously used in other marsupial IVM studies (Mate and Rodger 1993, Mate and Buist 1999) and could be investigated in the future for use in maturing Common Wombat oocytes from stimulated animals.

The size of the follicle has been suggested to be important when mature Tammar Wallaby oocytes, finding that significantly more oocytes matured from larger follicles (Mate and Rodger 1993). Oocytes were collected from all follicles ≥1mm from the FSH/LH stimulated wombats. As oocytes were not delineated by size of the follicles, this may be a reason for the low percentage of oocytes matured in this study.

Some Common Wombat immature oocytes parthenogenetically divided. Parthenogenetic division has been reported immediately after follicular aspiration in one *in vivo* study of the domestic cat (Pushett *et al* 1997). Parthenogenesis has been observed in many other species including humans (Winston *et al* 1991), rabbits (Garcia-Ximenez and Escriba 2002), goats (Ongeri *et al* 2001) and cows (Lechniak *et al* 1997; Neuber *et al* 2002).

Although viable offspring were not produced, embryos can form parthenogenetically. However, recent studies have found that using nuclear transfer techniques that the haploid cells from parthenotes can be injected into oocytes to produce live young (Garcia-Ximenez and Escriba 2002). Parthenogenetic division has also been observed in the Grey Short-tailed Opossum (Selwood and Vandeberg 1992). Following collection of oocytes from follicles of unstimulated post-ovular opossums, 8% of maturing oocytes (in DMEM) became 2-cell parthenogenetic embryos.

The ooplasm of mature MII oocytes of the Common Wombat was found to be very dark. This made viewing of the nuclei difficult without the use of staining techniques.

6.4.2 ICSI of MII oocytes collected 24hrs after pFSH/pLH treatment

ICSI has not been previously attempted on wombat oocytes, and is only the second attempt on marsupial oocytes (Magarey and Mate 2000) and both studies have performed ICSI after FSH/LH treatment. The only potential sign of fertilisation, of injected MII oocytes recovered from stimulated Common Wombats, occurred following the second superovulation attempt of the first wombat. Three oocytes appeared to divide by 48hrs after ICSI (Figure 6.4).

At the 2-cell division, vesicles, could be seen to be extruded into the perivitelline space from the newly forming cells. These vesicles were likely to be yolk-filled as observed by Selwood and Vandeberg (1992) during real division of Grey Short-tail Opossum zygotes. The division of these injected oocytes is similar to the parthenogenetic development observed of opossum oocytes (Selwood and Vandeberg 1992). Initially it considered that the wombat oocytes had potentially been fertilised and cleavage had begun, although parthenogenetic division was suspected (West *et al* 2002). It now seems more likely after comparison with the other studies (Selwood and Vandeberg 1992; Renfree and Lewis 1996; Selwood 1996; Frankenberg and Selwood 1998; Selwood 2001), especially in the absence of greater numbers, or naturally produced embryos, that the globular structures (Figure 6.4) represent fragmentation or yolk extrusion after parthenogenetic division.

Further work is therefore required to investigate normal embryo development in the Common Wombat.

Failure to fertilise MII oocytes by ICSI may be attributed to a number of factors including the viability of the MII oocyte, the preparation of sperm, the ICSI protocol, and the subsequent culture conditions (Catt 1996; Mansour 1998; Tesarick and Mendoza 1999). ICSI has been successfully used in many eutherian species including humans and domestic species (Catt 1996, Mansour 1998). In some species such as the human, special treatment of the spermatozoa are not required prior to injection (Mansour 1998; Sathananthan *et al* 1997; Tesarick and Mendoza 1999). In other species such as the hamster and mouse, fertilisation rates are significantly improved by initiation of the acrosome reaction prior to injection (Yanagimachi 1998; Lacham-Kaplan and Trounson 1995). Sperm acrosomes contain a variety of enzymes that may be harmful to the oocyte (Tesarick and Mendoza 1999). Previous studies have already found that in marsupials capacitation of the sperm is the main difficulty in *in vitro* fertilisation (IVF) attempts (Mate *et al* 1998). The acrosome reaction maybe the key to successful fertilisation by ICSI in marsupials, such as the Common Wombat, and further work should investigate this.

Like the acrosome reaction of the sperm, specific activation protocols for the oocyte were not used for the Common Wombat, and these may also be necessary. Naturally, oocyte activation is initially triggered by the sperm binding to the oocyte plasma membrane. ICSI has been demonstrated in humans to induce similar changes upon insertion of the injection pipette, however inappropriate activation may lead to abnormal embryo development (Tesarik and Sousa 1995a; Tesarik and Mendoza 1999). Other studies have used activation techniques following ICSI have reported significant improvements in fertilisation rates (Tesarik and Sousa 1995b). However in other species such as the mouse, activation methods have not affected fertilisation rates (Lacham-Kaplan and Trounson 1995).

Additional factors associated with the artificially induced maturation of the oocytes may also effect their ability to be fertilised and other methods of fertilisation may be required to investigate this. As an alternative to ICSI, in vitro fertilisation (IVF), artificial insemination (AI) and natural matings have been studied in marsupials following hormonal stimulation treatment. IVF has not yet produced live offspring in a marsupial. Fertilisation has been achieved in the Grey Short-tailed Opossum (Moore and Taggart 1993), although not yet in the Common Brushtail Possum (Mate *et al* 2000; Sidhu *et al* 2001). AI has also resulted in successfully fertilised oocytes, and embryo's have been collected (Molinia *et al* 1998a). Fertilisation following the natural mating of hormonal stimulation has been moderately successful in marsupials and several studies have reported collection of fertilized embryos (Renfree *et al* 1988; Rodger *et al* 1992b; Rodger *et al* 1993). It is the only technique so far that has resulted in production of live young, and this has only been achieved in one species, the Fat-tailed Dunnart (Rodger *et al* 1992b). Further studies in the wombat should consider assessing viability via AI and natural mating.

Gardener *et al* (1996) have demonstrated that the nutrient requirements of developing marsupial embryos vary from that of eutherian species. Signs of degeneration of the injected MII oocytes were found, suggesting that the TCM 199 maturation and culture media used in this study was not optimal for Common Wombat oocytes. TCM-199 has been successfully used in eutherian species, particularly the cow achieving maturation rates of approximately 80% after 24hrs IVM (A. French *pers comm*). Approximately 47% of immature oocytes mature to MII status in TCM-199 after 36 hrs in the domestic cat, although higher maturation rates of 60% were achieved in EMEM after 32hrs (Pushett 2000). Other marsupial studies have previously predominantly used Dulbecco's or Eagle's minimum essential medium (DMEM or EMEM) (Selwood 1987; Mate and Rodger 1993; Renfree and Lewis 1996; Mate and Buist 1999; Magarey and Mate 2000), although more appropriate media's may need to be based on the species own oviduct fluid, (Sidhu *et al* 2001; Smith *et al* 2001). Further studies are therefore required to examine appropriate maturation conditions.

6.4.3 ICSI of MII oocytes following IVM

Some of the mature MII oocytes were found to have hardened zona pellucida after 24hrs IVM, and it was difficult to pierce them with the injection pipette during ICSI. This may have been due the duration in maturation media. Prolonged *in vitro* culture is recognised in other species to cause hardening of the zona pellucida (Smitz *et al*2001).

Most injected MII oocytes degenerated following ICSI and culture, further suggesting that additional studies need to investigate appropriate maturation and culture media's for Common Wombats. Pronuclei were however found in 3 injected MII oocytes (11% of IVM MII oocytes), and did not necessarily indicate fertilisation had occurred. Magarey and Mate (2000) found that even when MII oocytes are not injected, a small percentage (7%; n=75) form 2 pronuclei. Sham-injected MII oocytes were also found to contain pro-nuclei (13%; n=71) 17-19 hrs after the oocytes were pierced with an injection pipette (Magarey and Mate 2000). Abnormalities were found in both non-injected and sham-injected MII oocytes finding both degenerate oocytes and oocytes with a single pronuclei. The major difference between sham-injected and sperm-indicated MII oocytes was that a much greater percentage (40%; n=100) contained 2 pronuclei (Magarey and Mate 2000) and less oocytes were degenerate or contained only a single pronuclei. An additionally abnormality identified was that some sperm-injected oocytes also contained 3 pronuclei.

The first sign of fertilisation, formation of 2 pro-nuclei, was reported to occur by 17-19 hrs after ICSI in the Tammar Wallaby (Magarey and Mate 2000). In the Common Wombat, we are unsure as to what stage pro-nuclei would have formed as, we were looking for signs of cleavage and nuclear staining was not undertaken until 42 - 90 hrs after ICSI.

Ultimately fertilisation and viability of MII oocytes is assessed by production of live young, that when mature, are capable of reproducing. Fertilisation, and viability can be examined earlier by chromosomes analysis, by techniques such as chromosome painting (Lopes *et al* 1998; Magli *et al* 2000). Chromosome painting has already been developed for a number of marsupials including the Southern Hairy-nosed Wombat (De Leo *et al*

1999). Although this technique is yet to be used to establish fertilisation in a marsupial. A paint has not yet been produced for the common wombat to enable determination of fertilisation.

6.5 Conclusion

IVM of the immature oocytes collected after FSH/LH treatment has been found to approximately double the yield of MII oocytes that can other wise be collected from stimulated Common Wombats. Following ICSI, most MII oocytes colleted after superovulation treatment and IVM, degenerated in culture. Further research is required to develop more appropriate maturation and culture systems for the wombat. Fertilisation was not confirmed during this study. Most importantly, further research is required to achieve and assess fertilisation following ICSI, or by other techniques.

Chapter 7

General discussion

This study has 1) defined the oestrous cycle of the common wombat in Victoria and 2) demonstrated for the first time that multiple MII oocytes can be produced in response to superovulation treatment. The mean cycle length of ~ 47 days with wide variation (18–38 days) (Chapter 3) is in agreement with the general marsupial pattern (Tyndale-Biscoe and Renfree 1978) that the estimated gestation length of 30 days (Green and Rainbird 1987a) and the luteal phase (~ 28 days) represent ~ 60% of the cycle length. Wide variation is also observed in other species and may be related to sampling techniques used or follicle growth. The common wombat in Victoria apparently has a longer oestrous cycle than the same species in Tasmania (~ 33 days; Peters 1977). This difference was considered significant despite low numbers of animals in both studies. Minimum body weight of cycling adult Victorian animals (~23.5kg) differed from the Tasmanian figure of 16.8kg (Peters 1977). Such inherent differences may be related to geographic isolation (for 9,000 – 10,000 years) or environmental factors. They support the distinction of subspecies of the Common Wombat: *Vombatus ursinus hirsutus*, and found on the Australian mainland (including Victoria) and *Vombatus ursinus tasmaniensis* found in Tasmania (McIlroy 1995). Further study is required to investigate differences in the reproductive cycle of the third recognized subspecies, *Vombatus ursinus ursinus*, now extinct from all Bass Strait islands except Flinders Island (McIlroy 1995).

The relatively high progesterone concentrations during the luteal phase of the cycle (Section 3.3.1) were similar to those of the Koala (Johnston *et al* 2000). Other marsupial species are reported to have lower progesterone concentrations (Section 3.4.1). Further characterisation of this apparent difference and its significance is needed.

Oestradiol concentrations were very low compared with other studies (Section 3.4.2). The suitability of the assay for wombats and the possibility that oestradiol is not the major oestrogen require further study.

Progesterone and oestradiol concentrations were consistent with the changes in vaginal cytology. A reliable pattern was identified with a high percentage of superficial type epithelial cells observed during the follicular phase and a low percentage of superficial type cells during the luteal phase. This general pattern has also been observed in all other marsupials studied (Section 1.3.5).

Monitoring progesterone, oestradiol and vaginal cytology ½ - 3 times per week gave a clear indication of the stages of the oestrous cycle in these captive experimental wombats. Progesterone concentrations gave the most detailed picture. Characteristics of the pouch and urogenital opening were not helpful in determining the stage of the oestrous cycle. Pouch inspection enabled lactational status to be determined. Protrusion of the urogenital opening was more often associated with follicular phase (~ oestrus) than the luteal phase, but was an unreliable indicator of the oestrous cycle stage.

Complimentary studies in wombats are being conducted in NSW (McCallum *pers com*). These will add valuable information since there is a need for more wombats to be examined over time and in different locations. No study has yet been undertaken to investigate oestrous cycles in free ranging wombats, which may show different reproductive characteristics from captive wombats. Faecal studies show promise (Paris *et al* 2002; Banks 2001) for defining the stage of the oestrous cycle in free ranging animals.

A protocol of FSH/LH treatment for the common wombat was established. The treatment successfully stimulated follicular development (Section 4.3.2). Field and captive observations (McIlroy 1973, Presidente 1982, Green and Rainbird 1987, Triggs 1996, Boer 1998) of only single young, and ovarian histology (Moritz *et al* 1998) strongly suggests that the Common Wombat is monovular. The superovulation treatment gave a yield of ~ 5.5 MII oocytes per wombat. Immature oocytes collected from FSH/LH treated

wombats could be matured in TCM 199 media (Section 6.3.1). Other medias, such as those used in other marsupial studies may latter increase this maturation rate (Section 6.4.1).

In any programme of assisted reproduction involving embryo production and transfer, the production of MII oocytes is an essential step. No other studies of superovulation in the wombat have been reported. In order to optimize the ovarian stimulation protocol, future studies will need to examine variations in dose, duration and types of hormones administered, and the effects of season, reproductive state and intrinsic factors. Other methods of obtaining mature oocytes, may involve IVM of oocyte from un-stimulated wombats, or xenotransplantation of ovarian tissue (Cleary *pers com*).

ICSI of collected oocytes was unsuccessful. Only 6 possibly fertilizations occurred (early cleavage (n=3); 2 x pronuclei formation (n=3)) of 57 injected oocytes although this could have been parthenogenetic division (Section 6.4.2). Chromosomal analysis would have been helpful to distinguish these two possibilities (Section 6.4.3). Viability of oocytes will ultimately be confirmed by the production of live young that are themselves capable of reproduction. Artificial insemination and mating have achieved successful fertilization rates in marsupials after superovulation (Rodger *et al* 1992b; Rodger *et al* 1993; Molinia *et al* 1998a;). These approaches are recommended in future work with wombats.

Management of over abundant species is assisted by knowledge of their reproductive characteristics (Rodger 1990; Hinds et al 1996; Mate et al 1998). The work on the oestrous cycle constitutes a step forward in our understanding of wombat reproduction. In endangered species habitat protection and management are essential. In the case of the Northern Hairy-nosed Wombat there is a need for additional measures to assist reproduction artificially. The work on ovarian stimulation and oocyte harvest provides a basis for continued efforts to save this endangered species.

Appendices

Appendix 1. Wombat feed

Barastoc Blue Ribbon Maintenance Horse Mix

Manufactured by: Ridley Agriproducts Pty Ltd,
70 – 80 Bald Hill Road, Pakenham, Vic 3810

Manufacturers analysis:

Min Crude Protein	8.5%
Min Crude Fat	2.0%
Max Crude Fibre	22.0%
Max Added Salt	0.5%
Min Calcium	1.0%
Min Phosphorus	0.4%
Max Fluorine	0.04%

Compounded from:

Cracked Maize, Black Sunflower Seeds, Lucerne Chaff, Wheaten Chaff, Cereal Chaff, Molasses, Salt, Limestone, and Nutribit pellets.
Nutribit pellets: cottonseed meal or sunflower meal, peas or lupins or faba beans, wheat or oats or barley or triticale, bran or pollard or rice pollard, limestone, rock phosphate, salt, vitamins A, D3, E, K3, B12, Thiamine, Riboflavin, Pyridoxine, Niacin, Calcium pantothenate, Folic acid, Biotin, Ferrous sulphate, Zinc oxide, Manganous oxide, Copper sulphate, Cobalt sulphate, Potassium iodide, Sodium selenite antioxidant.

Appendix 2. Vaginal smear staining protocols

Shorr's Stain Method
After collecting vaginal smears, each slide was allowed to dry for at least 5 minutes. Slides were then stained according to the following protocol.

Reagent	Immersion time (seconds)
70% Ethanol	10
Distilled H_20	10
Haemotoxylin (Lillie Mayer)	120
Tap H_20 - rinse well	20 (at least)
Bierbrich'e Scarlet	120
Tap H_20 - rinse well	20 (at least)
Fast Green	60
Tap H_20 - rinse well	20 (at least)
70% Ethanol	10
95% Ethanol	10
100% Ethanol	10
100% Ethanol	60
Xylol	10
Xylol	60

After 60 seconds, in Xylol (AnalaR), the smears were mounted using Depex mounting medium (Gibco BRL) and a 22 x 50mm; Menzel-Glaser cover slip.

Diff-Quik Stain Method
After collecting vaginal smears, each slide was allowed to dry for at least 5 minutes. Slides were then stained according to the following protocols:

Reagent	Immersion time
Diff-Quick Solution 1 (a Methanol fixative)	Dip for 5 seconds
Diff-Quick Solution 2 (containing Xanthene dye)	Dip for 10 seconds
Diff-Quick Solution 3 (containing Thiazine dye)	Dip for 10 seconds

The slide was then immediately rinsed under cold running water and allowed to dry. Slides were mounted using Depex mounting medium (Gibco BRL) and a cover slip. After the 9 April 2001, cover slips and mounting medium were not used since it the smears could be read satisfactorily without them.

Appendix 3. Progesterone assay validation

Progesterone Assay Validation

The concentration of progesterone in the plasma from the peripheral circulation was determined using the Chiron ACS: 180 Progesterone Assay: an automated kit, non-extraction, competitive immunoassay. Assay validation was undertaken in accordance with Chard (1987). Financial restrictions prevented replicate samples. For this reason, the progesterone concentrations presented in this study should be considered an estimate of the true values. Despite this, validation of the assay was strongly supported by tests for sensitivity, specificity, linearity, within-assay and between-assay variation.

Sensitivity: Manufactures of the kit assay, cite maximum and minimum detection levels as 0.35nmol/L (0.11ng/ml) and 190.8 nmol/L (60ng/ml). Any values detected outside this range were rejected.

Specificity: The manufacturers reported the assay as highly specific to progesterone (4-Pregnene-3,20-dione; $C_{21}H_{30}O_2$; molecular weight 314.5) with very low cross-reactivity with other compounds. The highest cross-reactivity was found to be 0.95% with Corticosterone (**TableA3.1**).

Linearity: A mean recovery of 76% ± 3.91 (range: 47.1 to 101.4%) was determined following the serial dilution of 3 wombat plasma samples within the range of 84.6nmol/L to 123.8nmol/L. Serially diluted wombat plasma samples were linear when the observed concentration was compared the expected concentration (Figure A3.1).

Assay variation: Within-assay variation was found to be 6.1% ± 2.2 (range: 2 - 9.6%) when 3 wombat plasma samples, 3.7, 50.9 and 104.6 pg/ml were measured 5 times during the same assay. Between-assay variation was found to be 9.1% ± 5.6 (range: 2.3 - 20.2%) when 3 wombat plasma samples, 4.2, 50.1 and 110.35 pg/ml were measured during 5 different assays.

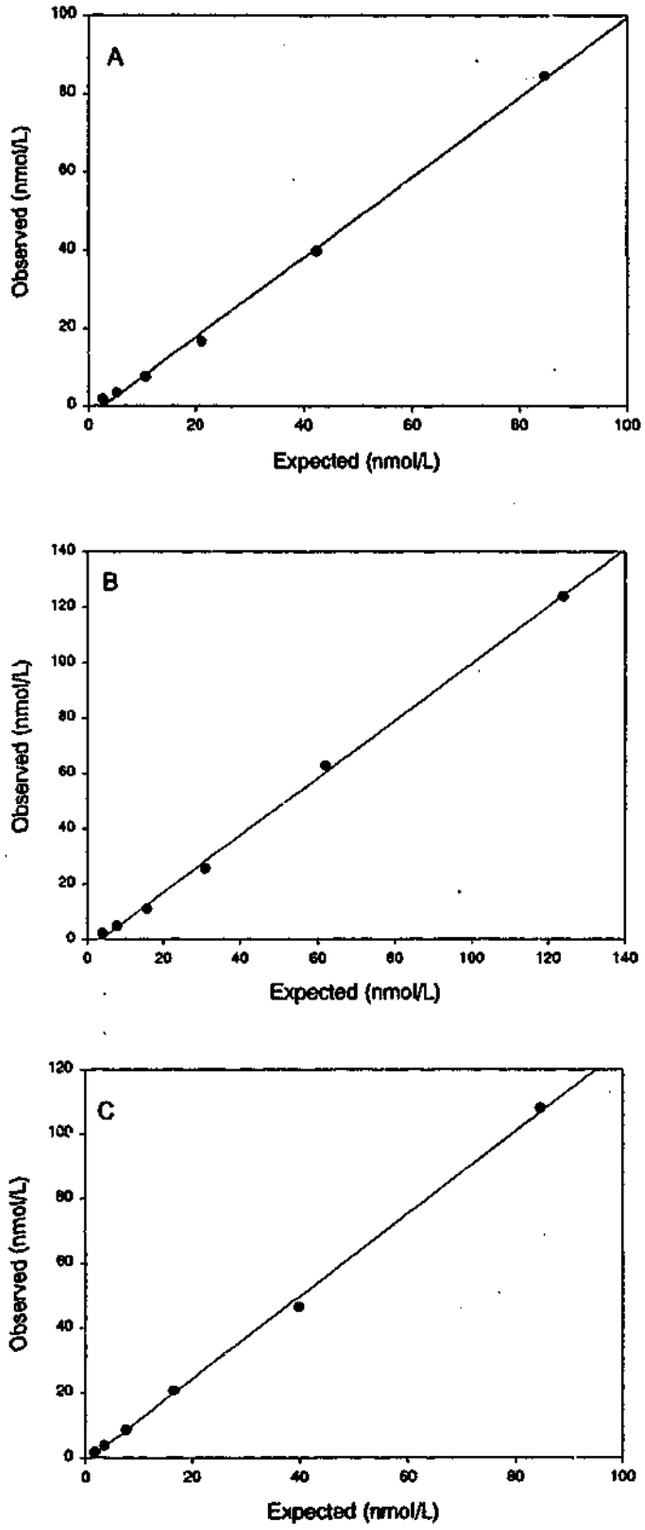


Figure A3.1. Results of serial dilution of Common Wombat plasma collected from cycling adults, assayed using the Chiron ACS: 180 Progesterone Assay kit. Observed progesterone concentration compared to the expected progesterone concentration. A, B and C show the respective results of serially diluting plasma samples determined to have a progesterone concentration of 80.3, 123.8 and 108 pg/ml.

Table A3.1 Cross-reactivity of the Chiron ACS: 180 Progesterone Assay (as determined by the manufacturers (Chiron Health Care Pty Ltd).

The ACS:180 Progesterone Assay shows high specificity for progesterone. The following compounds were spiked into Multi-Diluent 3, and the apparent progesterone value was determined. The percent cross-reactivity is the apparent result compared to the amount added.

Compound	Amount Added (ng/mL)	% Cross-reactivity
Cortisol	1000	ND*
11-deoxycorticosterone	1000	0.08
Corticosterone	1000	0.95
Testosterone	1000	ND
Aldosterone	1000	ND
Pregnenolone	1000	0.46
Androstenediol	1000	ND
17α-hydroxyprogesterone	1000	0.31
11-deoxycortisol	1000	ND
Danazol	10,000	ND
Prednisolone	1000	ND
17β-Estradiol	100	ND
Estrone	100	ND
Estriol	100	ND
Clomiphene	100	ND
Bromocryptine	100	ND

* ND = Not Detectable.

Appendix 4. Estradiol assay validation

Estradiol Assay Validation

The concentration of estradiol in the plasma from the peripheral circulation was determined using the Estradiol MAIA assay (BioChem ImmunoSystems; Code 12264; a manual, extraction radioimmunoassay; modified to be more sensitive for detection of estradiol in bovine plasma. Samples were assayed in duplicate, although financial limitations restricted the study to samples collected during 3 natural oestrous cycles (as determined by progesterone concentrations), and during the times of superovulation, including the samples collected in between the two superovulation attempts of wombats 6 and 13. The assay was validated according to Chard (1987).

Sensitivity: Standards were serially diluted to determine the minimum detection level of Estradiol MAIA assay, which was found to be 0.25pg/ml. Any values detected below this were considered to be below the detection level (<0.25pg/ml).

Specificity: The manufacturers reported the assay as highly specific to estradiol (estradiol-17β; $C_{18}H_{24}O_2$; molecular weight 272.4) and a very low cross-reactivity with other compounds. The highest cross-reactivity's found were 2.5% with 17β-Estradiol-3-Benzoate and 1.77% with Estrone (**Table A4.**).

Linearity: A mean recovery of 85.4% ± 10.0 (range: 43.6 to 134.2%) was determined following the serial dilution of 2 wombat plasma samples of 11.4pg/ml and 17.5pg/ml. Serially diluted wombat plasma was found to be linear when the observed concentration was compared against the expected concentration (Figure A4.1).

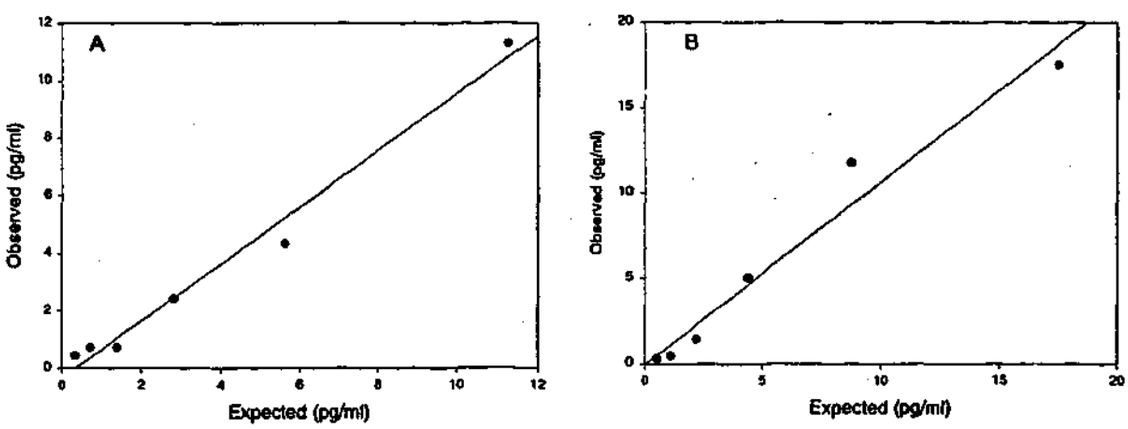


Figure A4.1. Results of serial dilution of Common Wombat plasma collected during pFSH/pLH treatment, assayed using the Estradiol MAIA kit. Observe estradiol concentration is compared to the expected estradiol concentration.

Spiking recovery: A mean recovery of 93.1% ±8.9 (range: 71.3 to 120%) was obtained after 2 wombat plasma samples, within the range of 2.16 to 7pg/ml, were spiked with 0, 5, 15 or 25pg/ml of estradiol (Estradiol MAIA Standards; BioChem ImmunoSystems).

Assay Variation: Within-assay co-efficient of variation was 18.1% ± 3.3 (range: 14.4 – 24.8) when standard samples, between 4.3 and 21.4 pg/ml were measured 6 times during the same assay. Between-assay co-efficient of variation was found to be 17.4%

± 4.0 (range: 13.4 - 21.5) when 2 standard samples, between 12.9 and 13.3pg/ml were measured during 2 different assays. Further assays using wombat plasma should be undertaken to increase the sample size.

The low oestradiol concentrations found during the Estradiol MAIA assay indicate that further tests should be undertaken to investigate the major oestrogen compounds of the Common Wombat.

Table A4.1 Cross-reactivity (specificity) of the Estradiol MAIA assay (as determined by the manufacturers (BioChem ImmunoSystems).

11.5 Specificity

The specificity has been evaluated by the interference of the following compounds, according to the Abraham's method (x/y x100) where x and y are respectively the weight of estradiol and of interfering compound that causes a decrease of 50% binding capacity.

Antigen	Cross-reactivity
Estradiol	100%
Estrone	1.77%
Estriol	0.47%
Testosterone	0.0033%
Aldosterone	0.007%
Progesterone	absent up to 100 γ/ml
Deoxycorticosterone	0.00003%
Androstenedione	0.0001%
Dehydroepiandrosterone	absent up to 100 γ/ml
DHT	0.0002%
Cortisolo	absent up to 50 γ/ml
ß-Estradiol-17ß-Glucoronide	absent up to 1 γ/ml
ß-Estradiol-3-Sulfate-17 Glucoronide	absent up to 1 γ/ml
17ß-Estradiol-3ßD-Glucoronide	0.14%
17ß-Estradiol-3 Sulfate	0.07%
ß-Estradiol-Dipropionate	0.19%
17ß-Estradiol-3 Glucoronide-17 Sulfate	absent up to 1 γ/ml
17ß-Estradiol-3-Benzoate	2.5%
Clomiphene	absent up to 50 γ/ml
17ß-Estradiol-3-disulfate	absent up to 1 γ/ml
DHEA-sulfate	0.0004 1%

Appendix 5. Aspiration and maturation culture media

TCM 199 Aspiration Media

Under sterile conditions, a TCM-199 Hepes solution was made by combining the ingredients as listed in table 5A.1. The osmolarity of the TCM-Hepes solution was adjusted to 275 – 285Osm and then filtered using a sterile 20ml syringe (Becton Dickson) and attached 0.22µm Millipore filter into a sterile 1L glass container.

Table 5A.1. TCM 199 Hepes solution

Sigma Medium 199	100ml
Tissue Culture Water	900ml
L-Glutamine	0.1461g
Hepes	6.5g
Pyruvic acid	0.22g
$NaHCO_3$	0.168g
Stock Solution (total volume)	**1000ml**

The TCM 199 Hepes solution was then used to make the TCM 199 aspiration media as described in Table 5A.2. The aspiration media was then gently mixed and then filtered using a sterile 10ml syringe (Becton Dickson) and attached 0.22µm Millipore filter. The media was then placed into a fridge at 4°C and used within 2 weeks. Aspiration media was warmed in a water bath at 35°C for a minimum of 4 hours before use.

Table A5.2. TCM 199 Aspiration Media

TCM-199 Hepes solution	48.5ml
FCS (CSL)	1ml
Penicillin/streptomycin	250µl
Heparin (Sigma)	0.3ml
Media (total volume)	**50ml**

TCM-199 Maturation Media

Under sterile conditions, a 0.4% stock solution of L-glutamine/ TCM-199 Bicarbonate solution was made as summarized in Table A5.3.

Table A5.3. TCM-199 Bicarbonate-High Osmolarity-Antibiotic Free/L-Glutamine Solution

Sigma Medium 199	50ml
L-Glutamine	0.2ml
Total volume	**50.2ml**

From the stock solution, TCM 199 Maturation media was made as described in Table A5.4. The 5µl of pLH (Vetrapharm) and 20µl of pFSH (Vetrapharm) was added using a sterile 20µl pipette tip and manual Gilson pipette on the evening before use. The maturation media was then filtered using a 10ml syringe (Becton Dickson) and a 0.22µm Millipore filter. After filtering the media was placed into a fridge at 4°C and used within 4 days. Before use aliquots were placed into an incubator at 35°C in 5% CO_2 to equilibrate for a minimum of 12h.

Table A5.4. TCM Maturation Media

TCM-199 Bicarbonate/ L-Glutamine	8.925ml
FCS (CSL)	1ml
Penicillin/streptomycin	50µl
Porcine LH	5µl
Porcine FSH	20µl
Maturation Media (total volume)	**10ml**

Appendix 6. Sperm collection and preparation for ICSI

Sperm Collection and Storage

Spermatozoa were collected from the epididymis of six shot male wombats from the Myrtleford area of Victoria by Bickell (2001). The spermatozoa were then cryo-preserved in 0.2M Trehalose and 10% glycerol or 0.3M Trehalose and 8% glycerol, and stored in liquid nitrogen at -196°C, as described by Bickell (2001).

Sperm Preparation for ICSI

A sperm thawing protocol designed by Bickell (2001) was followed as summarised in Table A6.1. Dry ingredients were weighed and then dissolved in Tissue culture water (CSL) by filling the Falcon tubes up to 10ml. After adding each of the ingredients listed in order (Table A6.1), 4ml of the 8.54ml of tissue culture water was initially added to the sperm Talp tube. A 1ml aliquot of the solution was transferred into an eppendorf tube and the pH was tested and the required volume of NaOH or HCl was added to achieve a pH of between 7 – 7.4. The volume in the falcon tube was then made up to 10ml with tissue culture water. The osmolarity (270 – 300mOsm) was tested and the sperm Talp solution was filtered, using a 0.22um Millipore filter and 10ml syringe, into a sterile 15ml falcon tube and stored at 4°C.

A straw containing wombat spermatozoa was removed from the cryo chamber and exposed to room temperature for 10 seconds. The straw was then plunged into a 35°C water bath for 30 seconds. One end of the straw was opened into an eppendorf tube containing 200µl pre-warmed sperm TALP at 35°C. The other end of the straw was cut to expel the sperm into the sperm Talp. The sperm and sperm Talp were then gently mixed and maintained at 35°C until ICSI.

Table A6.1. Sperm Talp. (All ingredients were supplied by Sigma unless otherwise noted).

Sperm TALP ingredients	MM	Stock	Volume/10ml
NaCl (AnalaR)	100	1 M	1ml
KCl	3.1	1 M	31ul
$NaHCO_3$ (AnalaR)	25	1 M	250ul
NaH_2PO_4	0.290	1 M	2.9ul
Na Lactate (60% Syrup)	21.6	60 %	31.5ul
HEPES	10	1 M	100ul
$CaCl_2$	2	1 M	20ul
$MgCl_2$	1.5	1 M	15ul
BSA (Gibco BRL)	0.5	1 %	5ul
Tissue culture water (CSL)			8.54ml

References

Al-Kamali AA, Boland MP, Crosby TF and Gordon I (1985) Reduced superovulation response in the ewe following repeated gonadotrophin treatment. Vet. Rec. 116(7): 180-181.

Banks S (2001) Microsatellite analysis of dispersal patterns and population genetics of the Common Wombat (*Vombatus ursinus*). Honours Thesis Monash University, Clayton.

Bennett JH, Breed WG, Hayman DL and Hope RM (1990) Reproductive and genetic studies with a laboratory colony of the dasyurid marsupial *Sminthopsis crassicaudata*. Aust. J. Zool. 37: 207-22.

Bickell C (2001) Evaluation of novel preservation strategies for spermatozoa. Masters Thesis, Monash University, Clayton, Victoria.

Boer M (1998) Observations on reproduction in the Common Wombat Vombatus ursinus in captivity. In: *Wombats*. Eds: RT Wells and PA Pridmore. Surrey Beatty & Sons, Chipping Norton. pp129-146.

Boland MP and Gordon I (1982) Effect of repeated anterior pituitary extract treatment on the ovulatory response in the ewe. Vet. Rec. 111(17): 391-392.

Bradshaw FJ and Bradshaw SD (1992) Estradiol-17 beta secretion by newly formed corpus luteum of a Macropodid marsupial, the quokka (*Setonix brachyurus*). Gen. Comp. Endocrinol. 87 (3): 425-435.

Brannian JD, Griffin F, Papkoff H and Terranova PF (1988) Short and long phases of progesterone secretion during the oestrous cycle of the African elephant (*Loxodonta africana*). J. Reprod. Fertil. 84(1): 357-365.

Brown AS (1987) Infertility in the Female Koala. PhD thesis. The University of Queensland, Brisbane.

Brown JL, Wasser SK, Wildt DE and Graham LH (1994) Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured non-invasively in faeces. Bio. Reprod. 51: 776-786.

Cake MH, Owens FJ and Bradshaw (1980) Differences in concentration of progesterone in plasma between pregnant and non-pregnant quokka (*Setonix brachyurus*). J. Endocrin. 84: 153-158.

Caraty A, Evans NP, Fabre-Nys CJ and Karsch EJ (1995) The preovulatory gonadotrophin releasing hormone surge: a neuroendocrine signal for ovulation. J. Reprod. Fertil. Suppl. 49: 245-255.

Catt JW (1996) Intracytoplasmic sperm injection (ICSI) and related technology. Animal Reproduction Science 42: 239-250.

Chard T (1987) Laboratory techniques in biochemistry and molecular biology: an introduction to radioimmunoassay and related techniques. 3rd Edition. Elsevier Science Publishers B.V. (Biomedical Division), Amsterdam.

Chikazawa K, Araki S and Tamada T (1986) Morphological and endocrinological studies on follicular development during the human menstrual cycle. J. Clin. Endocrinol. Metab. 62: 305.

Clark MJ and Poole WE (1967) The reproductive system and embryonic diapause in the female grey kangaroo *Macropus giganteus*. Aust. J. Zool. 15: 441-459.

Clarke IJ and Doughton BW (1983) Effect of various anaesthetics on resting plasma concentrations of luteinizing hormone, follicle-stimulating hormone and prolactin in ovariectomized ewes. J Endocrinol. 98(1):79-89.

Conder P (1970) Breeding of the common wombat in captivity. Victorian Naturalist 87: 322.

Connell BJ (1999). Monitoring reproductive status in tammar wallaby Macropus eugenii using faecal steroid analysis. Honors thesis, Department of Zoology, University of Melbourne, Parkville.

Crawford JL, Shackell GH, Thompson EG, McLeod BJ and Hurst PR (1997) Preovulatory follicular development and ovulation in the brushtail possum (Trichosurus vulpecula) monitored by repeated laparoscopy. J. Reprod. Fertil. 110: 361-370.

Crossman DG, Johnson CN and Horsup AB (1994) Trends in the population of the Northern Hairy-nosed Wombat *Lasiorhinus krefftii* in the Epping Forest National Park, central Queensland. Pac. Conserv. Biol. 1:141-149.

Curlewis JD Axelson M and Stone SM (1985) Identification of the major steroids in ovarian and adrenal venous plasma of the brushtail possum (Trichosurus vulpecula) and changes in the peripheral plasma levels of oestrogen and progesterone during the reproductive cycle. J. Endocrin. 105: 53-62.

Curlewis JD and Stone SM (1986) Effects of oestradiol, the oestrous cycle and pregnancy on weight, weight metabolism and cytosol receptors in the uterus of the brush-tail possum (*Trichosurus vulpecula*). J. Endocr. 73: 263-278.

De Leo AA, Guedelha N, Toder R, Voullaire L, Ferguson-Smith MA, O'Brien PC and Graves JA (1999) Comparative chromosome painting between marsupial orders: relationship with a 2n = 14 ancestral marsupial karyotype. Chromosome Research 7(7): 509-517.

Desauliniers DM, Goff AK Betteridge KJ, Rowel JE and Flood PF (1989) Reproductive hormone concentrations in faeces during the oestrous cycle and pregnancy in cattle (*Bos taurus*) and muskoxen (*Ovibos moschatus*). Can. J. Zool. 67: 1148-1154.

Desta B (1988) Complete separation of nine equine oestrogens by high-performance liquid chromatography. J Chromatogr. 435(2):385-90.

Domanski E, Przekop F, Chomick L and Ostrowska A (1989) Effects of stress on the coures of the oestrous cycle and the release of luteinizing hormone; the role of endorphin in these processes. Acta. Physiol. Pol. 40(1): 64-73.

Draincourt MA (2001) Regulation of the follicular dynamics in farm animals. Implications for manipulation of reproduction. Theriogenology 55: 1211-1239.

Drummond AE and Findlay JK (1999) The role of estrogen in folliculogenesis. Mol. Cell. Endocrinol. 25;151(1-2):57-64.

Duckworth JA, Scobie S, Jones DE and Selwood L (1998) Determination of estrus and mating in captive female brushtail possums, *Trichosurus vulpecula* (Marsupialia: Phalangeridae), from urine samples. Aust. J. Zool. 46: 547-555.

Evans SM, Tyndale-Biscoe CH and Sutherland RL (1980) Control of gonadotrophin secretion in the female Tammar Wallaby (Macropus eugenii). J. Endocr. 86:13-23.

Fadem BH and Rayne RS (1985) Characteristics of the oestrous cycle and the influence of social factors in grey short-tailed opossums (*Monodelphis domestica*). J. Reprod. Fertil. 73: 337-342.

Fielden ED and Hayman DL (1981) Repeated superovulation and embryo collection in cattle. In: Embryo Transfer in Cattle, Sheep and Goats; Australian Society for Reproductive Biology. Eds: JN Shelton, AO Trounson, NW Moore and JW James. Union Offset Company Pty Ltd, ACT. pp 20-23.

Findlay L, Ward KL and Renfree MB (1983) Mammary gland lactose, plasma progesterone and lactation in the marsupial Macropus eugenii. J. Endocr. 97: 425-436.

Flannery, T. (1996) Tree kangaroos: a curious natural history. Reed Publishing. Chatswood, N.S.W.

Fletcher TP (1983) Endocrinology of reproduction in the dasyurus marsupial *Dasyuroides byrnei* (Spencer). PhD thesis, Latrobe University, Melbourne.

Fletcher TP (1989) Plasma progesterone and body weight in the pregnant and non pregnant kowari, *Dasyuroides byrnei* (Marsupialia: Dayuridae). Reprod. Fertil. Dev 1: 65-74.

Flint AP and Renfree MB (1982) Oestradiol-17 beta in the blood during seasonal reactivation of the diapausing blastocyst in a wild population of tammar wallabies. J Endocrinol. 95(3):293-300.

Folman Y, Rosenberg M, Ascarelli I, Kaim M and Herz Z (1983) The effect of dietary and climatic factors on fertility, and plasma progesterone and oestradiol-17β levels in dairy cows.

Forcada F, Abecia JA, Lozano JM and Zuniga O (2000) Repeated superovulation of high-prolificacy Rasa Aragonesa ewes before culling as an inexpensive way to obtain high quality embryos. Livestock Production Science 66: 263-269.

Frankenberg S and Selwood L (1998) An ultrastructure study of the role of an extracellular matrix during normal cleavage in a marsupial, the brushtail possum. Molecular Reproduction and Development 50: 420-433.

Garcia-Ximenez F and Escriba MJ (2002) Viable offspring derived from cryopreserved haploid rabbit parthenotes. Theriogenology. 57: 1319-1325.

Gardener DK Selwood L and Lane M (1996) Nutrient Uptake and culture of *Sminthopsis macroura* (Striped-faced Dunnart) embryos. Reprod. Fertil. Dev. 8: 685-690.

Gaughwin MD and Wells RT (1978) General features of reproduction on the hairy-nosed wombat (*Lasiorhinus latifrons*) in the blanchetown region of South Australia. Aust. Mamm. Soc. Bull. 5(1): 46-47.

Gemmell RT (1987) Effect of melatonin and removal of pouch young on the seasonality of births in the marsupial possum, Trichosurus vulpecula. J Reprod Fertil. 80(1):301-7

Gemmell RT (1989) The persistence of the corpus luteum of pregnancy into lactation into lactation in the marsupial bandicoot, *Isoodon macrourus*. Gen. Comp. Endocrinol. 75 (3): 355-362.

Gemmell RT and Sernia C (1995) Effect of changing from a short-day to long-day photoperiod on the breeding season of the brushtail possum (Trichosurus vulpecula). J Exp Zool. 273(3):242-6.

Geshi M, Yonai M, Sakaguchi M, Nagai T (1999) Improvements of in vitro co-culture systems for bovine embryos using a low concentration of carbon dioxide and media supplemented with β-mercaptoethanol. Theriogenology. 51: 551-558.

Gilbert CL, Hunter MG, Southee JA and Wathes DC (1990) Immunoctyochemical localization of oxytocin in corpora lutea and luteinized cysts from anoestrous ewes stimulated with gonadotrophin releasing hormone. Cell Tissue Res. 262: 157-164.

Glazier AM and Molinia FC (1998) Improved method of superovulation in monovulatory brushtail possums (Trichosurus vulpecula) using pregnant mares' serum gonadotrophin-luteinizing hormone. J Reprod Fertil. 113(2):191-5.

Glazier AM (1998) Seasonal variation in ovarian response to pregnant mares serum gonadotrophin in the brushtail possum (Trichosurus vulpecula). Reprod Fertil Dev. 10(6):499-503.

Green RH and Rainbird JL (1987a) The Common Wombat *Vombatus ursinus* (Shaw, 1800) in Northern Tasmania – Part 1. Breeding growth and development. Records of the Queen Victoria Museum. 91: 1-19.

Green RH and Rainbird JL (1987b) The Common Wombat *Vombatus ursinus* (Shaw 1800) in Northern Tasmania –Part 2. The Bass Strait population, *V. ursinus Rec. Queen Vic. Mus.* 92: 1-7

Greenwald GS and Terranova PF (1988) Follicular selection and its control. In *The Physiology of Reproduction*. Eds: E Knobil and J Neil *et al*. Raven Press, Pty Ltd, New York.

Godfrey G. (1969) Reproduction in a laboratory colony of the marsupial mouse *Sminthopsis larapinta* (Marsupialia:Dasyuridae). Australian Journal of Zoology 17: 637-654.

Halse S and Rose RW (1988) Variation in the basal body temperature of the common brushtail possum (*Trichosurus vulpecula*) in Tasmania. Mammalia. 52: 225-231.

Hamilton R (1997) Faecal steroid analysis as a technique for monitoring reproductive status in the hairy-nosed wombat. Honors thesis, Department of Anatomy, Monash University, Clayton.

Hamilton RA, Stanton PG, O'Donnell L, Steele VR, Taggart DA, Temple-Smith PD. (2000) Determination of seasonality in southern hairy-nosed wombats (*Lasiorhinus latifrons*) by analysis of fecal androgens. Biology of Reproduction: 63(2):526-31.

Handesyde KA, McDonald IR, Than KA, Michaelides J and Martin RW (1990) Reproductive hormones in the koala. In: *Biology of the Koala* pp203-210. Eds AK Lee, KA Handesyde and GD Sanson. Surrey Beatty & Sons Pty Ltd, New South Wales.

Harder JD and Fleming MW (1981) Estradiol and progesterone profiles indicate a lack of endocrine recognition of pregnancy in the opossum. Science. 212(4501): 1400-2.

Harder JD, Hinds LA, Horn CA and Tyndale-Biscoe CH (1984) Oestradiol in follicular fluid and utero-ovarian venous and peripheral plasma during parturition and post-partum oestrus in the tammar *Macropus eugenii*. J. Reprod. Fertil. 72: 551-558.

Harder JD, Hinds LA, Horn CA and Tindale-Biscoe CH (1985) Effects of removal in late pregnancy of the corpus luteum, Graafian follicle or ovaries on plasma progesterone, oestradiol, LH parturition and post partum oestrus in the tammar, *Macropus eugenii*. J. Reprod. Fertil. 75: 449-459.

Harding HR (1969) Studies on the periovulatory changes in the ovary, uterus and vagina of the marsupial *Trichosurus vulpecula*, and the effect of PMSG. Honours Thesis, University of NSW, Sydney Australia.

Hartmen GC (1923) The oestrus cycle of the opossum. Amer. J. Anat. 32: 353-421.

Hatasaka HH, Schaffer NE, Chenette PE, Kowalski W, Hoecht BR, Meehan TP, Went AC, Valle RF, Chatterton RT and Jeyendran RS (1997) Stratergies for induction and oocytes retrieval in the lowland gorilla. J. Assit. Reprod. Genet. 14(2): 102-110.

Hayman DL, Smith MJ and Rodger JC (1990) A comparative study of chiasmata in male and female *Bettongia penicillata* (Marsypialia). Genetica. 83: 45-49

Hickford DE, Merry NE, Johnson MH and Selwood L (2001) Induced ovulation, mating success and embryonic development in the stripe-faced dunnart, *Sminthopsis macroura*. Reproduction. 122(5): 777-83.

Hilderbrandt TB, Hermes R., Jewgenow K and Gotitz F (2000) Ultrasonography as an important tool for the development of reproductive technologies in non-domestic species. Theriogenology. 53: 73-84.

Hill JP and O'Donoghue CH (1913) The reproductive cycle in the marsupial *Dasyurus viverrinus*. Quart. J. Micro. Sci. 59:133-74.

Hillier SG (1994) Current concepts of the roles of follicle stimulating hormone and luteinizing hormone in folliculogensis. Human Reprod. 9(2):188-191.

Hillier SG (2001) Gonadotrophin control of ovarian follicular growth and development. Mol. Cell. Endorcinol. 179(1-2): 39-46.

Hinds L A and Tyndale-Biscoe C H (1982a). Prolactin in the marsupial, *Macropus eugenii*, during the oestrus cycle, pregnancy and parturition. Biol. Reprod. 26: 391-398.

Hinds LA and Tyndale-Biscoe CH (1982b) Plasma progesterone levels in the pregnant and non-pregnant tammar, *Macropus eugenii*. J. Endocrin. 93:99-107.

Hinds LA (1983) Progesterone and prolactin in marsupial reproduction. PhD Thesis. Australian National University, Canberra.

Hinds LA Evans SM and Tyndale-Biscoe CH (1983) In-vitro secretion of progesterone by the corpus luteum of the tammar wallaby, Macropus eugenii. J. Reprod. Fert. 67: 57-63.

Hinds LA (1989) Progesterone Concentrations through pregnancy and the estrous cycle in the Eastern Quoll, *Dasyurus viverrinus*. General and Comparative Endocrinology 75: 110-117.

Hinds LA, Fletcher TP and Rodger JC (1996) Hormones of oestrus and ovulation and their manipulation in marsupials. In: Marsupial Gametes and Embryos. Reprod. Fertil. Dev. Vol. 8: 661-672.

Hirshfield AN (1989) Rescue of atretic follicles in vitro and in vivo. Biol. Reprod. 40(1): 181-190.

Hodges J. (1998) Endocrinology of the ovarian cycle and pregnancy in the Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephant. Anim. Reprod. Sci. 53: 3-18.

Horn CA, Fletcher TP and Carpenter S (1985). Effects of oestradiol-17β on peripheral plasma concentrations of LH and FSH in ovarectomised tammars (Macropus eugenii). J. Reprod. Fertil. 73: 585-592.

Horsup A and Davidson C (1994) *Recovery Plan for the Northern Hairy-nosed Wombat,* Lasiorhinus krefftii (2nd ed.) Report to Australian Nature Conservation Agency.

Horsup A (1997) Recovery Plan for the Northern Hairy-nosed Wombat (*Lasiorhinus krefftii*). Department of Environment, Queensland.

Horsup A (1998) A trapping survey of the Northern Hairy-nosed Wombat *Lasiorhinus krefftii*. In: *Wombats*. Eds: RT Wells and PA Pridmore. Surrey Beatty & Sons, Chipping Norton. pp147-155.

Howard JG, Baraone MA, Donoghue AM and Wildt DE (1992) The effect of pre-ovulatory anaesthesia on ovulation in laparoscopically inseminated domestic cats. J. Reprod. Fertil. 96(1): 175-186

Hoyle SD Horsup AB Johnson CN, Crossman DG and McCallum HI (1995) Live trapping of the Northern Hairy-nosed Wombat *Lasiorhinus krefftii*: population size estimates and effects on individuals. Wildl. Res. 22: 741-755.

Hughes RL, Thomson JA and Owen WH (1965) Reproduction in natural populations of the Australian ringtail possum, *Pseudocheirus peregrinus* (Marsupialia: Phalangeridae), in Victoria. Aust. J. Zool. 13: 383-406.

Hughes HE and Dodds TC (1968). Handbook of Diagnostic Cytology. E. and S Livingston Ltd., Edinburgh.

Hughes R and Green R (1998) Placentation and adaptations for survival at birth in the common wombat *Vombatus ursinus*. In: *Wombats*. Eds. Wells R., Pridmore P. Surrey Beatty & Sons, Chipping Norton NSW. pp86-108.

Ireland JJ, Mihm M, Austin E, Diskin MG, Roche JF (2000) Historical perspective of turnover of dominant follicles during the bovine estrous cycle: key concepts, studies, advancements and terms. J. Dairy Sci. 83(7):1648-1658.

Johnston SD, McGowan MR, O'Callaghan P, Cox R and Nicolson V (2000) Studies of the oestrous cycle, oestrus and pregnancy in the koala (Phascolarctos cinereus). J Reprod Fertil. 120(1):49-57.

Johnson CN and Crossman DG (1991) Dispersal and social organization in the Northern Hairy-nosed Wombat *Lasiorhinus krefftii*. J. Zool. (London). 225: 605-615.

Johnson CN and Gordon G (1995) Northern Hairy-nosed Wombat. In: *The Mammals of Australia*. Ed: R Strahan. Reed Books, Chatswood, NSW. pp200-201.

Johnson CN (1998) The evolutionary ecology of wombats. In: *Wombats*. Eds: RT Wells and PA Pridmore. Surrey Beatty & Sons, Chipping Norton. pp34-41.

Johnson MH and Everitt BJ (2000) E ential Reproduction. 5th edition. Blackwell Science Ltd

Jones SM and Rose RW (1992) Progesterone levels in the pregnant Tasmanian bettong (*Bettongia gaimardi*) General and Comparative Endocrinology. 87: 178-182.

Jungnickel MK, Molinia FC, Harman AJ and Rodger JC (2000) Sperm transport in the female reproductive tract of the brushtail possum, Trichosurus vulpecula, following superovulation and artificial insemination. Anim Reprod Sci. May 31;59(3-4):213-28.

Jungnickel MK and Hinds LA (2000) Hormonal profiles in the tammar wallaby, *Macropus eugenii*, following FSH/LH superovulation. Reprod Fertil Dev. 12(7-8): 457-64.

Kafi M and McGowan MR (1997) Factors associated with variation in the superovulatory response of cattle. Anim Reprod Sci. 48(2-4): 137-57.

Kaneko H, Wantanabe G, Taya K and Sasamoto S (1992) Changes in the peripheral levels of bioactive and immunoreactive inhibin, estadiol-17β, progesterone, luteinizing hormone, and follicular stimulating hormone associated with follicular development in cows induced to superovulate with equine chorionic gonadotrophin. Biol. Reprod. 47(1): 76-82.

Keisler DH and Keisler LW (1989) Formation and function of GnRH induced subnormal corpora lutea in cyclic ewes. J. Reprod. Fertil. 87(1): 265-273.

Kemper Green CN, Hawkins DA, Rocha A, Tanner JW, Harms PG, Forrest DW and Welsh Jr. TH (1996) Temporal aspects of ovarian follicular growth and steroidogenesis following exogenous follicle-stimulating hormone in Angus heifers. Anim. Reprod. Science. 45:157-176.

Kilen SM and Schwartz NB (1999) Estrus Cycle. *In:* Encyclopedia of Reproduction Vol. 2 *Eds.* Knobil E and Neill J D. Academic Press, San Diego.

Kingston J (1994) Goodfellow's Tree Kangaroo. Draft Management Plan. Melbourne Zoo.

Kirkpatrick JF, Kasman LH, Lasley BL and Turner JW (1988) Pregnancy determination in uncaptured feral horses. J. Wildl. Management. 52 (2): 305-308.

Kirsch JA. (1977) The six-percent solution: second thoughts on the adaptedness of the marsupialia. Am Sci.65(3):276-88.

Lacham-Kaplan O and Trouson A (1995) Intracytoplasmic sperm injection in mice: increased fertilisation and development to term after induction of the acrosome reaction. Human Reprod. 10(10): 2642-2649.

Lechniak D, Cieslak D and Sosnowski J (1997) Cytogenetic Analysis of bovine parthenotes after spontaneous activation in vitro. Theriogenology. 49: 779-785.

Lemon M (1972) Peripheral plasma progesterone during pregnancy and the oestrus cycle in the tammar wallaby, *Macropus eugenii*. J. Endocr. 55: 63-71.

Lemon M and Saumande J (1972) Oestradiol-17β and progesterone after induction of superovulation in cattle. J. Reprod. Fertil. 31: 501-502.

Lima SB, Verreschi IT and Ribeiro Neto LM (2001) Reversed-phase liquid chromatographic method for estrogen determination in equine biological samples. J Chromatogr Sci.39(9):385-7.

Lintern-Moore S, Moore GP, Tyndale-Biscoe CHA and Poole WE (1976) The growth of the oocyte and follicle in the ovaries of monotremes and marsupials. Anat Rec. 185(3):325-32.

Lopes S, Jurisicova A and Casper RF (1998) Gamete specific DNA fragmentation in unfertilized human oocytes after intracytoplasmic sperm injection. Human Reprod. 13(3): 703-708.

Lu Y, Bently GR, Gann PH, Hodges KR and Chatterton RT (1999) Salivary oestradiol and progesterone levels in conception and non-conception cycles in women: evaluation of a new assay for salivary oestradiol. Fertil. Steril. 71(5): 863-868.

Lubbadeh WF, Graves CN and Spahr SL (1980) Effect of repeated superovulation on ovulatory response of dairy cows. J. Anim. Sci. 50(1): 124-127.

Lyne AG, Pilton PE and Sharman GB (1959) Oestrous cycle, gestation period and parturition in the marsupial *Trichosurus vulpecula*. Nature. 183: 622-623.

Lyne AG and Hollis DE (1979) Observations of the corpus luteum during pregnancy and lactation in the marsupials *Isodon macrourus* and *Perameles nasuta*. Australian J. Zool. 27: 881-899.

Magarey GM and Mate KE (2000) Intracytoplasmic Sperm Injection of Tammar Wallaby Oocytes. [Abstract] Proceedings of the 14th annual conference of the Australian Society for Reproductive Biology. p.88.

Magli MC, Jones GM, Gras L, Gianaroli L, Korman I and Trounson AO (2000) Chromosome mosaicism in day 3 aneuploid embryos that develop to morphologically normal blastocysts in vitro. Human Reprod. 15(8): 1781-1786.

Mansour R (1998) Intracytoplasmic sperm injection: state of the art technique. Human Reprod. Update. 4(1): 43-56.

Marks C. (1998a) Courtship and mating in a pair of free-ranging Common Wombats *Vombatus ursinus*. In: *Wombats*. Eds: RT Wells and PA Pridmore. Surrey Beatty & Sons, Chipping Norton. pp125-128.

Marks CA (1998b) Review of the humaneness of destruction techniques used on the Common Wombat *Vombatus ursinus* in Victoria. In: *Wombats*. Eds: RT Wells and PA Pridmore. Surrey Beatty & Sons, Chipping Norton. pp287-297.

Mate KE and Rodger JC (1993) In vitro maturation of oocytes from a marsupial, the tammar wallaby (Macropus eugenii). Mol. Reprod. Dev. 34: 329-336.

Mate K (1996) Cytoplasmic Maturation of the Marsupial Oocyte during the Periovulatory Period. Reprod. Fertil. Dev. 8: 509-519.

Mate KE Molinia FC and Rodger JC (1998) Manipulation of the fertility of marsupials for conservation of endangered species and control of over-abundant populations. Anim. Reprod. Sci. 53: 65-76.

Mate KE and Buist JB (1999) Timing and regulatory aspects of oocyte maturation in vitro in the tammar wallaby (*Macropus eugenii*). Reprod. Fertil. Dev. 11: 247-254.

Mate KE, Sidhu KS, Molinia FC, Glazier AM and Rodger JC (2000) Sperm binding and penetration of the zona pellucida in vitro but not sperm egg fusion in an Australian marsupial, the brushtail possum (*Trichosurus vulpecula*). Zygote 8: 189-196.

McGregor F, Wells R (1998). Population status of the Southern Hairy-nosed Wombat *Lasiorhinus latifrons* in the Murraylands of South Australia. In: *Wombats*. eds. Wells R., Pridmore P. Surrey Beatty & Sons, Chipping Norton NSW. pp218-227.

McIlroy JC (1973) *Aspects of the ecology of the Common Wombat* Vombatus ursinus (Shaw 1800). Ph.D. Thesis. Australian National University, Canberra.

McIlroy JC (1990). The common wombat. Wildl. Aust. 11: 5-7.

McIlroy JC (1995) Common Wombat *Vombatus urinus*. In: *The Mammals of Australia*. Ed: R Strahan. Reed Books, Chatswood, NSW. pp204-205.

McLeod BJ, Hunter MG, Crawford JL and Thompson EG (1999) Follicle development in cyclic, anoestrous and FSH-treated brushtail possums (*Trichosurus vulpecula*). Anim Reprod Sci. 57(3-4):217-27.

McNab B (1986) Food habits, energetics, and the reproduction of marsupials. Journal of Zoology, London 208: 595-614.

Merchant JC (1976) Breeding biology of the agile wallaby, *Macropus agilis* (Gould) (Marsupialia: Macropdidae), in captivity. Australian Wildlife Research. 3: 93-103.

Merchant JC (1979) The effect of pregnancy on the interval between one oestrous cycle and the next in the tammar wallaby, *Macropus eugenii.* J. Reprod. Fertil. 56: 459-463.

Merchant JC and Calaby JH (1981) Reproductive biology of the red-necked wallaby (Macropus rufogriseus banksianus) and Bennett's wallaby (M. r. rufogriseus) in captivity. J. Zool. (London). 194: 203-217.

Molinia FC, Gibson RJ, Brown AM, Glazier AM and Rodger JC (1998a) Successful fertilization after superovulation and laparoscopic intrauterine insemination of the brushtail possum, Trichosurus vulpecula, and tammar wallaby, Macropus eugenii. J Reprod Fertil. 113(1):9-17.

Molinia FC, Gibson RJ, Smedley MA and Rodger JC (1998b) Further observations of the ovarian response of the tammar wallaby (Macropus eugenii) to exogenous gonadotrophins: an improved method for superovulation using FSH/ LH. Animal Reproduction Science 53: 253-263.

Molinia FC Glazier AM and Myers JV (2000) Superovulation of the Brushtail Possum during seasonal anoestrous with porcine FSH/LH. Proceedings of the 31st Annual Conference of the Australian Society for Reproductive Biology. [Abstract] p104

Molinia FC Glazier AM and Myers JV (2001) Egg nuclear maturation following superovulation of the Brushtail Possum during seasonal anoestrous. Proceedings of the 32nd Annual Conference of the Australian Society for Reproductive Biology. [Abstract no. 83].

Moore HDM and Taggart DA (1993) In vitro fertilisation and embryo culture in the grey short-tailed opossum, *Monodelphis domestica*. J. Reprod. Fertil. 98: 267-274.

Moritz K, Green RH and Selwood L (1998). A histological analysis of the reproductive cycle of the common wombat *Vombatus ursinus. In*: Wombats, Ch. 8: 75-85. *Eds.* Wells R T and Pridmore P A. Surrey Beatty and Sons, Chipping Norton.

Murphy BD and Martinuk SD (1991) Equine chorionic gonadotrophin. Endocr. Rev. 12(1): 27-44.)

Nelsen O, White L. (1941) A method for inducing ovulation in the anoestrous opossum (*Didelphys virginiana*). Anatomical Records 81: 529-535.

Nelsen O, Maxwell N. (1941) Induced oestrus and mating in the opossum, *Didelphys virginiana*. Anatomical Records 81: 105.

Neuber E, Lutetjens CM, Chan AWS and Schatten GP (2002) Analysis of DNA fragmnetation of in vitro cultured bovine blastocysts using TUNEL. Theriogenology. *In press*.

Nicholson PJ (1963) Wombats *Timbertop magazine* no. 8. Geelong Grammar.

O'Donoghue CH (1916) On the corpora lutea and interstitial tissue of the ovary in the marsupial. Quart. J. Micro. Sci. 61: 433-479.

Ongeri EM, Bormann CL, Butler RE, Melican D, Gavin WG, Echelard Y, Krisher RL and Behboodi E (2001) Development of goat embryos after in vitro fertilisation and parthenogenetic activation by different methods. Theriogenology. 55: 1933-1945.

Othen LS, Bellem AC, Gartley CJ, Aucland K, King WA, Liptrap RM and Goodrowe KL (1999) Hormonal control of oestrus cyclicity and attempted superovulation in wood bison (Bison bison athabascae). Theriogenology. 52 (2): 313-323.

Paris MCJ, White A, Reiss A, West M, Schwarzenberger F (2002) Faecal progesterone metabolites and behavioural observations for the non-invasive assessment of oestrous cycles in the Common Wombat (*Vombatus ursinus*) and the Southern Hairy-nosed Wombat (*Lasiohinus latifrons*). Anim. Reprod. Sci. *In press*.

Parkes AS (1932) The reproductive process of certain mammals. II. The size of the Graafian follicle at ovulation. Proc. R. Soc. B. 109: 185-196.

Pedersen T & Peters H (1968) Proposal for a classification of oocytes and follicles in the mouse ovary. J Reprod Fertil. 17:555-557.

Peters DG (1977) The oestrous cycle, anoestrous and body temperature in the Tasmanian wombat, *Vombatus ursinus*. PhD thesis, The University of Tasmania, Hobart.

Peters DG and Rose RW (1979) The estrus cycle and basal body temperature in the common wombat (*Vombatus ursinus*). J. Reprod. Fertil. 57: 453-460.

Phillips LG, Simmons LG, Bush M Howard JG and Wildt DE (1982) Gonadotrophin regimen for inducing ovarian activity in captive wild felids. J. Am. Vet. Med. Assoc. 181(11): 1246-1250.

Pilton PE and Sharman GB (1962) Reproduction in the marsupial *Trichosurus vulpecula*. J. Endocrinology. 25:119-136.

Pollard I and Cairncross KD (1977) Ultrastructural changes in the adenohypophysis adrenal gland activity, and the desynchronization of the oestrous cycle following unpredictable stress in the rat. Aust. J Biol. Sci. 30(6): 559-572.

Poole WE and Catling PC (1974) Reproduction in the two species of grey kangaroo, *Macropus giganteus* (Shaw) and *M. Fuliginosus* (Desmarest). I. Sexual maturity and oestrus. Aust. J. Zool. 22: 227-302.

Poole WE, Merchant JC, Carpenter SM and Calaby JH (1985) Reproduction growth and age determination in the yellow-footed rock-wallaby, *Petrogale xanthopus* Gray, in captivity. Aust. Wildlife Research. 12: 127-136.

Poole WE, Westcott M and Simms NG (1992) Determination of oestrus in the female tammar, *Macropus eugenii*, by analysis of cellular composition of smears from the reproductive tract. Wildlife research. 19: 35-46.

Presidente PJA (1982) Common Wombat Vombatus ursinus: maintenance in captivity, blood values, infectious and parasitic diseases. In: The management of Australian Mammals in Captivity. Ed: DD Evans. The Zoological Board of Victoria, Melbourne.pp 133-143.

Pushett DA, Gunn IM and Trounson AO (1997) Retrieval of parthenotes-like embryos from the ovaries of domestic cats. Theriogenology. 47(1): 404.

Pushett D (2000) Assisted reproduction and subsequent embryo development to form embryos for transfer and cryobanking in endangered cats using the domestic cat as a model. Honours thesis, Monash University, Clayton, Victoria

Renfree M and Tyndale- Biscoe CH (1973) Intra-uterine development after diapause in the marsupial *Macropus eugenii*. Developmental Biology 32: 28-40.

Renfree MB, Shaw G, Fletcher TP (1988) Superovulation in a macropod marsupial Macropus eugenii. Proc. 23rd Ann. Conf. Aust. Soc. Reprod. Biol., Vol. 8: 725-742.

Renfree MB and Lewis AM (1996) Cleavage in vivo and in vitro in a marsupial Macropus eugenii. Reprod. Fertil. Dev. 8: 725-742.

Renfree MB and Shaw G (1999). Marsupials. *In:* Encyclopedia of Reproduction Vol. 3 *Eds.* Knobil E and Neill J D. Academic Press, San Diego.

Renfree MB and Blanden DR (2000) Progesterone and Oestrogen receptors in the female genital tract throughout pregnancy in tammar wallabies. Journal of Reproduction and Fertility. 119: 121-128.

Reynolds HC (1952) Studies on reproduction in the opossum (*Didlephis virginiana virginiana*). University of California Publications in Zoology. 52: 223-84.

Rodger JC (1990) Prospects for the artificial manipulation of marsupial reproduction and its application in research and conservation. Aust. J. Zool. 37: 249-258.

Rodger JC, Giles I and Mate KE (1992) Unexpected growth after follicular antrum formation in four marsupial species. J. Reprod. Fertil. 96 (2): 755-763.

Rodger JC and Mate KE (1988) A PMSG/GnRH method for the superovulation of the monovulatory brush-tailed possum (Trichosurus vulpecula). J Reprod Fertil. 83(2):885-91.

Rodger JC, Giles I and Mate KE (1992) Unexpected oocyte growth after follicular antrum formation in four marsupial species. J Reprod Fertil.96(2):755-63.

Rodger JC, Breed WG and Bennett JH (1992) Gonadotrophin-induced oestrus and ovulation in the polyovulatory marsupial Sminthopsis crassicaudata. Reprod Fertil Dev. 4(2):145-52.

Rodger JC, Cousins SJ, Mate KE and Hinds LA (1993). Ovarian function and its manipulation in the tammar wallaby, *Macropus eugenii*. Reprod. Fertil. Dev. 5 (1): 27-38.

Rose RW and McCartney DJ (1982) Reproduction in the red-bellied pademelon *Thylogale billardierii* (Marsupialia). Australian Wildlife Research. 9: 27-32.

Rose RW and Jones SM (1996) The association between basal body temperature, plasma progesterone and the oestrus cycle in a marsupial, the Tasmanian bettong (*Bettongia gaimardi*). J. Reprod. Fertil. 106: 67-71.

Saiha O and Balen AH (2000) New concepts in superovulation strategies for assisted conception treatments. Curr. Opin. Obstet. Gynecol. 12(3): 201-206.

Sathananthan AH, Szell A, Ng SC, Kausche A, Lacham-Kaplan O and Trounson A (1997) Is the acrosome reaction a prerequisite for sperm incorporation after intra-cytoplasmic sperm injection (ICSI)? Reprod. Fertil. Dev. 9: 703-709.

Saumande J (1980) Concentrations of luteinizing hormone, oestradiol-17β and progesterone in the plasma of heifers treated to induce superovulation. J. Endocrinology. 84:425-437.

Schwarzenberger F, Palme R, Bamberg E and Mostl E (1997) A review of progesterone metabolite analysis for invasive monitoring of reproductive function in mammals. International Journal of Mammalian Biology. Suppl 2: 214-221.

Selwood L (1980) A time table of embryonic development of the dasyurid marsupial Antechinus stuartii (Macleay). Aust. J. Zool. 28:649-668.

Selwood L (1987) Embryonic development in culture of two dasyurid marsupials S. *crassicaudata* (Gould) and S. macroura (Spencer) during cleavage and balstocyst formation. Gamete Research. 16: 355-370.

Selwood L and Woolley P (1991) A timetable of embryonic development and ovarian and uterine changes during pregnancy in the stripe-faced dunnart J. Reprod. Fertil. 91: 213-227.

Selwood L and Vandeberg JL (1992) The influence of incubation temperature on oocyte maturation, parthenogenetic and embryo development in vitro of the marsupial *Monodelphis domestica*. Animal Reproduction Science. 29: 99-116.

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

Selwood L (2001) Mechanisms for pattern formation leading to axis formation and lineage allocation in mammals: a marsupial perspective. Reproduction. 121: 677-683.

Sharman GB (1955) Studies on marsupial reproduction. II. The oestrus cycle of *Setonix brachyurus*. Aust. J. Zool. 3: 156-161.

Sharman GB and Calaby JH (1964) Reproductive behaviour in the red kangaroo, Megaleia rufa, in captivity. C.S.I.R.O. Wildlife Research. 9: 58-85.

Shaw G and Renfree MB (1984) Concentrations of oestradiol-17β in plasma and corpora lutea throughout pregnancy in the tammar *Macropus eugenii*. J. Reprod. Fertil. 72:29-37.

Shorey CD and Hughes RL (1973) Development function and regression of the corpus luteum in the marsupial *Trichosurus vulpecula*. Aust. J. Zool. 21: 477-489.

Sidhu KS, Mate KE, Molinia FC, Berg D, Rodger JC (2001) In vitro sperm-egg binding, penetration and fusion in an Australian marsupial species, the Brushtail Possum (*Trichosurus vulpecula*). Proceedings of the 32nd Annual Conference for the Society of Reproductive Biology. [Abstract 22].

Sirois J and Fortune JE (1988) Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. Biol. Reprod. 39: 308-317.

Sloane MA, Sunnicks P, Alpers D, Beheregaray LB and Taylor AC (2000) Highly reliable genetic identification of individual northern hairy-nosed wombats from

single remotely collected hairs: a feasible censusing method. Molecular Ecology. 9: 1233-1240.

Smith JF, Berg DK, McDonald RM, Beaumont S, Sidhu KS, Molinia FC and Duganzich DM (2001) Effect of possum synthetic oviduct and conditioned media on the motility and viability of Brushtail Possum (*Trichosurus vulpecula*). Proceedings of the 32nd Annual Conference for the Society of Reproductive Biology. [Abstract 22].

Smith MJ and Godfrey GK (1970) Ovulation induced by gonadotrophins in the marsupial, Sminthopsis crassicaudata (Gould). J Reprod Fertil. 22(1):41-7.

Smitz J, Nogueira D, Albano C, Cortvrindt R, Devroey P. (2001) Improving in vitro manipulation of oocytes in the human taking lessons from the experiences in animal species. Reprod. Domest. Anim. 36 (1): 11-17.

Stewart F and Tyndale-Biscoe CH (1982) Prolactin and luteinizing hormone receptors in marsupial corpora lutea: relationship to control of luteal function. J. Endocrinol. 92 (1): 63-72.

Stewart EA. (2001) Gonadotrophins and the uterus: is there a gonad independent pathway? J Soc Gynecol. Investig. 8(6) 319-326.

St John B. (1998) Management of Southern Hairy-nosed Wombat *Lasiorhinus latifrons* of South Australia. In: *Wombats.* eds. Wells R., Pridmore P. Surrey Beatty & Sons, Chipping Norton NSW. pp228-242

Stott AV (1998) Problems with Southern Hairy-nosed Wombats Lasiorhinus latifrons in the agricultural lands of the far west of South Australia. In: *Wombats.* Eds: RT Wells and PA Pridmore. Surrey Beatty & Sons, Chipping Norton. pp280-286.

Strahan R (1995) The Mammals of Australia. Reed Books, Chatswood, NSW.

Sutherland RL, Evans SM and Tyndale-Biscoe CH (1980) Macropod marsupial luteinizing hormone: validation of assay procedures and changes in concentration in plasma during the oestrus cycle in the female tammar wallaby (*Macropus eugenii*). J. Endocrinology. 86: 1-12.

Taggart D., Steele V., Schultz D., Dibben R., Dibben J., Temple-Smith P. (1998). Semen collection and cryopreservation in the Southern Hairy-nosed Wombat *Lasiorhinus latifrons*: implications for conservation of the Northern Hairy-nosed Wombat *Lasiorhinus krefftii*. In: *Wombats.* eds. Wells R., Pridmore P. Surrey Beatty & Sons, Chipping Norton NSW. pp180-191.

Taylor A, Sherwin W, Wayne R. (1994) Genetic variation of microsatellite loci in a bottlenecked species: the Northern Hairy-nosed Wombat *Lasiorhinus krefftii.* Molec. Ecol. 3: 277-290.

Taylor A, Horsup A, Johnson C, Sunnucks P, Sherwin B. (1997) Relatedness structure detected by microsatellite analysis and attempted pedigree reconstruction in an endangered marsupial, the northern hairy-nosed wombat, *Lasiorhinus krefftii.* Molecular Ecology 6:9-19.

Taylor RJ (1993) Observations of the behaviour and Ecology of the Common Wombat *Vombatus ursinus* in northeast Tasmania. Australian Mammalogy. 16(1) 1-7.

Tesarik J and Sousa M (1995a) Key elements of a highly efficient intracytoplasmic sperm injection: Ca^{2+} fluxesand the oocyte cytoplasmic dislocation. Fertil. Steril. 64: 770-776.

Tesarik J and Sousa M (1995b) More than 90% fertilisation rates after intracytoplasmic sperm injection and artificial induction oocyte activation with calcium ionophore. Fertil. Steril. 63: 343-349.

Tesarik J and Mendoza C (1999) In vitro fertilization by intracytoplasmic sperm injection. BioEssays. 21: 791-801.

Thornburn GD, Cox RI and Shorey CD (1971) Ovarian steroid secretion rates in the marsupial *Trichosurus vulpecula*. J. Reprod. Fertil. 24:139.

Treagust DF, Folk GE Randall W and Folk MA (1979) The circadian rhythm of body temperature of unrestrained opossums, *Didelphis virginia*. J. Thermal Biology. 3: 251-255.

Torres S and Sevellec C (1987) Repeated superovulation and surgical recovery of embryos in the ewe. Reprod. Nutr. Dev. 27(4): 859-863.

Towers PA, Shaw G and Renfree MB (1986) Urogenital vasuclarture and local steroid concentrations in the uterine branch of the ovarian vein of the female tammar wallaby (*Macropus eugenii*). J. Reprod. Fertil. 78 (1): 37-47.

Triggs B, Goldingay R. (1996) *The Wombat*: Common wombats in Australia. University of New South Wales Press. Sydney, NSW.

Trounson A, Anderiesz C and Jones G (2001) Maturation of human oocytes in vitro and their developmental competence. Reproduction. 121: 51-75.

Tyndale-Biscoe CH, Hearn JP and Renfree MB (1974) Control of reproduction in Macropodid marsupials. J. Endocr. 63: 589-614.

Tyndale-Biscoe CH and Hawkins J (1977) The corpora lutea of marsupials, aspects of function and control. In: *Reproduction and Evolution*. Eds: JH Calaby and CH Tyndale-Biscoe pp 245-252 Canberra: Australian Academy of Science.

Tyndale-Biscoe CH and Rodger JC (1978) Differential transport of spermatozoa into the two sides of the genital tract of a monovular marsupial, the tammar wallaby (Macropus eugenii). Journal of Reproduction and Fertility 52: 37-43.

Tyndale-Biscoe CH, Hinds LA, Horn CA and Jenkin G (1983) Hormonal changes at oestrus, parturition, and post-partum oestrus in the Tammar Wallaby (Macropus eugenii). J. Endocrinology. 96: 155-161.

Tyndale-Biscoe CH and Hinds LA (1984) Seasonal patterns of circulating progesterone and prolactin and in response to bromocriptine in the female tammar *Macropus eugenii*. Gen. Comp. Endocrinol. 53:58-68.

Tyndale-Biscoe H and Renfree M (1987) Reproductive physiology of marsupials. Cambridge University Press, Cambridge.

Van Niekerk FE and Van Niekerk CH (1997) The effect of dietary protein on reproduction in the mare. IV Serum progesterone, FSH, LH and melatonin concentrations during the anovulatory transitional and ovulatory periods in the non-pregnant mare. J. S. Afr. Vet. Assoc. 68(4): 114-120.

Walker MT and Gemmell RT (1983) Plasma concentrations of progesterone, oestradiol-17β and 13,14-dihydro-15-oxo-prostaglandin $F_{2\alpha}$ in the pregnant wallaby (*Macropus rufogriseus rufogriseus*). J. Endocrin. 97: 369-377.

Waring H, Sharman GB, Lovat D and Kahan M (1955) Studies on marsupial reproduction. General features and techniques. Aust. J. Zool. 3: 34-43.

Web R, Campbell BK, Garverick HA, Gong JG, Gutierrez CG and Armstrong DG. (1999) Molecular mechanisms regulating follicular recruitment and selection. J Reprod Fertil Suppl 54: 33-48.

Wells RT (1978) Field observations of the hairy nosed wombat *Lasiorhinus latifrons* (Owen). Aust. Wild. Res. 5: 299-303.

Wells RT (1995) Southern Hairy-nosed Wombat. In: *The Mammals of Australia*. Ed: R Strahan. Reed Books, Chatswood, NSW. pp202-203.

Welsh TH and Johnson BH (1981) Stress induced alteration in secretion of corticosteroids, progestins, luteinizing hormones and testosterone in bulls. Endocrinology. 109: 185-190.

West M, Lacham-Kaplan O, Bickell C, Cleary M, Galloway D, Edwards G, Trounson A & Paris M (2002) Successful superovulation of the common wombat and cleavage following ICSI of resulting oocytes. Theriogenology. 57(1): 594.

Williams LM and Menkhorst PW (1995) Common Wombat. In: *Mammals of Victoria; distribution, ecology and conservation.* Ed: PW Menkhorst. Oxford University Press, Melbourne.

Winston N, Johnson M, Pickering S and Bruade P (1991) Parthenogentic activation and development of fresh and aged human oocytes. Fertil. Steril. 56(5): 904-912.

Wolfe BA and Wildt DE (1996) Development to blastocyst of domestic cat oocytes matured and fertilized in vitro after prolong cold storage. J. Reprod. Fertil. 106: 135-141.

Woodford J (2001) *The secret life of Wombats.* Text publishing. Melbourne, Victoria.

Woolley PA (1971) Maintenance and breeding of laboratory colonies of *Dasyuroides byrnei* and *Dasycercus cristicauda*. International Zoo Yearbook. 11:351-354.

Woolley P. (1990) Reproduction in *Sminthopsis macroura* (Marsupialia:Dasyuridae): The Female. Australian Journal of Zoology 38: 187-205.

Yamauchi N, Saada H, Soloy E, Dominko T, Kikuchi K and Nagai T (1999) Effects of hormones and osmolarity in the culture media on germinal vesicle breakdown of porcine oocytes. Theriogenology. 52: 153-162.

Yanagimachi R (1998) Intracytoplasmic sperm injection experiments using the mouse as a model. Human Reprod. 13(suppl 1): 87-98.

Young CE and McDonald IR (1982) Oestrogen receptors in the genital tract of the Australian Marsupial *Trichosurus vulpecula* General and Comparative Endocrinology. 46:417-427.