Ovarian follicle growth and yolk formation in the New World marsupial *Monodelphis domestica*.

By

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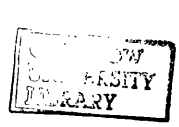

DECLARATION

I declare that this thesis is my own composition and that all of the research described has been carried out by myself. None of the material presented in this thesis has been submitted for any other degree. All animal procedures were carried out under licence from the Home Office: Project Licence 60/01168; Personal Licence 60/04231.

Liza Butcher

March 1995

DEDICATION

This work is dedicated to my Mother, my Father (affectionately known as Boyo) and to Pop.

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ABSTRACT

The adult *Monodelphis domestica* ovary was found to be similar to that of the eutherian mammal possessing follicles in various stages of development, corpora lutea and interstitial tissue. Polyovular follicles, containing between two and eleven oocytes, were found in approximately 25% of the individuals observed.

Folliculogenesis proceeded in an identical manner to that in the eutherian but oocyte growth continued throughout follicular development. It therefore did not conform to the strict biphasic pattern of follicle growth previously described in eutherian and marsupial species.

Oestrous cycles were monitored and, contrary to previous studies, female *M. domestica* isolated from males continued to exhibit oestrous cycles. All females appeared to exhibit repetitive cycles of ovarian follicle growth accompanied by changes in the reproductive tract. If exposed to the stimulus of a male, a group of large follicles completed their development and ovulation occurred. In the absence of such stimulus, these follicles became atretic and were replaced by a successive wave of follicles.

At the ultrastructural level, the growth and development of the oocyte was accompanied by an increase in the number of its organelles and a change in their distribution which reflected the metabolic activity involved during such growth. Apart from the conventional organelles found within oocytes, lamellar complexes and two unusual forms of mitochondria were observed at different stages of development.

Smooth endoplasmic reticulum and Golgi bodies appeared to be responsible for the formation of multivesicular bodies which were first observed in the oocytes of primary follicles. As the multivesicular bodies increased in size they became more flocculent in nature until they formed transparent vesicles which exhibited no polarity. These then coalesced to form a large vesicular mass, occupying most of the central region of the oocyte, which was identical to the "yolk" mass described in other marsupial species.

Histochemical analyses revealed that in smaller oocytes the cytoplasm consisted mostly of carbohydrate and protein but during development this was replaced by the yolk vesicles. The nature of

these vesicles remained undetected by histochemical staining and it was only by means of molecular probe labelling that lipid was detected.

Autoradiographical analysis revealed that metabolites were transported from the circulation into the oocytes and that this uptake was fairly uniform in all developmental stages of oocyte.

For the first time in a marsupial, individual preantral follicles were cultured *in vitro*. Although follicles grew for up to 8 days, and sometimes exceeded the size where antrum formation was observed *in vivo*, definite antrum formation was not achieved.

CONTENTS

Chapter 1

General Introduction.

1.1. INTRODUCTION

1.1.1. Origin of marsupials.

Within the Class Mammalia there are three distinct lineages: the monotremes which lay eggs, the marsupials which give birth to relatively undeveloped young and the eutherians which give birth to more developed young. The once traditional view that marsupials were primitive or inferior compared to eutherian mammals has gradually changed and it is now acknowledged that they merely represent an alternative path from eutherian evolution.

Fossil evidence has shown that eutherians and marsupials shared a common ancestor and that their differentiation occurred more than 100 million years ago. The earliest fossil records which are undoubtedly marsupial are 70 - 80 million years old and come from the Milk River formation of Alberta, Canada (Fox, 1971). Thus, it is strongly believed that marsupials originated in western North America, some time during the Cretaceous, radiated throughout North and South America and then dispersed across the globe by various routes. Although several theories have been proposed, with varying amounts of fossil evidence, the dispersive history of the marsupials is yet to be truly resolved. From the available evidence the following account appears to be the most probable. From North America, one group of didelphids reached Europe, probably via Greenland, and survived there until competition from eutherians led to their demise. Others radiated from South America island-hopping along the archipelago of West Antarctica, across East Antarctica and thence into Australia. The discovery of a marsupial fossil in Antarctica (Woodburne & Zinmeister, 1982) strongly supports the theory of southern route of dispersal. Following their separation there was adaptive radiation of the South American and Australian species and different patterns of evolution occurred on the two continents. South America already had a variety of eutherian species and so the marsupials entered the vacant niches available to carnivores and insectivores. Lacking any eutherian competitors the Australian marsupials were not subject to similar constraints and so showed much greater diversification.

The marsupials form a distinctive group which have long been accepted as standing apart from the main body of the Class and the study of these animals began early in the 16th Century with the discovery of the opossum by Spanish explorers of South America (Clemens, 1968). Originally, biologists grouped the marsupials with a variety of eutherian mammals which showed similar

characteristics so Linnaeus classified the opossum with armadillos, hedgehogs, pigs and shrews because of their common possession of sharp teeth (Tyndale-Biscoe, 1971). It was not until 1816, that de Blainville recognised that, despite their many similarities with the carnivores and rodents, marsupials could be distinguished from other mammals by their anatomical and physiological features of reproduction.

Despite their name (marsupial being derived from the Greek word for purse *marsupion*) not all marsupials possess a pouch and there is considerable variation between the pouch of different species. The most developed form is found in marsupials which climb (phalangers), dig (bandicoots and wombats, in which the pouch opens to the rear), hop (kangaroos) or swim (yapoks), whilst some small terrestrial species (short-tailed opossums) are pouchless (Kirsch, 1977a). In certain marsupials the pouch merely consists of folds of skin around the mammae and in many species a pouch is only developed when the mother is suckling.

Marsupials are currently found in two widely separated regions of the world - Australasia and the Americas. There are about 250 living species, assigned to 16 families, with approximately two-thirds of them being Australasian. The Americas are inhabited by just three families - the Didelphidae, Caenolestidae and Microbiotheriidae - of which the Didelphidae is the largest containing some 70 species.

Although several Australian and American marsupials have been kept and bred under captive conditions, few have proved suitable as laboratory animals. Before *M. domestica* was introduced to captivity, attempts were made to utilise several other Didelphids such as the Virginia opossum (*Didelphis virginiana*: 1969; Jurgelski, 1974; Harder & Fleming, 1982), mouse opossums (*Marmosa elegans,* and *M. mitis*: Barnes, 1968; *M. robinsoni*: Godfrey, 1975), woolly opossum and grey four-eyed opossum (*Caluromys derbianus* & *Philander opossum*: Barnes, 1968). All of these showed various limitations such as low reproductive and survival rates, aggressive behaviour, cannibalism, disease susceptibility and dietary problems.

1.1.2. Marsupial reproductive anatomy.

The unusual reproductive anatomy of the female marsupial has been known for more than 250 years since Tyson dissected and described the genital tract of an opossum (Tyndale-Biscoe, 1966).

Unlike eutherians the anal and urogenital openings are enclosed within a common sphincter, the ureters are located medially, rather than laterally, relative to the genital ducts and the reproductive tract is bifid with two vaginae and uteri. It is the relationship of the urinary and genital tracts, seen in both sexes, that differentiates marsupials from eutherians. In marsupials the urinary ducts pass between the genital ducts whereas in eutherians they pass laterally. In female eutherians the two oviducts can fuse together in the midline, to form a single vagina and uterus, whereas in marsupials fusion is impeded by the ureters and cannot occur. In male eutherians each pronephric duct must loop around the inside of the ureter to reach the testis, while in marsupials this loop does not exist.

The unusual vaginal apparatus consists of two lateral vaginae, which at the anterior end form the vaginal culs de sac which leads to the two uteri. Posteriorly, the vaginae open into the urogenital sinus which also receives the urethra (Tyndale-Biscoe & Renfree, 1987). At parturition the birth canal, or median vagina, is formed which connects the vaginal culs de sac and the urogenital sinus. In most marsupials the median vagina closes back up and must be reformed before each birth. This condition is seen in all species of Caenolestidae, Dasyuridae, Didelphidae, Peramelidae, Potoroinae and most Phalangeridae. In the Macropodidae and Tarsipedidae though, the median vagina becomes lined with epithelium after the first birth and so forms a permanent birth canal (Sharman, 1970).

1.1.3. Mammalian ovulation.

Classification of mammalian species into either induced or spontaneous ovulators has been a common practice for many years but it is now accepted that such a strict classification must be treated with some caution (Weir & Rowlands, 1973; Milligan, 1982) as allocation to one group, or the other, may be quite arbitrary. Several eutherian species, known as spontaneous ovulators, have converted to induced ovulation under certain conditions (Jöchle, 1975) and spontaneous ovulations have been reported in many of the species (rabbit: Walton & Hammond, 1928; Pincus, 1948; llama: England *et al.*, 1969; alpaca: Fernandez-Baca *et al.*, 1970; Bravo & Sumar, 1989; pine vole: Kirkpatrick & Valentine, 1970; montane vole: Gray *et al.*, 1974; short-tailed field vole: Milligan, 1974; mink: Moller, 1974 and lion: Schmidt *et al.*, 1979) generally considered as induced ovulators.

In spontaneous ovulators there is a set hormonal pattern and the LH surge occurs at a particular time which is independent of any direct external stimuli. Spontaneous ovulations therefore tend to recur

at regular intervals in the unmated female. With induced ovulators, ovulation occurs following a surge in LH secretion caused directly by some form of external stimulus. There is, however, a considerable variation between species in their response to stimulation. Although coitus readily induces ovulation in most species, olfactory, visual, tactile or acoustic stimuli can also be effective. Rabbits may ovulate following mounting by other females (Staples, 1967) and mink may ovulate following brief non-sexual contact with a male (Enders, 1952).

Since spontaneous ovulators exhibit regular oestrus cycles it has often been assumed that induced ovulators do not show cycles and either remain in a state of constant oestrus or at least show long periods of continued oestrus (Weir & Rowlands, 1974). Although some individuals of a species may sometimes exhibit a prolonged oestrus period this seems to be the exception rather than the rule. Studies of the duration and periodicity of oestrus in induced ovulators suggest that there is considerable variability both between species as well as between individuals within the same species. Some induced ovulators exhibit no cyclic changes in vaginal, ovarian or sexual behaviour (montane vole: Gray *et al.*, 1974; woodchuck: Sinha Hikim *et al.*, 1991; 1992 and musk shrew: Fortune *et al.*, 1992) whereas others do show cyclic variations (rabbit: Hill & White, 1933; Hamilton, 1951; cat: Shille *et al.*, 1979; mink: Enders, 1952; camel and alpaca: Bravo & Sumar, 1989 and field vole: Milligan, 1974).

Among the many marsupials studied, the phenomenon of male-induced oestrus and ovulation has only been observed in two species: the South American grey short-tailed opossum (*Monodelphis domestica*: Fadem & Rayve, 1985) and the Australian brush-tailed bettong (*Bettongia penicillata*: Hinds & Smith, 1992). However, males have been found to have an effect on the frequency and synchrony of oestrus in several marsupial species which are spontaneous ovulators (*Sminthopsis crassicaudata*: Smith *et al.*, 1978; *Antechinus stuartii*: Dickman, 1985; Scott, 1986; *Caluromys philander*: Perret & Ben M'Barek, 1991).

1.1.4. Detection of oestrous cycles.

The vagina, or the urogenital tract, can be a useful source of information on the reproductive state of an animal. Ideally, the vagina, sloughed-off cells and the mucus show characteristic changes which can be sampled and categorized into various stages. Under the influence of ovarian oestrogens, the vaginal epithelium proliferates and forms a cornified layer of cells which is then shed - therefore, a

smear with cornified cells indicates follicular activity. The appearance of leucocytes in a smear is associated with progesterone secretion and indicates luteal activity (Parkes, 1926). Techniques for obtaining samples from the reproductive tract can be modified and applied to most mammals - apart from biopsy, sampling methods include swabbing, scraping, flushing and aspiration of fluids (D'Souza, 1978).

With laboratory rodents such as the mouse and rat, the various stages of the oestrous cycle can be readily identified using vaginal smears. In proestrus there is active growth in the reproductive tract and the cells sloughed off from the vaginal epithelium are predominantly nucleated. During oestrus the epithelium of the reproductive tract has proliferated and the vaginal epithelium has developed a deep layer of cornified cells which are shed in the smear. This is followed by metoestrus during which there are degenerative changes in the reproductive tract and the smear consists mostly of cornified cells and leucocytes. During dioestrus smears typically contain fewer cells than at other stages and consist mostly of leucocytes and a few nucleated cells.

Since the initial study of the guinea pig oestrous cycle by Stockard & Papanicolaou (1917), exfoliative vaginal cytology has been used to determine the reproductive state of various mammalian species including the rat (Long & Evans, 1922), mouse (Allen, 1922; Whitten, 1958) rabbit (Hamilton, 1951), cat (Shille *et al.*,1979), mink (Enders, 1952; Travis *et al.*, 1978) and various voles (Milligan, 1974; Gray *et al.*, 1974).

Smears have also been used to follow the oestrous cycle of many marsupials such as the Virginia opossum (*Didelphis virginiana*: Hartman, 1923; Jurgelski & Porter, 1974; Feldman & Ross, 1975; Harder & Fleming, 1982; Rodger & Bedford, 1982); common and white-eared opossums (*D. marsupialis* and *D. albiventris*: Tyndale-Biscoe & Mackenzie, 1976); mouse opossum (*Marmosa robinsoni*: Godfrey, 1975); marsupial mouse (*Sminthopsis larapinta*: Godfrey, 1969); brush-tailed possum (*Trichosurus vulpecula*: Lyne *et al.*, 1959; Pilton & Sharman, 1962); mountain possum (*T. caninus*: Smith & How, 1973); woolly possum (*Caluromys philander*: Perret & Atramentowicz, 1989) ; potoroo (*Potorous tridactylus*: Hughes, 1962); bandicoots (*Isoodon macrourus* and *Perameles nasuta*: Lyne, 1976); common wombat (*Vombatus ursinus*: Peters & Rose, 1979); bettong (*Bettongia lesueur*: Tyndale-Biscoe, 1968); Rottnest Island wallaby (*Setonix brachyurus* Sharman, 1955); and grey kangaroos (*Macropus giganteus* Shaw and *M. fuliginosus*: Poole & Catling, 1974). Like many non-

domestic species though, marsupial smears do not always conform to the expectations of what should happen and may be highly idiosyncratic.

Most marsupial workers have adopted the phrase "vaginal" smear but strictly speaking this is incorrect since, due to the marsupial reproductive anatomy, smears are actually taken from the urogenital sinus. The more accurate term "urogenital sinus" smear has therefore been employed in this study.

Although vaginal / urogenital sinus smearing can often be very valuable, the procedure does have its limitations. Not all species show the classic stages of vaginal cornification and even if the phenomenon is characteristic of a certain species, not every animal will show changes to the same degree. Therefore, one must always consider individual variation between animals of the same species and between the cycle of one individual. The technique itself is also not without its disadvantages - frequent vaginal smearing using cotton swabs has been reported to result in an abnormally high occurrence of cornified smears in rats (Emery & Schwabe, 1936; Astwood, 1939) and mice (Wade & Doisy, 1935).

It was Allen in 1922 who first noted that, in the mouse, the appearance of the vagina changed during the oestrous cycle. Visual assessment of the vagina as a method for identifying murine oestrous stages was subsequently found to be as reliable as vaginal smearing, less stressful to the animal and avoided the problems associated with mechanical manipulation during smearing (Bingel & Schwartz, 1969a; Champlin *et al.*, 1973). Although suitable for mice, visual assessment is of limited use in species, such as the rabbit (Carlyle & Williams, 1961) and mink (Enders, 1952), which often show vulval swelling but will mate and ovulate when there are no external signs of stimulation.

1.1.5. Oogenesis in the mammalian ovary.

The adult ovary contains many oocytes derived from primordial germ cells (PGCs) which colonize the ovary during embryonic development. These PGCs are the sole source of adult germ cells and are initially detected in the endoderm of the yolk sac. During their migration to the gonadal ridges, the PGCs have been observed to undergo mitosis in both eutherian (Zamboni & Merchant, 1973) and marsupial species (Alcorn, 1975; Ullmann, 1981a). It is possible to distinguish migrating PGCs from most of the surrounding somatic cells by several criteria. Their nuclei are spherical and their cytoplasm

is clear in appearance due to a paucity of organelles and in some eutherian (mouse: Clark & Eddy, 1975) and marsupial species (*Macropus eugenii*: Alcorn, 1975; *Isoodon macrourus*: Ullmann, 1981a) the nuclear envelope is characterized by a rim of heterochromatin which disappears upon arrival at the gonadal ridges.

Upon reaching the gonadal ridges, the PGCs divide mitotically to form a population of oogonia which also proliferate by mitosis. Once the oogonia have completed their last mitotic division they become primary oocytes and enter the first meiotic prophase. The stage of development at which meiosis begins varies between species and can be immediate or delayed (Byskov, 1986). In immediate meiosis, the oogonia enter meiosis simultaneously with, or shortly after, the onset of gonadal sexual differentiation, as is the case in the mouse, rat and human. Alternatively, in species with delayed meiosis, such as the cow, pig and sheep, there is a time delay separating gonadal differentiation and the onset of meiosis. In contrast to most eutherians where ovarian development occurs prenatally, ovarian growth and differentiation in marsupials occurs postnatally during the so-called pouch life of the young.

Irrespective of the timing of meiosis, primary oocytes enter a prolonged resting phase during prophase known as the dictyate stage (Franchi *et al.*, 1962) and remain in this state until stimulated, shortly before ovulation in most species, to resume meiosis. It is whilst they are arrested in meiotic prophase that primary oocytes undergo considerable growth and development and become enclosed by granulosa cells to form follicles.

In eutherians, the growth of the oocyte and its follicle have been found to follow a biphasic pattern (Brambell, 1928; Parkes, 1931). During the first phase, growth occurs synchronously whereas the second phase, which coincides with antrum formation, is characterized by follicle growth alone. Studies conducted on marsupials have reported that they conform to the same biphasic pattern with the only difference from the eutherians being the larger size of the marsupial oocyte and follicle (Lintern-Moore *et al.*, 1976).

1.1.6. Ultrastructure of the ovarian follicle.

Compared to eutherian studies, very little attention has been paid to the ultrastructure of marsupial oocytes and such studies have only focussed on a certain stage of development. In her study of the the primordial oocyte of the bandicoot (*Isoodon macrourus*) Ullmann (1978) described various

unique structures including a paranuclear complex; a vesicle-microtubule complex and an aggregate of tubular cisternae. Subsequent studies on the oocytes from Graafian follicles of this species (Lyne & Hollis, 1983) found inclusions similar to some of those described by Ullmann (1978) but astonishingly, there has been no work covering the expanse of oocyte development between these two stages. This also holds true for other marsupial species where the entire process of oocyte and follicle development has been neglected.

1.1.7. Yolk.

Yolk is a characteristic features of many oocytes and acts as a major nutritional reserve for developing embryos. This is a morphological term, rather than a reference to a specific chemical substance, as the shape, size and consistency of yolk components are extremely variable between species. With regard to its chemical nature, the three main components of yolk that can be distinguished are carbohydrate yolk, fatty yolk and protein yolk. The question of yolk in eutherian oocytes is an intriguing one as there is still some debate as to whether they do actually contain yolk - of course, the answer to this depends on the definition being adopted. Descriptions such as those of Waddington (1962) were quite precise in stating that yolk was only one part of the oocyte reserve that consisted of platelets or lumps of protein-lipoid substance. However, such classification appears to have been based on non-mammalian oocytes and other definitions have been more general in classing any form of storage reserve as yolk (Selwood, 1992).

The amount of yolk also varies considerably between animal classes and on this basis eggs can be classified into different groups: those containing only a small amount of yolk are described as microlecithal, those with a moderate amount are mesolecithal and those with a large quantity are megalecithal. Most mammalian oocytes are microlecithal and marsupials appear to be intermediate in the amount of yolk they possess between the monotremes and eutherians.

Within this grouping, marsupials are commonly referred to as possessing "yolky" eggs but the formation of this yolk has never been studied. The only aspects of yolk which have been described are the polarity of the yolk, once it has been formed, and its extrusion during embryonic development (Hill, 1910; Hartman, 1916; Selwood & Young, 1983; Breed & Leigh, 1988; Baggott & Moore, 1990).

1.1.8. Marsupial gestation and placentation.

Embryonic development in marsupials has a number of features that distinguish it from that of eutherians. Following fertilization the zygote passes rapidly down the oviduct in 24 hours or less (Hartman, 1923; Rodger & Bedford, 1982; Baggott & Moore, 1990) during which time it acquires a mucoid coat (Hill, 1910; Hartman, 1916; Hughes & Shorey, 1973). The embryo then becomes surrounded by a tough, proteinaceous material consisting of interwoven fibres (Krause & Cutts, 1983) known as the shell membrane. This shell membrane persists for at least the first two thirds of gestation and thus isolates the embryo from direct contact with maternal tissues. Rupture of the shell membrane marks the beginning of organogenesis and close contact between the fetal and maternal tissues. At this time the yolk sac elaborates and, with the chorion, makes contact with the uterine epithelium to form the choriovitelline placenta (Krause & Cutts, 1984a).

The choriovitelline placenta forms the major organ of physiological exchange in most marsupials and it is only in the bandicoot family (Peramelidae) that formation of the yolk sac placenta is followed by the appearance of a functional chorioallantoic placenta during the last quarter of gestation (Flynn, 1923; Padykula & Taylor, 1976).

1.1.9. The grey, short-tailed opossum: *Monodelphis domestica.*

Following its fairly recent introduction to the laboratory this marsupial species has been the subject of many and varied biomedical studies. However, despite a substantial amount of work conducted on aspects of female reproduction there are several important areas which have either been neglected or remain unclear.

When this study commenced the morphology of the adult ovary, at either the light or electron microscope level, had not been described; there were differing reports on the oestrous cycles exhibited and no information regarding how the oestrous and ovarian cycles interrelate with one another. Although various groups had loosely described the *Monodelphis domestica* oocyte as containing yolk no attempts had been made to follow its formation or analyse any of its constituents.

1.1.10. Objectives of this study.

Due to the large gaps in the knowledge concerning certain aspects of female reproduction in not just this species but other marsupials, both American and Australian, the aims of this study on *Monodelphis domestica* were as follows:

1. To describe the general morphology of the adult ovary.
2. To study histologically and ultrastructurally the process of folliculogenesis.
3. To monitor the oestrous cycles and observe how they correlated with the ovarian cycles.
4. To establish a culture system, capable of supporting individual preantral follicles, for studying folliculogenesis *in vitro*.
5. To follow the pathway of yolk formation and describe any polarity.
6. To identify the major constituents of yolk.

Chapter 2

The grey short-tailed opossum, *Monodelphis domestica*

2.1. INTRODUCTION

2.1.1. General description and history.

The grey short-tailed opossum (Fig. 2.1), *Monodelphis domestica* (Wagner, 1842) is one of seventeen species of the pouchless *Monodelphis* genus (Burnett, 1830) distributed from eastern Panama southward to central Argentina. Like most of the American opossums, *M. domestica* is a member of the Didelphid family from which all other New World and Australian marsupials are thought to be derived (Clemens, 1968; Keast, 1977). This species is omnivorous, nocturnal in nature and gains its specific name from its tendency to occupy human dwellings. An adult weighs between 80 and 155 g (Fadem *et al.*, 1982) and is intermediate in size between a mouse and a rat. It is the least well adapted member of its group to arboreal life and, although able to climb reasonably well, is usually found on the ground.

Despite its low basal metabolic rate, this animal has a high aerobic metabolic capacity (Dawson & Olson, 1988) which allows it to cope with marked temperature changes. A mean body temperature of 33.2°C (Christian, 1983), 32.6°C (Dawson & Olson, 1988) and 32.3°C (Kraus & Fadem, 1987) has been recorded, which can fluctuate from 30.1°C - 34.7°C depending on the ambient temperature (Dawson & Olson, 1988).

In 1978 representatives of the National Zoological Park in Washington D.C. captured nine *M. domestica*, from the state of Pernambuco in Northeastern Brazil, which unlike other marsupial species, survived and bred prolifically in the laboratory. Due to its small size, tractability, easy maintenance and high fecundity under normal laboratory conditions, this species was the first practical marsupial for laboratory research and it was soon anticipated that it would become a valuable asset in biomedical research (VandeBerg, 1983). More animals have subsequently been caught, in various regions of Brazil, and introduced to the colony.

2.1.2. Reproductive biology.

Sexual maturity, in both sexes, is reached at 4 - 5 months (Fadem & Rayve, 1985) and animals breed throughout the year (Fadem *et al.*, 1982; VandeBerg, 1983). Females of up to 28 months of age have produced litters and males of 39 months have sired young but the majority of animals become reproductively senescent at an earlier age than this. None of the animals in captivity have been reported to survive beyond 4 years of age.

Unlike the majority of marsupials studied *M. domestica* is an induced ovulator (Fadem & Rayve, 1985) and, as in many eutherian species (Milligan, 1980), oestrus and ovulation are influenced by olfactory and social factors - females housed separately from males are reputed to become sterile and oestrus can be activated by direct access to male pheromonal cues. Fadem (1989) found that oestrus, following pheromonal stimulation, was associated with an increase in oestradiol secretion but the first hormonal evidence confirming that *M. domestica* is an induced ovulator was only recently provided by Hinds *et al.* (1992) who monitored progesterone and LH levels. They also observed that oestrus was indicated by a dramatic change in the appearance of the urogenital opening and fluctuations in body weight.

Several research groups have observed that females separated from males fail to exhibit oestrous cycles. In 1981, Maliniak & Taft reported that isolated females could become reproductively inactive if kept solitary for long periods, whilst Fadem & Rayve (1985) found that urogenital sinus smears typical of oestrus were more common in females living in the same cage as a male than those housed individually. In another study, Fadem (1985) reported that only 18% of isolated females, housed in a different room from males, showed signs of oestrus. Monitoring body weight, vulval swelling and progesterone concentrations, rather than smears, Hinds *et al.* (1992) found that none of the females isolated from males for 35 days showed any evidence of oestrus.

Oestrus activation has been studied by several workers with variable results. In 1985, Fadem found that 57% of females allowed sensory contact with a male exhibited an oestrus period but in a subsequent study, whereby females were put into cages previously occupied by either an intact or castrated male, she found that oestrus was induced in 100% and 25% of the females respectively (Fadem, 1987). Somewhat surprisingly it was also noted that exposure to another intact female's cage induced oestrus in 25% of the females.

The pheromonal cues responsible for oestrus induction were found to be in scent marks left by the males on cage walls (Fadem & Cole, 1985) rather than soiled shavings or bedding (Fadem, 1989). Scent marking in *M. domestica* is sexually dimorphic with males more likely to mark and at higher frequencies than females (Fadem & Cole, 1985). Following a study on castrated and testosterone replaced males (Fadem *et al.*, 1989) scent marking, along with precopulatory behaviour, was subsequently found to be related to testosterone secretion. Further confirmation was provided by Hinds

et al. (1992) with the observation that the introduction of an intact or vasectomized male was equally effective in stimulating females.

Stonebrook & Harder (1992) observed that male pheromonal stimuli were necessary for the maturation of the reproductive organs and initiation of the first oestrus in pubertal females. It was also noted (Cothran *et al.*, 1985) that females housed singly past the normal age of puberty attained significantly lower adult weights than those paired with a male at 6 months of age.

These all-important male pheromonal cues were found to be mediated via the main olfactory epithelium of the female, with the vomeronasal organ appearing to have a subsidiary role in promoting the transition from pro-oestrus to oestrus (Pelengaris *et al.*, 1991).

Following exposure to a male, or his cage (Fadem, 1987), smears from the urogenital sinus have indicated that females enter oestrus after an interval of 3 - 7 days (Fadem, 1985); 4 - 11 days (Fadem, 1987; 1989) or 5 - 8 days (Stonebrook & Harder, 1992). The median time between pairing and the onset of behavioural oestrus was observed by Baggott *et al.* (1987) to be 8.5 days. Once a female has entered oestrus, laparoscopic investigation has shown that ovulation can occur without mating taking place (Baggott *et al.*, 1987). Stonebrook & Harder (1992) observed that ovulation did not take place in oestrus females not allowed physical contact with males so it would appear that, although ovulation is not dependent on copulation, some form of physical contact with a male is necessary.

Fadem & Rayve (1985) have recorded an oestrus period of 3 - 12 days whilst Stonebrook & Harder (1992) have narrowed it down to 3 - 5 days. Oestrous cycles of 26 days (Falconnier & Kress, 1992); 28 days (Maliniak & Taft, 1981) and 32 days (Fadem & Rayve, 1985; Fadem, 1989) have been observed although shorter, possibly anovulatory, cycles of 14 days have also been reported (Fadem & Rayve, 1985).

2.1.3. Breeding systems.

Polygamous breeding groups of 1 male with 3 to 5 females have proved unsuccessful, due to other females interfering with copulation, so Fadem *et al.* (1982) tried housing animals in male - female pairs. However, when animals were kept in permanent pairs the males tended to exhibit decreased breeding activity. To avoid this reduction in interest, and the male-female aggression displayed by unfamiliarized animals, a procedure for swapping males was devised. This entailed removing the

original male and exchanging him, for 4 - 6 hours on 3 consecutive days, with a new male held within a wire cage (Trupin & Fadem, 1982). After the 3 days of gradual familiarization the original male was removed and the new male placed freely with the female.

An improved breeding procedure was subsequently developed whereby the female was kept within a holding cage, in the male's cage, and after a 2 day familiarization period released into the male's cage (Fadem, 1985). Following the observation that the majority of conceptions occurred within 13 days of pairing (Fadem, 1985) it was decided that animals need only be paired for 14 days (Kraus & Fadem, 1987). More recently another regime was devised whereby the female was placed directly into the male's cage (Maitland, 1992). This was found to be more efficient and appeared to be less stressful for the animals than when one of them was restrained within a holding cage.

2.1.4. Mating behaviour.

Courtship behaviour of the male includes scent-marking and strategies for reducing female aggressiveness such as avoiding face-to-face confrontation. A non-receptive female typically runs away from the male and if pursued will often hiss, make open-mouthed threats and strike at the male. Female receptivity lasts approximately 36 hours with copulation often occurring on two consecutive nights (Trupin & Fadem, 1982). Once she is receptive, the male quickly mounts the female, grasping her midsection tightly with his forepaws and her ankles with his hindfeet, and in most cases copulation occurs with the animals lying on their right sides (Trupin & Fadem, 1982; Maitland, 1992).

2.1.5. Gestation and early life.

Gestation periods of 14 - 15 days (Fadem *et al.*, 1982, Fadem & Rayve, 1985; VandeBerg, 1983), and 15 days (Maliniak & Taft, 1981) have been observed although Hinds *et al.* (1992) suggest that it may be longer. Litters range in size from 2 - 14 with an average of 7 (Cothran *et al.*, 1985; Maitland, 1992).

Like all marsupials, the young are born in a very immature state of development- each one measuring approximately 1 cm in length (Fadem *et al.*, 1982) and weighing 100 mg (VandeBerg, 1983; Kraus & Fadem, 1987), or less. Maitland (1992) managed to weigh several neonates before they had attached to the teat, prior to suckling, and recorded a minimum weight of 60 mg. Immediately after

birth, the young migrate to the teats (Fig. 2.2) and remain attached for approximately 14 days (Fadem *et al.*, 1982) after which time they are able to detach from the mother and suckle at will, usually in the nest.

The pattern of sodium and potassium concentrations in *M. domestica* milk is apparently unusual in that it is the reverse to that found in other marsupials studied, ie. there is a higher concentration of potassium throughout early lactation but more sodium during the latter stages (Green *et al.*, 1991).

Although nest-building is performed by both males and females there is a sex difference in nest construction which is dependent on the ambient temperature (Fadem *et al.* 1986). At 24°C both male and females were observed to build nests but very few males, compared to females, did so when the temperature was raised to 27°C. Whatever the temperature, females were also found to use more nest material than males. An intact nest appears to be important to ensure close proximity of the dam and litter (Fig. 2.3) and to maintain the body temperature of the poikilothermic young as disruption of a nursing female's nest can cause loss or runting of the litter (Fadem *et al.*, 1982). Homeothermy is not developed until the youngsters are approximately 25 days of age (Fadem *et al.*, 1988).

Gould (1984) observed that whilst still attached to the nipple, pups emit ultrasonic sounds which presumably emerge through the nostrils. These ultrasonic calls are of high intensity and have a repetition rate exceeding that observed in rodents. Between 18 and 25 days of age pups can open their mouths and emit a more intense vocalization, the lower frequencies of which can be heard by humans.

By about 3 weeks of age the pups are fully furred and at 4 - 5 weeks their eyes are open and they begin to eat solid food. At this stage they are often seen out of the nest riding on the mother's back (Fig. 2.4). Pups can be weaned at 7 weeks, separated from their mother and housed individually. If the young are not removed from their mother's cage, lactation can continue for as long as 75 days postpartum (Crisp *et al.*, 1989).

2.2. MATERIALS & METHODS

2.2.1. Research colony and maintenance.

The Glasgow colony of *M. domestica* is derived from 19 animals (10 males, 9 females) acquired from Manchester University in 1989, 23 females from Southampton University in 1992 and an additional 15 animals (4 males, 11 females) from Manchester University in 1993.

Housing consisted of plastic rat cages, measuring 56 x 38 x 18 cm, with wood shavings for floor coverage and shredded tissue paper supplied for bedding. Following separation from a mate, females were given plastic nest boxes (20 x 16 x 8 cm) and plenty of nesting material. The animal house was maintained at 24°C with a 14 hour light : 10 hour dark photoperiod and a humidity of approximately 35%.

Animals were fed with a reconstituted cat food (SDS powdered carnivore meat) or a commercial, tinned cat food (Whiskas, Pedigree Pet Foods). Twice weekly the veterinary vitamin supplement SA-37 (Intervet Labs. Ltd.) and fresh fruit, such as banana, were given. Water was available *ad libitum* from sipper tube bottles.

Animals used in this study were either killed by asphyxiation, with carbon dioxide or ether, or given an intraperitoneal injection of Euthatal (RMB Animal Health Ltd.). They were weighed on a Sartorius Basic balance to the nearest 0.1g and the body and tail lengths measured with a ruler to the nearest mm.

2.2.2. Husbandry.

Occasional cases of necrotic ears or tails, probably due to low humidity, were treated successfully with Dermisol multicleanse solution (Beecham Animal Health) and Dermisol cream S.A. (Beecham Animal Health). Eye infections, seen in two females, responded to Orbenin ophthalmic ointment (Beecham Animal Health) but tended to be recurrent.

Although the animals showed no symptoms, a health screen of the colony, in 1992, revealed that *Salmonella braenderup*, *S. montevideo*, *S. kedougou* and *S. mbandaka* were present in some individuals. To eliminate the possibility of infection from dietary sources, their diet was changed from the reconstituted cat food (SDS powdered carnivore meat) to a commercial feline canned diet (Whiskas). Following daily subcutaneous injections of 2.5% Baytril (Bayer plc) at a dose rate of 10 mg/kg/day, for 7 days, none of the animals were shedding Salmonellae. However, when retested a month later, *S. kedougo* was recovered from several individuals. Since infected animals showed no ill effects and treatment was proving unsuccessful, it was decided that the animals would not undergo further treatment. Measures to protect handlers and other animals from infection were therefore instituted -

workers entering the animals' room wore protective gowns and shoe covers and all cages were decontaminated in Virkon disinfectant (Antec International Ltd.) prior to leaving the room.

The commercial cat food appeared to be far from ideal for maintaining weight or condition as a significant weight loss, of up to 25% in some large males, and an obvious increase in aggression was observed. These factors were overcome by increasing the amount of food, but only to the level where each animal was fed approximately 30 to 50% of its body weight each day.

Breeding success was found to be very sensitive to physical disturbance: during the course of this study, the colony was relocated to different premises on two occasions which significantly disrupted their breeding. In the first location, there was a breeding rate of 61% and weaning rate of 58% (ie. 61% of the pairings produced a litter and 58% of these survived to be weaned). This rate fell to 43% and 36% respectively after the first move and following the last move, the breeding rate dropped to 20% and the weaning rate to 14 %. The loss of young prior to weaning was invariably due to cannibalism by the mother.

Due to the limited number of animals available, inbreeding has occurred to a certain extent, but does not appear to have caused many problems. Congenital eye defects have occurred with two females born without one eye and one male with both eyes missing. The blind male does not appear to be at all debilitated - he is able to find food and water and climb as well as any of the sighted animals.

2.2.3. Breeding.

The breeding system of Maitland (1992) was applied whereby the female was placed directly in to the male's cage and the pair left together for 14 days. After this period the female was returned to her own cage, given a nest box and checked for a litter over the next 14 days.

2.2.4. Monitoring of oestrous cycles.

Smears from the urogenital sinus were taken from three different groups of adult females: group 1 consisted of 8 singly caged females housed in the same room as males, group 2 contained 6 singly caged females isolated from males for 3 months and group 3 consisted of 6 females paired with intact males. Each group contained a mixture of virgin and parous females. Smears were taken daily over an 8

week period from groups 1 and 2, and from five days prior to pairing until oestrus was indicated with group 3 females. In total, over 750 smears were examined.

2.2.5. Urogenital sinus smearing.

Smears were obtained by lavage of the urogenital sinus with 0.9% saline using a plastic Pasteur pipette with the tip flamed to a smooth, reduced aperture. Animals were restrained in a reverse hold in the palm of one hand and the pipette tip inserted approximately 3mm into the urogenital sinus, and 0.1 ml of saline injected and withdrawn. The fluid was spread onto a chrome-alum gelatin subbed slide, fixed in 95% ethanol and allowed to air dry. Staining was either by the Papanicolaou method (Appendix A.1.5) or with haematoxylin and eosin (Appendix A.1.6). To eliminate the possibility of contamination between animals, a new pipette was used for each smear.

2.2.6. Urogenital opening.

In every smeared female, and other females paired without being smeared, the urogenital opening was examined daily for any change in appearance.

2.2.7. Statistical analysis.

The mean oestrous cycle and oestrus period lengths between group 1 and 2 females were compared with a non-paired t-test. The results are presented as means ± SEM.

2.2.8. Examination of reproductive system.

At the end of the experimental period, when smears indicated that an individual was in oestrus, the animal was sacrificed. The reproductive tract was examined macroscopically and the ovaries fixed in aqueous Bouin's fixative and embedded in paraffin wax for light microscopy. Longitudinal, serial sections were cut at 5μm and stained with haematoxylin and eosin or Mallory's triple stain (Appendix A.1.1. & A.1.2). A total of 8 animals, with 2 - 3 from each group, was studied.

2.3. OBSERVATIONS

2.3.1. General features.

In our colony, sexual dimorphism was usually apparent in adults with males being significantly larger than females. Adult females weighed from 60 -100 g (75.4 ± 1.7g, n=43), had a total head and body length of 120 - 150 mm (132.0 ± 3.1 mm, n=20) and an additional tail length of 60 - 85 mm (78.7 ± 2.6 mm, n=20). Males weighed from 70 - 150 g (110.3 ± 2.7g, n=38), had a total head and body length of 140 - 180 mm (158.3 ± 5.2 mm, n=20) with the tail measuring 80 - 100 mm (92.0 ± 4.3 mm, n=20).

The short coat was grey-brown with lighter underparts, and was either smooth or slightly rough-haired. Adult males possessed a suprasternal scent gland which was visible as a sparsely-furred oval patch often amber-tinged from its secretions. The prehensile tail was lightly-furred and able to support the animal's entire body weight. The manus and pes had five separate digits, the first of which was opposable, and all of the digits apart from the halluces had short, curved claws. The naked palm and sole were granular with well-developed pads. The adult dental formula was found to be: I 4/4, C 1/1, P 3/3, M 4/4. However, an unusual feature was the presence of a single incisor in the middle of the upper jaw which was accommodated in a space on the lower jaw.

2.3.2. Reproductive anatomy.

Female *M. domestica* have 13 nipples which are arranged in a circular pattern on the lower abdomen (Fig. 2.5). The openings of the urogenital sinus and the rectum are enclosed within a single sphincter. The urogenital sinus leads to the posterior vaginal sinus and then to the 2 lateral vaginae - these open into 2 uteri via separate cervices. The oviducts are convoluted and measure about 10 mm in length. The ovaries are discoid structures measuring approximately 3 mm in diameter and are located on the dorsal aspect of the uteri partially enclosed within a bursa (Fig. 2.6).

2.3.3. Analysis of urogenital sinus smears.

Microscopic examination of smears, from each experimental group, revealed several cell types. These were: i) nucleated, cornified and enucleated superficial cells with a polygonal outline, small nucleus and pale cytoplasm, ii) nucleated and cornified intermediate cells with an elongated form and

folded edges, iii) nucleated parabasal cells with a large nucleus and dense cytoplasm and iv) polymorphonuclear leucocytes. Occasionally, circular anuclear structures were observed which have previously been identified as anal gland secretion (Fadem & Rayve, 1985). (See Figs. 2.8 - 2.12).

Although each cell type was often found in any one smear there tended to be a subtle variation in their abundance according to the stage of the oestrous cycle. During dioestrus, leucocytes were the predominant cell type with a few nucleated and cornified epithelial cells. With the onset of pro-oestrus, nucleated epithelial cells began to increase in number although leucocytes and cornified epithelial cells were still observed. At oestrus, cornified and enucleated epithelial cells showed their greatest abundance with the other cell types still present but usually with a lower occurrence.

Females in group 1 exhibited an oestrous cycle length ranging from 24 - 28 days (26.4 ± 0.9 days) with an oestrus period of 7 - 11 days (8.8 ± 0.7 days). Isolated females in group 2 showed an oestrous cycle length of 24 - 33 days (28.0 ± 1.9 days) and an oestrus period of 6 - 10 days (7.4 ± 0.6 days). Statistically, there was no significant difference in the length of the oestrous cycle ($t=0.80$, $df=5$, $p=0.46$)or oestrus period ($t=-0.39$, $df=10$, $p=0.70$) between the two groups. Unpaired females therefore, exhibited an average oestrous cycle length of 27.2 ± 1.0 days and oestrus period of 8.1 ± 0.5 days. Females from group 3 were found to enter oestrus, 3 - 7 days (5.4 ± 0.6 days) after being paired with an intact male.

With two females from group 1 and one individual from group 2 there were no oestrus periods detected - this was partly due to the fact that for several days a scant smear was obtained with too few cells for reliable diagnosis. In both of these unpaired groups oestrus could only be identified retrospectively with all of the data being examined in context, so smears were of limited use for indicating an impending oestrus period.

Smears from group 3 females were much easier to interpret, with more distinct variations in cell abundance, than the other two groups and oestrus could readily be identified. It was also noticed that cellular changes seen in the smears tended to lag behind the actual changes occurring in the ovary and higher reproductive tract - in three females, with smears that indicated they were in proestrus, examination of the tract and ovary revealed that they had already entered oestrus (see 2.3.6.). With the openings of the urogenital sinus and rectum being in such close proximity, within the same sphincter, there was always the chance of inadvertantly taking a rectal smear. By inserting the pipette tip into the

anterior portion of the sphincter at an angle of about 45° to the abdomen it was possible to avoid this and obtain consistent urogenital sinus smears. Even so, anal gland secretion varied dramatically between animals with certain individuals producing copious amounts which often contaminated their smears.

2.3.4. Appearance of urogenital opening.

In some of the paired females the urogenital opening became marginally more pink and moist than usual and occasionally slightly enlarged. This was often accompanied by abdominal swelling in the mammary area.

2.3.5. Reproductive tract and ovaries.

The appearance of the reproductive tract and ovaries quickly confirmed the reproductive state of the animal. During dioestrus the unstimulated reproductive tract was pale in colour and the width across the lateral vaginae was about 1.5 cm (Fig.2.7a). At oestrus the tract was deep red in colour and very oedematous. Although the entire tract increased in size, it was most obvious in the lateral vaginae which swelled to more than twice the width observed during dioestrus (Fig.2.7b). Both ovaries were found to contain between four and eight large, healthy, antral follicles (Fig.2.13). Corpora lutea were never found in either the isolated females or those in the same room as males - they were only present in females paired with a male (Fig.2.14).

2.3.6. Puberty and gestation.

Ovaries from a supposedly prepubertal female (118 days old) were found to contain large, healthy, antral, follicles. Although not conclusive, this suggests that puberty in females may occur before 4 months of age. Results also suggest that the gestation period can be longer than the expected 14 days, as young have been born 17 and 21 days after the male and female were separated. The number of pups in litters that were successfully weaned ranged from 1 - 13 and although more than 13 pups were sometimes born - one female gave birth to 16 pups - with only 13 nipples this is the maximum number that can be suckled.

Fig. 2.1. The grey, short-tailed opossum (*Monodelphis domestica*).

Fig. 2.2. Newborn *M. domestica* attached to teats.

Fig. 2.3. Female with young in nest.

Fig. 2.4. Female with youngsters attached.

Fig. 2.5. Mammary region.

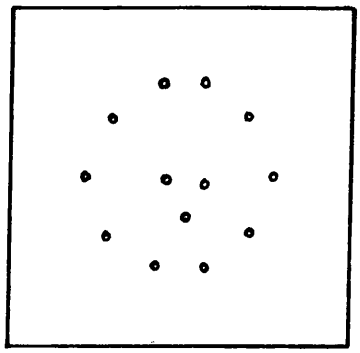


Fig. 2.6. Schematic view of reproductive tract, with bladder deflected, showing urogenital sinus (ugs), lateral vaginae (lv), median vagina (mv), cervices (c), uteri (u), ovaries (o) and oviducts (ov).

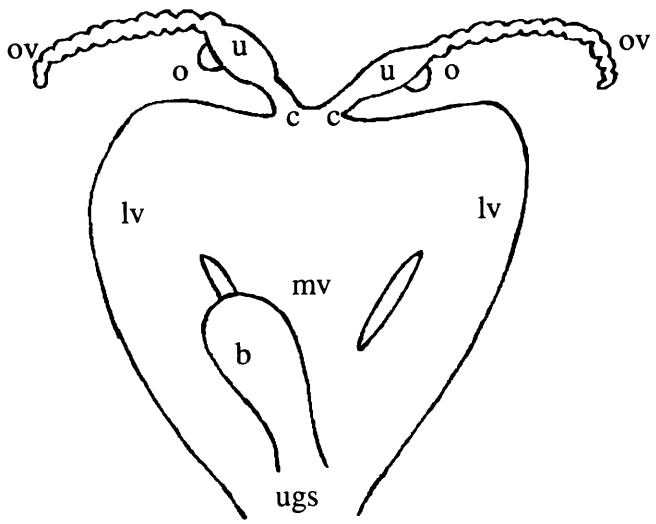
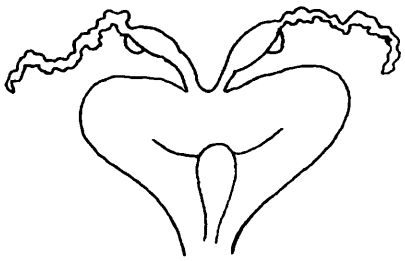


Fig.2.7a. Dioestrus state.

Fig.2.7b. Oestrus state.



All figures apart from Fig.2.6. are drawn to size.

Fig. 2.8. Smear with nucleated superficial cells (ns), cornified superficial (cs) cells and a few clumps of leucocytes (l). (x75). Typically seen during proestrus.

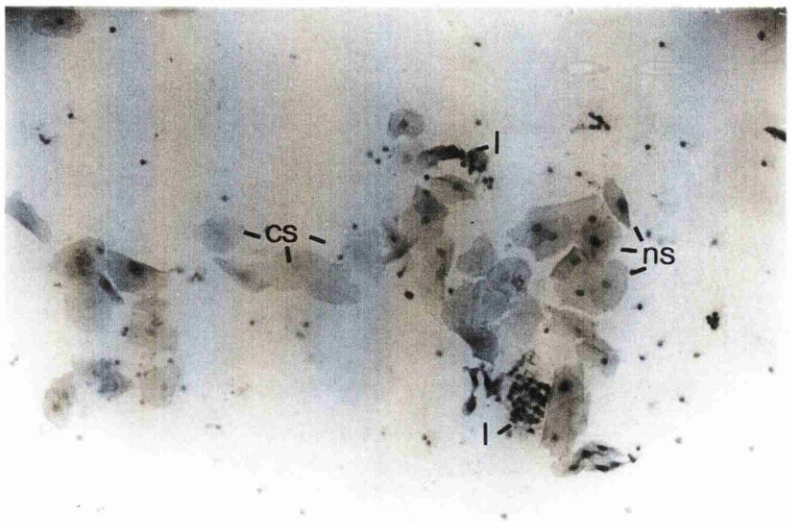


Fig. 2.9. Smear showing nucleated parabasal cells. (x75). Typically seen during proestrus.

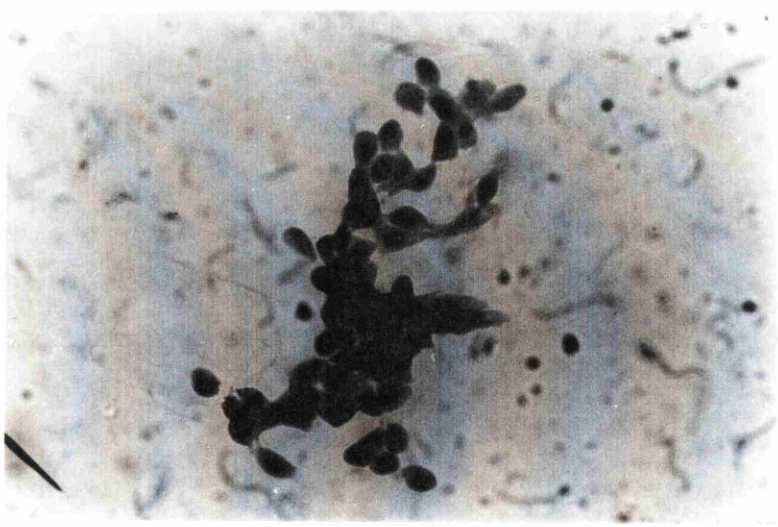


Fig. 2.10. Smear consisting mostly of cornified superficial (cs) cells (x75). Typically seen during oestrus.

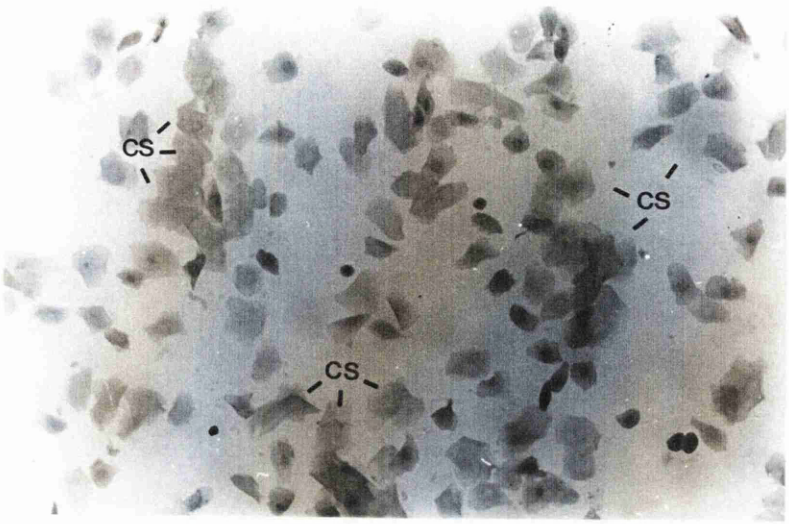


Fig. 2.11. Smear with central clump of cornified superficial cells (cs) and abundant leucocytes (x75). Typical of metoestrus.

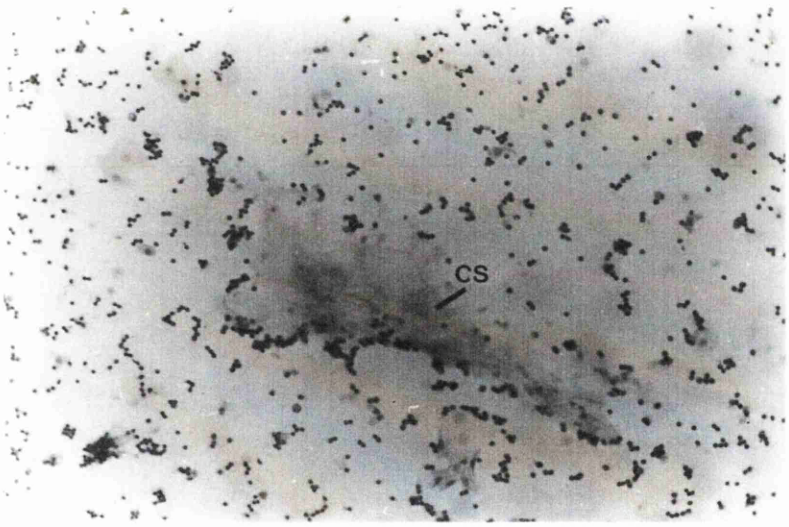


Fig. 2.12. Anal gland secretion (x30).

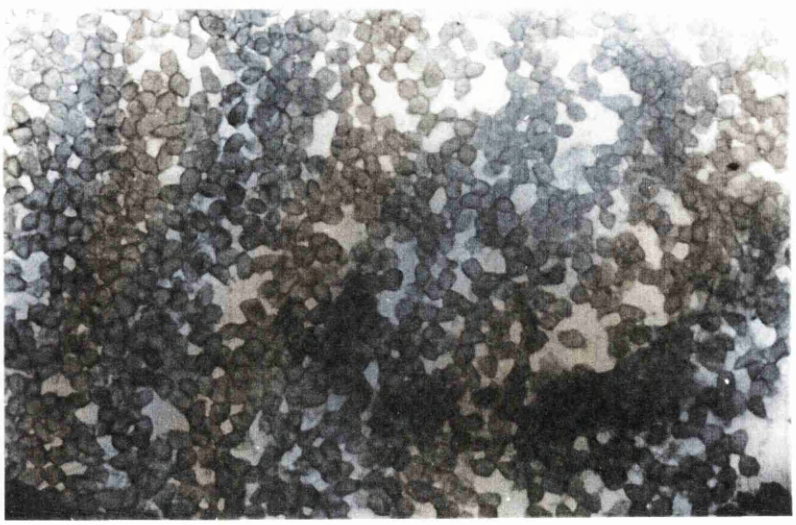


Fig. 2.13. Ovary during oestrus with four, large antral follicles (x12).

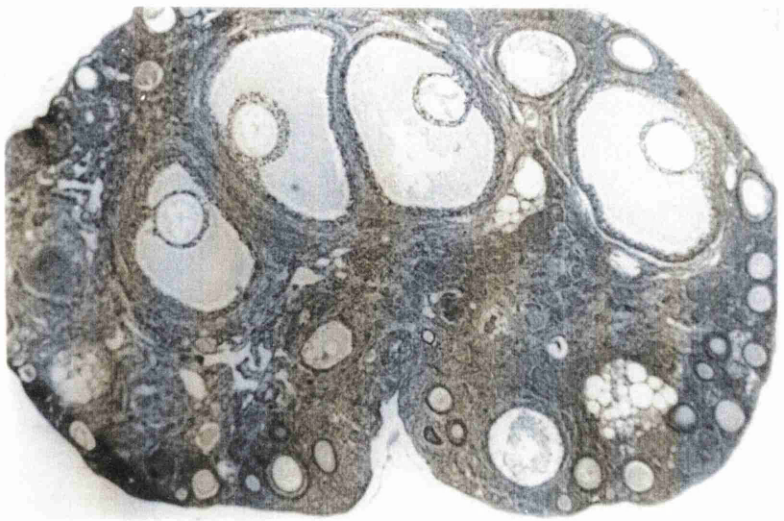
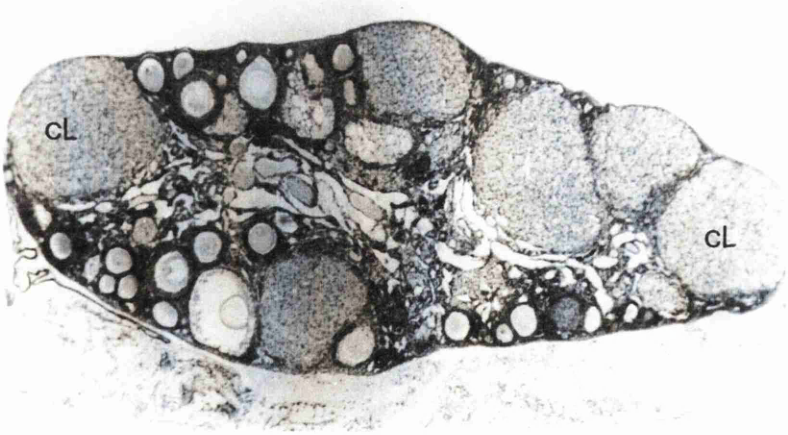


Fig. 2.14. Ovary with six corpora lutea (CL) visible (x12).



2.4. DISCUSSION.

2.4.1. Body size.

Fadem *et al.* (1982) stated that adults weighed 80 - 155 g, had a head and body length of 170 - 200 mm and an additional tail length of 60 - 80 mm. Although the weight range is similar to that found in our colony, as is the combined body and tail length, Fadem's opossums appear to be considerably more "short-tailed" than the Glasgow colony. Animals in another British colony of *M. domestica* (Moore, pers. comm.) are also considerably larger and heavier than our animals. However, size variation between *M. domestica* stocks has previously been reported (Cothran *et al.*, 1985; VandeBerg, 1990), where there was a 30% difference in body weight between two separate populations of wild-caught animals.

2.4.2. Husbandry.

Commercial cat food proved to be inadequate in maintaining weight or condition, which was also observed by Fadem *et al.* (1982) who tried feeding their animals on commercial dry and canned cat food. Similar dietary problems have been observed in mink where a large amount of research has been conducted to demonstrate the effect of different feeds on reproductive success. The commonly found high prenatal mortality was reduced by altering the diet (Broxup, 1968) and nutritional causes were found to account for 58% of the cases of anovulation (Sundqvist *et al.*, 1989).

The extent to which the dietary replacement affected our animals' breeding is unknown, but the fact that it was considerably lower than when they were previously relocated, without a change in diet, indicates that it was probably a major factor. In April 1994, after I had completed my experimental work with the colony, the animals were weaned from the commercial cat food back on to their original scientific carnivore diet and I am told that their reproductive success has shown a great improvement.

2.4.3. Suitability of urogenital sinus smearing for monitoring oestrous cycles.

Smears have been utilized to follow *M. domestica* oestrous cycles, by several workers, with varying results. Fadem & Rayve (1985) and Stonebrook & Harder, (1992) were able to follow the oestrous cycle and obtained results similar to those of this study. However, Baggott *et al.* (1987) found that smearing alone could not accurately monitor the timing of reproductive events but could be

correlated with behavioural observations. Hinds *et al.* (1992) were unable to obtain consistent smears and found the technique to be unsatisfactory for *M. domestica*.

Although the length of the oestrous cycle and oestrus period obtained in this study are in agreement with groups who have used smears successfully, the results concerning unpaired females differed. Unlike previous observations on isolated females (Maliniak & Taft, 1981) and females housed alone (Fadem, 1985), smears indicated that both groups continued to exhibit oestrous cycles which were not significantly different. There was no evidence of the short 14 day cycles reported by Fadem & Rayve (1985).

In this study, smears did not give the clear-cut indication of the stage of the oestrous cycle seen in laboratory rodents and deciphering them often proved tricky. This disparity is presumably due to the reproductive anatomy of the marsupial. It is the vaginal epithelium which shows the characteristic changes under hormonal influence and, although the injected saline will have reached the vaginae, some of the smear cells obtained will have come from the urogenital sinus. In eutherian species which have a urogenital sinus, (eg. hedgehog: Deanesly, 1934; porcupine: Mossman & Judas, 1949; elephant: Perry, 1953) the lower part of the tract is less responsive to hormonal changes and often contaminated with urinary tract cells (D'Souza, 1978). This is also likely to be the case in *M. domestica*. The often continual presence of cornified cells has also been observed in this (Fadem & Rayve, 1985) and other marsupials (Hughes, 1962; Tyndale-Biscoe, 1968; Peters & Rose, 1979) and has been suggested to be due to an influx of cornified cells from the lateral vaginae. However, due to their continued appearance, it would seem more likely that these cells come from the urinary tract rather than occasional samples from the higher reproductive tract. The prolonged presence of leucocytes observed in several individuals may have been due to contamination from the anal gland which, in the Virginia opossum (Hartman, 1923; Jurgelski & Porter, 1974), has been found to be leucocyte-rich. Because changes in smears lagged behind the actual changes occurring in the ovary and higher reproductive tract, they were not particularly accurate as far as timing was concerned.

The results from this study indicate that, despite the fact that *M. domestica* smears do not follow the textbook pattern, vaginal smears can be used to obtain a progressive picture of the oestrous cycle. In unpaired females, because oestrus could only be identified retrospectively, smears were of little use for indicating an impending oestrus period. In paired females however, the variation in exfoliated cells was

much more dramatic and smears were a suitable way of pinpointing when a female was about to enter oestrus. It has been reported that ovulation occurs approximately 18 hours after the first mating (Baggott *et al.*, 1987) so monitoring animals by time-lapse video may prove to be an easier way of predicting oestrus

2.4.4. Appearance of urogenital opening and reproductive tract.

The distinct changes in the colour and enlargement of the urogenital opening observed by Hinds *et al.* (1992) were not observed in this study. These authors did however acknowledge that such changes did not provide unequivocal evidence that ovulation will follow. Oedema and erythema of the vulva of the rabbit (Carlyle & Williams, 1961), ferret (Marshall, 1933), marten (Enders & Leekley, 1941) and mink (Enders, 1952) has also been noted although females may mate and ovulate when the vulva appears normal. From this study it would seem that visual assessment of the urogenital opening in *M. domestica* has very little value in terms of predicting the reproductive state of a female.

The most striking change affecting the reproductive tract was the dramatic swelling of the lateral vaginae. They began to swell during proestrus and continued until oestrus when they were extremely vascular and distended. Similar changes in the reproductive tract were also observed in *D. virginiana* by Hartman (1923).

2.4.5. Oestrous cycles.

Urogenital sinus smears and examination of the reproductive system indicated that females have repetitive cycles of follicle growth and regression which are anovular without the presence of a male. It appears that animals enter oestrus in the usual manner with the reproductive tract undergoing preovulatory swelling and large antral follicles being formed in the ovaries. If the female is stimulated by a male, these large follicles will complete maturation and be ovulated but otherwise they will become atretic and replaced by the next wave of follicles. This would correlate with the findings of Hinds *et al.* (1992) that there were no changes in plasma progesterone, indicative of active corpora lutea, seen in females isolated from males.

Thus it would seem that *M. domestica* behaves in a similar manner to certain eutherian induced ovulators, such as the rabbit (Hill & White; 1933, Hamilton, 1951), mink (Enders, 1952), cat (Wildt *et*

al., 1978; Shille *et al.*, 1979), vole (Milligan, 1974), camel and alpaca (Bravo & Sumar, 1989), which also show cyclic variations in vaginal smears and histology of the reproductive tract. However, unlike many of these species, which can sometimes exhibit spontaneous ovulation, this was not the case with *M. domestica* as corpora lutea were never observed in unpaired animals.

2.4.6. *Monodelphis domestica*: a successful laboratory marsupial.

In 1983, VandeBerg correctly predicted that *M. domestica*'s value in biomedical research would rapidly increase. Since then this species has been the object of many and varied studies such as sexual differentiation and development of the gonads (Fadem & Tesoriero, 1986; Baker *et al.*, 1990; Moore & Thurstan, 1990; Maitland, 1992; Fadem *et al.*, 1992) and the brain (Fadem & Harder, 1992 a,b; Elmquist *et al.*, 1992); IVF and embryology (Baggott & Moore, 1990; Moore & Taggart, 1993); genetics (Merry *et al.*, 1983; Holmes *et al*, 1992; Van-Oorschot *et al.*, 1992 a,b); haematology (VandeBerg *et al.*, 1986; Cothran *et al.*, 1990; Manis *et al.*, 1992); immunology (Croix *et al.*, 1989; Brozek *et al.*, 1992); carcinogenesis (Fadem *et al.*, 1988); neurology (Wang *et al.*, 1992; Brunjes *et al.*, 1992); cholesterol studies (Rainwater & VandeBerg, 1992) and anatomy (Morrison *et al.*, 1993). In fact, scientific publications concerning *M. domestica* have overtaken the combined number of those on *Sminthopsis crassicaudata* and *Macropus eugenii*, the two most commonly studied Australian marsupials (VandeBerg, 1990).

Chapter 3

Ovarian morphology and follicle growth

3.1. INTRODUCTION.

3.1.1. Ovarian morphology.

The comparative aspects of the structure of the ovary in numerous mammalian species and at different levels of maturity have been recorded and discussed extensively by Zuckerman (1962) and Mossman & Duke (1973). When examined microscopically, the adult ovary has been found to contain numerous follicles in various stages of development. For many years phrases such as primordial, primary and small follicles have been used to describe follicles with a single layer of granulosa cells; secondary and growing follicles those with several layers of granulosa cells and tertiary, Graafian or vesicular follicles for those in the later, antral stages of growth. Whilst these terms may have been adequate for describing follicles with regard to ovarian development it was decided that they were inadequate for follicle growth studies (Wischnitzer, 1966).

Subsequently, various classifications have been devised to describe the stages of oocyte and follicle development. The main characteristic for some authors has been the number of granuosa cell layers surrounding the oocyte (Mandl & Zuckerman, 1952; Wischnitzer, 1966). Others concentrated solely on the maximum follicle diameter (Bullough, 1942; Ingram, 1959) whilst some used a combination of the number of cell layers and follicle diameter as a guide (Knigge & Leathem, 1956). The most comprehensive classification appears to be that proposed by Pedersen & Peters (1968) which was based upon the size of the oocyte in follicles of different developmental stages, the size of the follicle as determined by the number of follicle cells and the overall morphology of the follicle. This classification has subsequently been adopted and expanded upon by various workers (Alcorn, 1975; Oakberg, 1979).

3.1.2. Follicle and oocyte growth.

Before oocyte growth commences, a few flattened, granulosa cells become arranged around the oocyte to form a primordial follicle. When a complete layer of granulosa cells has enclosed the oocyte the structure is described as a primary follicle and it is at this stage that oocyte growth begins. During subsequent follicle development an amorphous material begins to accumulate in the intercellular spaces between the oocyte and granulosa cells until a single layer, the zona pellucida, completely surrounds the oocyte. The origin of the zona pellucida has long been controversial and species differences may

account for the uncertainty concerning its formation. Evidence gathered from both morphological and biochemical studies (Dunbar, 1983; Tesoriero, 1984; Wolgemuth *et al.*, 1984) indicates the likelihood that both the oocyte and its follicle cells contribute, probably on a sequential basis, to the synthesis and secretion of the zona pellucida. The marsupial zona pellucida consists of a weakly acidic glycoprotein (Hughes, 1974) and varies in thickness from about 1μm to 6μm between species. It is therefore considerably thinner than in eutherians in which the zona pellucida is 10 μm to 30 μm thick (Austin, 1961). With further growth and development the follicle becomes multilayered and pockets of fluid appear in the spaces between the granulosa cells. These coalesce into a single large cavity to form the follicular antrum which surrounds the oocyte and its associated cells. During antrum formation the oocyte remains surrounded by two or more layers of granulosa cells which form what is known as the *cumulus oophorus*.

A biphasic pattern of oocyte and follicle growth in eutherian mammals was clearly established some 60 - 70 years ago (Brambell, 1928; Parkes, 1931) and has since been recorded in a wide variety of species (mouse: Brambell, 1928; rat, rabbit, ferret and pig: Parkes, 1931; baboon: Zuckerman & Parkes, 1932; bat: Wimsatt, 1944; rhesus monkey: Green & Zuckerman, 1947; goat: Harrison, 1948; human: Green & Zuckerman, 1951; hamster: Knigge & Leathem, 1956; Asian musk shrew: Dryden, 1969; impala: Kayanja, 1969; hyrax: Kayanja & Sale, 1973 and shrews: Kress, 1984 a,b). During the first phase, growth of the oocyte and follicle was found to be synchronous whereas the second phase, which coincided with antrum formation, was characterized by follicle growth alone.

In the Monotremes, oocyte growth in relation to follicle growth was found not to be biphasic. In these animals there is no antrum formed and both the oocyte and follicle undergo coincident size increases throughout the whole growth period (Lintern-Moore *et al.*, 1976).

Studies conducted on marsupials have concluded that oocyte and follicular growth conform to the biphasic pattern, the only difference from the Eutheria being the larger size of the marsupial oocyte and follicle (Lintern-Moore *et al.*, 1976). Alcorn's comprehensive research on the wallaby *Macropus eugenii* (1975) included a study of oocyte and follicle growth which he described as biphasic - he divided the data and produced separate linear regression expressions to describe the two growth phases. Lintern-Moore *et al.* (1976) and Lintern-Moore & Moore (1977) examined the ovaries of nine marsupial species, including *M. eugenii*, and similarly found a biphasic relationship between the

growth of the follicle and its oocyte. However Panyaniti *et al.* (1985), studying the ovaries of pouch young, adult and hypophysectomized adult *M. eugenii*, concluded that the relationship between oocyte and follicle diameter for each group was best described by an exponential curve.

More recently, two separate groups (Falconnier & Kress, 1992 and Rodger *et al.*, 1992) monitored oocyte and follicle development in *M. domestica* and both reached the conclusion that oocyte growth continued after antrum formation and therefore a biphasic growth pattern was not strictly followed.

3.1.3. Polyovular follicles.

The majority of follicles contain a single oocyte, but polyovular follicles have been recorded in various eutherians (cat, dog, rhesus monkey: Hartman, 1926; mouse: Engle, 1927; Ullmann, 1976; rat: Davis & Hall, 1950; Kent, 1960; hamster: Bodemer & Warnick, 1961; rabbit: Szöllösi, 1978; Al-Mufti *et al.*, 1988; ferret: Mainland, 1927; goat: Harrison, 1948; human: Gondos & Zamboni, 1969; Papadaki, 1978). They tend to be quite common in fetal and juvenile ovaries, usually disappearing with age, and when present in adults occur more often in particular species such as the domestic bitch (Telfer & Gosden, 1987), rabbit (Al-Mufti *et al.*, 1988) and certain inbred strains of mice (Ullmann, 1976; Tagami & Akimoto, 1978). In marsupials, they have been observed with varying abundance in *Dasyurus viverrinus* (O'Donoghue, 1912), *Didelphis virginiana* (Hartman, 1926; Morgan, 1943), *Potorous tridactylus* (Bates *et al.*, 1972; Dairi, 1988), *Macropus eugenii* (Alcorn, 1975) and *Isoodon macrourus* (Telfer & Gosden, 1987).

In Hartman's study (1926) he classified polyovular follicles into various types according to their shape and oocyte positioning. In the commonest form, designated as type I, the oocytes were separated by granulosa cells whilst in type 2 follicles the oocytes were in contact. The third type of polyovular follicle varied from the usual spherical form with the oocytes exhibiting a linear arrangement.

Bi and triovular follicles are the most frequently observed but up to 24 oocytes have been recorded in the rabbit (Al-Mufti *et al.*, 1988) and over 100 in the opossum *Didelphis virginiana* (Hartman, 1926). Although the majority of polyovular follicles are primordial or primary (Hartman, 1926; Engle, 1927; Bodemer & Warnick, 1961; Alcorn, 1975; Al-Mufti *et al.*, 1988) they do not appear to exhibit any developmental abnormality or be any less viable than normal follicles as they have been

observed to reach preovulatory maturity (O'Donoghue, 1912; Hartman, 1926; Engle, 1927; Bodemer & Warnick, 1961; Tagami & Akimoto, 1978; Al-Mufti *et al.*, 1988; Ullmann, pers. comm.). Al-Mufti *et al.* (1988), studying the rabbit, reported a relationship between the topographical situation of the oocyte inside the follicle and its potential to grow - centrally positioned oocytes were more likely to grow and reach the preovulatory stage than peripheral ones.

Several explanations concerning the formation of polyovular follicles have been suggested: division of a polynuclear oocyte within its follicle, concrescence of previously separate follicles and imperfect separation of Pflüger's egg tubes leading to a group of oocytes becoming enclosed by a common layer of granulosa cells (see Hartman, 1926). Several workers have produced evidence against the first possible mode of origin and have selected either the second (Papadaki, 1978) or third theory (Hartman, 1926; Telfer & Gosden, 1987) as the most probable. Whatever their mode of origin, it is generally accepted that polyovular follicles are accidents of development.

3.1.4. Atresia.

The word atresia (derived from the Greek *a* = not; *tretos* = perforated) was originally a medical term used to designate the closure or obliteration of a normal body orifice or passage. In the context of ovarian physiology it is now used to describe the process of follicular degeneration.

The majority of oocytes in the mammalian ovary are eliminated by atresia rather than ovulation and it has been estimated that more than 99% of human follicles become atretic and about 77% in the mouse (Byskov, 1978). Once a follicle enters its growth phase it either goes on to ovulate or becomes atretic; the chances are that it will become atretic since more follicles are recruited during a cycle than usually ovulate. However, follicles which have yet to enter the growing pool may also succumb to atresia (Ingram, 1962).

Classification of follicles as normal or atretic has been highly subjective and is usually only recognized after extensive morphological signs of degeneration are detectable (Ingram, 1962; Byskov, 1974). In the hamster (Knigge & Leathem, 1956), the histological course of atresia was found to be different in preantral and antral follicles. A method for identifying early atresia in large preantral to antral mouse follicles was devised by Byskov (1974). She used 5% pycnotic granulosa cells (in the

largest cross section) as a sign of definite but early atresia and proposed that atresia in the granulosa of such follicles occurred in three progressive phases.

In the canine ovary two processes of follicular atresia have been observed (Spanel-Borowski, 1981). Type A, which occurs predominantly in preantral follicles, involves striking necrotic changes in the oocyte and zona pellucida. Type B, the only form encountered in antral follicles, is typified by distinctive degenerative changes in the granulosa with an almost unchanged oocyte and zona pellucida.

Morphological evidence has led to the suggestion that follicular atresia may represent an example of apoptosis or programmed cell death (Hirshfield, 1989; Hurwitz & Adashi, 1992). By conventional histological staining, atretic nuclear pycnosis is all but identical with the apoptotic condensation of nuclear chromatin. Also, the condensed chromatin in atretic cells undergoes karyorrhexis, a process indistinguishable from apoptotic nuclear fragmentation, characterized by nuclear rupture and chromatin transformation into granules which have been referred to as atretic (apoptotic) bodies (Hay *et al.*, 1976).

The histochemical and biochemical characteristics of atresia have also been described (Lobel *et al.*, 1961; Guraya & Greenwald, 1964; Moor *et al.*, 1978; Carson *et al.*, 1979). A useful model was devised by Uilenbroek *et al.* (1980) which allowed the detection of biochemical changes taking place during atresia of preovulatory rat follicles, before any morphological signs became apparent. They concluded that prior to any visible signs of atresia the follicles show a reduction in oestradiol production and suggested that the enzymes involved in the conversion of progesterone to androgens became deficient in atretic preovulatory follicles.

3.1.5. The corpus luteum.

The first accurate and detailed description of the corpus luteum was provided by Regner de Graaf in 1672 (translated by Corner, 1943), and subsequently there have been numerous reviews on its structure. Although O'Donoghue (1963) claimed that the corpus luteum of the hyrax was derived exclusively from the *theca interna* of the mature follicle, it is generally accepted that the majority of the cells of the corpus luteum are luteinized granulosa cells from the ruptured follicle. In some cases they are supplemented with *theca interna* cells although their contribution varies greatly even between quite closely related species.

Corner recognized two cell types in the sow (1919) and considered the larger ones to be luteinized granulosa cells and the smaller ones *theca interna* cells. Harrison (1948) was of the same opinion in his examination of the goat ovary. Mossman & Duke (1973) identified two classes of cells in the corpora lutea of various mammals such as the Artiodactyla (even-toed hoofed mammals: cow, deer, llama); Perissodactyla (odd-toed hoofed mammals: horse) and Odontoceti (toothed whales: pilot whale and white whale). The corpora lutea of the European mole, beaver and African Springhare were also found to have two types of cells but these were not as distinctly different in size and morphology as those of the hoofed mammals and whales. However, with regard to the pocket gopher Mossman (1937) believed that it was only the granulosa cells which contributed to the corpora lutea.

The marsupial corpus luteum was first described by Sandes in 1903 whilst studying the ovaries of the native cat *Dasyurus viverrinus*. Although he found it difficult to distinguish between the parts played by the *theca interna* and *theca externa* he was of the opinion that the only contribution of the *theca interna* was to form the supportive, vascular connective tissue. The corpora lutea of various marsupials, both American and Australian, have been described by O'Donoghue (1912; 1916) who stated "there is no doubt whatever that the *theca interna* plays no part in the production of lutein cells".

In subsequent studies there have been some differences in opinion concerning the formation of the corpus luteum. In 1943, Morgan reported that the corpus luteum of the opossum *Didelphis virginiana*, consisted of both granulosa cells and *theca interna* cells which had undergone luteinization. Martínez-Esteve (1942) and Guraya (1968) however decided that it was solely the granulosa cells which became luteinized whilst the unchanged *theca interna* provided the connective element of the gland. This conclusion was also reached for *Sminthopsis larapinta* (Godfrey, 1969); *Trichosurus vulpecula* (Pilton & Sharman, 1962; Shorey & Hughes, 1973); *Trichosurus caninus* (Smith & How, 1973) and *Antechinus minimus maritimus* (Wilson, 1986). However, Sharman (1955) believed that in *Setonix brachyurus* there were luteal cells derived from the *theca interna* as did Kean *et al.* (1964) regarding *Trichosurus vulpecula*.

The actual pattern of follicle collapse and formation of the corpus luteum varies in the several species described. In some (*Perameles nasuta, Isoodon obesulus, Macropus rufogriseus, Phascolarctos cinereus*: O'Donoghue, 1916; *Macropus giganteus*: Clark & Poole, 1967; *Isoodon macrourus*: Lyne & Hollis, 1979), the thecal elements rapidly penetrate the basement membrane and fill the central cavity

with connective tissue and blood capillaries. In others such as *Didelphis albiventris* and *Trichosurus vulpecula* (O'Donoghue, 1916); *Antechinus stuartii* (Woolley, 1966) and *Macropus eugenii* (Tyndale-Biscoe, 1984) the basement membrane is not penetrated until several days after ovulation and the central cavity is almost filled by hypertrophic granulosa cells before the capillary network invades it.

3.1.6. Interstitial tissue.

Since ovarian interstitial cells were forst described by Pflüger in 1863, their origins and formation have been the subject of much controversy. Bouin (1902) referred to collections of interstitial cells as interstitial glands but, as discussed by Harrison & Weir (1977), the term interstitial tissue may be preferable since "gland" implies a more discrete structure than is usually found for groups of interstitial cells.

In the comprehensive literature on the origin of interstitial tissue in various orders (Brambell, 1956; Mossman & Duke, 1973) numerous views have been expressed concerning the cellular elements which produce these cells. In the hamster, Knigge & Leathem (1956) believed that the interstitial tissue developed from the granulosa cells of atretic follicles whereas Davis & Broadus (1968) described rabbit interstitial tissue as being produced by a luteal-like transformation of the stroma and *theca interna* of both atretic and recently ovulated follicles. From her studies on the ferret (1970), rabbit and guinea pig (1972), Deanesly came to the conclusion that the stroma gave rise to primary interstitial tissue whilst the theca of atretic follicles produced secondary interstitial tissue. The same conclusion was reached following the examination of various hystricomorph rodents (Weir & Rowlands, 1974) and the woodmouse (Brook & Clarke, 1989) although these authors did not categorize the interstitial tissue as either primary or secondary.

Extensive studies by Guraya (reviewed 1973) and his associates have been made on the distribution and origin of interstitial tissue, in various mammalian species, utilizing histochemical techniques for identifying lipids. Although the histochemistry of the interstitial tissue was similar in all of the species, they observed that the amount varied greatly between species as did its origin. In the rat and bat, interstitial tissue was found to originate from the theca of atretic preantral and antral follicles; in the cat it came from the theca and surrounding stromal tissue of normal and atretic follicles of all sizes and in the dog it was observed to form similarly to that in the cat but in addition also came from

the germinal epithelium (Guraya & Greenwald, 1964). The interstitial tissue of the hamster was traced to the theca of atretic preantral and antral follicles (Guraya & Greenwald, 1965) whilst cattle were believed to have three types of interstitial tissue formed from the theca and surrounding stromal tissue of atretic follicles; the granulosa cells of atretic primordial follicles and the remnants of embryonic glandular tissue found in the deeper medullary parts (Guraya, 1968).

Based on the origin, location, time of appearance, resemblance to other endocrine tissue and relation to the reproductive cycle, Mossman & Duke (1973) identified seven fairly distinct types of interstitial tissue: fetal, thecal, stromal, medullary cord, rete, gonadal adrenal and adneural. However, they did concede that once differentiated some of these may be anatomically indistinguishable from one another and may have similar functions whether or not they structurally resemble one another.

O'Donoghue (1916) examined the ovaries of sixteen marsupial species and divided them into two groups according to whether or not interstitial tissue was present. He was surprised to find that this grouping corresponded with the two main divisions of the Marsupialia - the Diprotodonts which had interstitial tissue and the Polyprotodonts which did not. He found nothing to indicate that interstitial tissue was formed from the *theca interna*, old corpora lutea or atretic follicles and regarded it as a tissue *sui generis* although there was the possibility that it originated from modified stromal cells.

Subsequent studies on various Polyprotodonts (*Didelphis virginiana, Perameles nasuta, Isoodon macrourus*) have concluded that interstitial tissue is present in this group. Martínez-Esteve (1942) stated that in *Didelphis virginiana*, interstitial tissue arose from the persistent *theca interna* of atretic antral follicles whilst Guraya (1964) described it as sparsely scattered patches of cells which stemmed from the theca of atretic, large, preantral follicles. In contrast, in *Perameles nasuta* and *Isoodon macrourus* (Hughes *et al.*, 1965) the interstitial tissue was found to have a medullary origin. In the Diprotodonts examined, this was also the case for *Pseudocheirus peregrinus* (Hughes *et al.*, 1965) while in *Macropus eugenii* Alcorn (1975) derived the interstitial tissue from the rete cords.

3.2. MATERIALS AND METHODS

3.2.1. Source of material.

Ovaries were obtained from two groups of adult females both of which contained a mixture of virgin and parous animals. One group consisted of 4 females which were not in oestrus and the other of

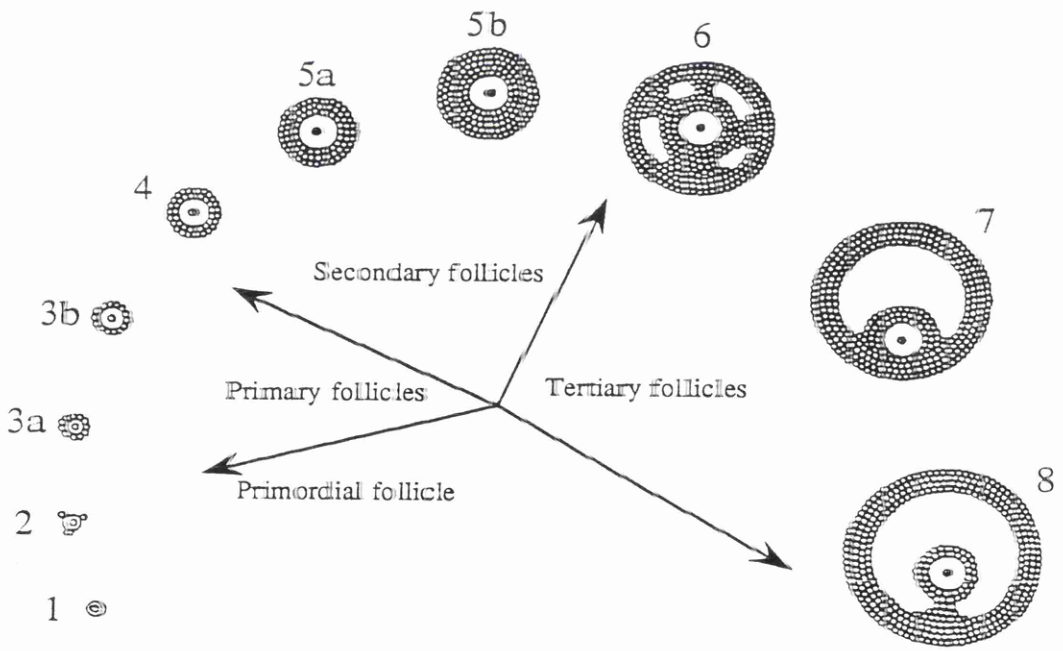
6 females (3 unpaired and 3 paired with a male) which were either in proestrus or oestrus as determined from urogenital sinus smears.

3.2.2. Preparation of paraffin wax sections.

Ovaries were fixed in either aqueous Bouin's fixative or 10% neutral buffered formalin for 24 hours, dehydrated through a graded ethanol series, cleared in histoclear and impregnated with paraffin wax. After being blocked in fresh paraffin wax, serial sections were cut longitudinally at 5 µm on a Leitz 1512 rotary microtome and mounted on poly-L-lysine coated glass slides. They were then stained with haematoxylin and eosin or Mallory's triple stain and examined under a Wild M20 light microscope.

3.2.4. Classification and measurement of follicles.

Follicles were staged using a classification adapted from that of Pedersen & Peters (1968).



Measurements of healthy follicles and oocytes were taken from paraffin wax sections, at x25 or x40 objective, using a calibrated ocular micrometer, and the mean diameters obtained from the

maximum diameter and the corresponding diameter at right angles. The basement membrane was taken as the outer limit of the follicle whilst the zona pellucida acted correspondingly for the oocyte. It was taken into account that in large, antral follicles (types 7 & 8) where the oocyte was displaced to one side, the maximum follicle and oocyte diameters would not usually be found in the same histological section. In order to always record the maximum diameters and ensure that a follicle was measured only once its appearance was followed and compared in serial sections. Polyovular follicles were not included in these measurements.

To distinguish between healthy and atretic follicles and follicles in various stages of atresia, some of the criteria employed in the mouse ovary (Byskov, 1974) were adopted. These were: the presence and percentage of pycnotic nuclei among the granulosa cells, the presence of polymorphonuclear leucocytes in the follicle, the presence of cavities in preantral follicles and disintegration of the basement membrane.

3.2.4. Statistical analyses.

An analysis of variance (1-way ANOVA) was conducted to test for differences in follicle and oocyte sizes between animals.

3.3. OBSERVATIONS.

3.3.1. General morphology.

The adult *Monodelphis domestica* ovary was found to be disc-shaped with a diameter of 2 - 4 mm and depth of approximately 1 mm depending on the stage of the oestrous cycle. It was enclosed within a flattened to cuboidal surface epithelium beneath which there was an incomplete *tunica albuginea ovarii*. The medullary region was relatively small and encompassed by a dense, well-developed cortex although there was no sharp delineation between the two zones. Connective tissue and large blood vessels were found in the medulla whilst the various follicle types, corpora lutea and interstitial cells were in the cortex. Primordial follicles tended to be bunched in groups, often intermingled with primary follicles and were located in the outer regions of the cortex. In contrast, primary, secondary and tertiary follicles were found throughout the cortex including the innermost area contiguous to the medulla.

3.3.2. Follicle types.

The smallest follicle found in the adult ovary was the type 2 primordial follicle (Fig.3.1). This consisted of a small, quiescent oocyte which was ovoid to spheroid in shape measuring about 22 μm in diameter. The germinal vesicle was spheroidal, with an irregularly dense chromatin network, and occupied a large proportion of the oocyte. A single nucleolus was often visible, with the immediately surrounding area appearing to contain considerably less chromatin. This chromatin-sparse region consisted of a few interlaced chromatin strands which merged into the much denser periphery of the germinal vesicle. The oocyte cytoplasm was mottled in appearance and when semithin resin sections were examined at high power, with the x100 objective, small, darkly-stained organelles were visible. A few flattened granulosa cells, with dense darkly-stained nuclei, were arranged around the oocyte and rested on a basement membrane.

The type 3a primary follicle consisted of a small oocyte which had commenced growing and was surrounded by a single layer of granulosa cells (Fig.3.1). The oocyte cytoplasm contained vesicles which remained unstained by haematoxylin or eosin - these were interspersed with the aforementioned organelles and showed no obvious pattern of arrangement. The ratio of germinal vesicle to cytoplasm was now about equal. The granulosa cells had become more cuboidal in form, which was reflected in their nuclei being less elongate in shape. Several theca cells which were small and flattened with dense. crescent-shaped nuclei had appeared around the follicle.

Progression to the 3b stage was characterized by an increase in the number of granulosa cells enclosing the oocyte and a change in their shape to a more columnar form (Fig.3.1). The zona pellucida had formed and a single layer of theca cells was more or less complete. The germinal vesicle:oocyte ratio had now shifted in favour of the oocyte.

In type 4 follicles the growing oocyte had two complete layers of granulosa cells (Fig.3.1). There was no significant change in the visible structure of the oocyte other than a slight increase in the size and number of cytoplasmic vesicles. The granulosa cell nuclei had become more elongated and a second layer of theca cells was beginning to form.

The type 5a follicle consisted of three complete layers of granulosa cells (Fig.3.1). The oocyte cytoplasm was filling with the vesicles which varied considerably in size. They showed no obvious polarity or size gradient but tended to occupy the central region of the oocyte, with the organelles

accumulated around the periphery. The granulosa cells were elongate in shape and several layers of theca had formed around the follicle.

The type 5b follicle contained a large oocyte enclosed by more than three layers of granulosa cells. The germinal vesicle was eccentric and tended to be located in the outer zone of the oocyte. In contrast to the single nucleolus observed in smaller oocytes, several prominent nucleoli were present in the 5b germinal vesicle. The vesicles were now very conspicuous and, apart from the extreme periphery, occupied most of the oocyte.

The type 6 follicle consisted of a large oocyte, with a diameter of about 150 μm, encircled by many layers of granulosa cells and several layers of theca (Fig.3.2). At this stage it was possible to distinguish that the theca had differentiated into two separate layers - the *theca interna* and *theca externa*. The fully developed *theca interna* cells were large and polygonal in shape whereas the *theca externa* consisted of a dense, concentric arrangement of smaller, elongated cells. Antrum formation had commenced and in one or more areas some of the granulosa cells were separated by follicular fluid.

With development into the type 7 follicle, the fluid-filled areas coalesced to form a single crescent -shaped antrum which surrounded the oocyte (Fig.3.2). The antral fluid was slightly granular in appearance and as it accumulated the oocyte was displaced to one side of the follicle where it was surrounded by just one to three layers of granulosa cells which formed the *cumulus oophorus*. Unlike the granulosa cells in the main body of the follicle, which were mostly columnar, the cumulus oophorus cells around the oocyte were flattened like those initially seen in the primordial follicle. The granulosa cells in the basal layers of the follicle also tended to have basally rather than centrally located nuclei.

In the type 8 follicle the oocyte, measuring approximately 170 μm, had moved back to a more central position due to the *cumulus oophorus* extending to form a stalk which projected towards the middle of the follicle (Fig.3.2). The antrum continued to increase in size, distending the follicle until eventually the granulosa and theca layers were compressed to just a few cells in thickness. Follicles which reached the preovulatory stage had a very well vascularized theca and had moved to the outermost region of the cortex where they could be seen bulging on the surface of the ovary. In the final stages, prior to ovulation, the granulosa cells of both the *cumulus oophorus* and follicle began to disperse and there was breakdown of the basement membrane.

3.3.3. Follicle and oocyte growth.

The measurements obtained indicate that the growth of the oocyte, in relation to its follicle, did not conform to a strict biphasic pattern as it continued to grow after antrum formation. There was also a striking variation in the occurrence and size of antral follicles during urogenital sinus oestrus between paired and unpaired animals. Although the size of the type 6 follicle and its oocyte was very similar in both paired and unpaired females, this stage was rarely found in the former group - most of their antral follicles had reached the type 7, type 8 or preovulatory stages. In contrast, the ovaries of unpaired females were not observed to have any preovulatory or healthy type 8 follicles. The most developed follicle reached in these ovaries was the type 7 which was significantly smaller ($F_{1,77}$ =10.76, p=0.002), as was its oocyte ($F_{1,77}$ =15.56, p=0.0001), than that found in paired animals. There was a significant increase in oocyte size in paired ($F_{1,151}$ =10.74, p=0.002) and unpaired ($F_{1,91}$ =20.97, p=0.0001) animals and follicle size in paired ($F_{1,51}$ =5.53, p=0.023) and unpaired ($F_{1,91}$ = 84.14, p=0.0001 respectively) between the type 6 and type 7 stage. During the development of a type 7 into a type 8 follicle, seen only in paired females, there was a significant change in follicle diameter ($F_{1,51}$ = 11.37, p=0.002) but not in oocyte diameter ($F_{1,151}$ = 10.74, p=0.69).

Table 3a. Mean follicle and oocyte diameters in unpaired females (± SEM).

Follicle type	No. measured	Follicle diameter (μm)	Oocyte diameter (μm)
2	75	26 (± 0.54)	22 (± 0.49)
3a	75	34 (± 0.76)	26 (± 0.62)
3b	75	57 (± 1.92)	41 (± 1.58)
4	75	109 (± 3.12)	79 (± 2.52)
5a	75	142 (± 3.61)	100 (± 3.02)
5b	75	184 (± 4.87)	112 (± 3.22)
6	49	305 (± 8.16)	146 (± 3.96)
7	27	360 (± 11.60)	156 (± 3.01)

Table 3b. Mean follicle and oocyte diameters in paired females (± SEM).

Follicle type	No. measured	Follicle diameter (μm)	Oocyte diameter (μm)
2	25	27 (± 0.53)	23 (± 0.42)
3a	25	36 (± 0.42)	25 (± 0.39)
3b	25	61 (± 2.10)	43 (± 1.73)
4	25	104 (± 2.76)	79 (± 2.61)
5a	25	161 (± 4.53)	114 (± 3.17)
5b	25	191 (± 5.78)	128 (± 5.18)
6	4	311 (± 9.24)	151 (± 4.71)
7	73	543 (± 18.12)	176 (± 2.24)
8	15	644 (± 16.05)	177 (± 9.15)

Thus there were significant differences between the antral follicles of paired and unpaired females when the absolute growth of the follicle and its oocyte were compared (Fig.3.3a). When one compares the growth of the oocyte relative to that of its follicle however (Fig.3.3b) there was very little difference between the two groups - if anything, in type 7 follicles the oocyte was slightly larger in relation to its follicle in the unpaired ovary.

3.3.4. Polyovular follicles.

Polyovular follicles were observed in 24% of the females examined and occurred in both ovaries (Fig.3.4). A few primordial polyovular follicles, with oocytes in direct contact with one another, were observed but the majority were primary or secondary follicles. In these, the oocytes were completely enclosed by granulosa cells and the structure appeared to be comprised of individual follicles sharing several granulosa cells and a common enveloping theca (Fig.3.5). A spherical cluster of follicles was typically seen although occasionally the elongate form with the linear oocyte arrangement occurred. The number of oocytes enclosed ranged from two to eleven with the majority being bi or triovular and there was considerable variation in oocyte size both within and between polyovular follicles.

Polynuclear oocytes were not observed in any of the sections examined.

3.3.5. Atretic follicles.

Atresia was found to occur at various stages of follicular development but was most common in antral and large preantral follicles. The first recognizable evidence was nuclear pycnosis of the granulosa cells and Byskov's (1974) three categories of atresia were identified in large follicles.

Follicles in stage 1 atresia had up to 20% pycnotic granulosa cells which were often fragmented. The basement membrane was intact and there were no polymorphonuclear leucocytes present. Stage 2 atretic follicles had a similar proportion of pycnotic cells as the previous stage but the basement membrane was no longer intact and leucocytes were seen among the granulosa cells. In stage 3, most of the parameters were similar to stage 2 except the percentage of pycnotic nuclei was only 5%.

Degenerative changes were first observed in the type 4 follicle - earlier stages did not appear to be affected. Preantral follicles showed fairly uniform nuclear pycnosis of their granulosa cells with the oocyte gradually becoming distorted from its normal spherical shape and the zona pellucida appearing

to thicken and become more eosinophilic. In antral follicles atresia was indicated by nuclear pycnosis of the mural granulosa cells lining the antrum: these eventually broke away and floated freely in the follicular fluid. The follicle layers gradually became thinner as the granulosa cells underwent pycnosis and the *cumulus oophorus* dispersed until the oocyte with its zona pellucida was left floating within the degenerating follicle. The follicle was then invaded by leucocytes and stromal tissue and the oocyte and zona pellucida disintegrated.

3.3.6. Corpora lutea.

Several corpora lutea, measuring up to 700 μm in diameter, were observed in the ovaries of one pregnant female (Fig.3.6). Two cell types were distinguishable, the majority of which were luteinized granulosa cells which were relatively large and polygonal with prominent spheroidal nuclei. Smaller, elongate cells with narrow darkly stained nuclei were sandwiched between the granulosa luteal cells forming ingrowths of connective tissue which reached the central cavity where it formed a plug. These smaller cells appeared to be theca cells, most of which were from the *theca interna* rather than the *theca externa*. In some areas around the periphery it was possible to see the infoldings of the ruptured follicle where there was an infiltration of *theca interna* cells and blood vessels (Fig.3.7).

From the limited material available from pregnant and lactating females it would appear that the corpus luteum begins to regress very shortly after parturition. In the one lactating animal obtained, with a 6 day old litter, only small remnants of the corpora lutea could be found. There was no evidence of accessory corpora lutea formed by preovulatory luteinization of granulosa cells.

3.3.7. Interstitial tissue.

Two types of interstitial tissue, described as either vacuolated or corded in this study, were found in varying amounts. The vacuolated form appeared similar to a corpus luteum - it was lightly stained and consisted of large, rounded cells with a relatively small nucleus (Fig.3.8). The vacuoles within them showed a large size variation. In some instances the cells formed a discrete structure and could be traced to a degenerating follicle but it was impossible to tell which part of the follicle they originated from. However, not every atretic follicle, either preantral or antral, appeared to produce or to be associated with this tissue. In other cases, small groups of vacuolated cells were scattered through the

ovary and their source could not be traced. Unless followed through serial sections, atretic oocytes with a heavily vacuolated cytoplasm could be mistaken for this type of interstitial tissue.

The corded form was more darkly stained and composed of smaller, oval cells with relatively large nuclei This tissue appeared more structured than the vacuolated type and was arranged in cords, often separated by connectice tissue and elements of the vascular system (Fig.3.8). Both types were observed during pro-oestrus, oestrus and early lactation whilst only the vacuolated interstitial tissue was present during pregnancy.

Fig. 3.1. Various types of preantral follicle (x75).

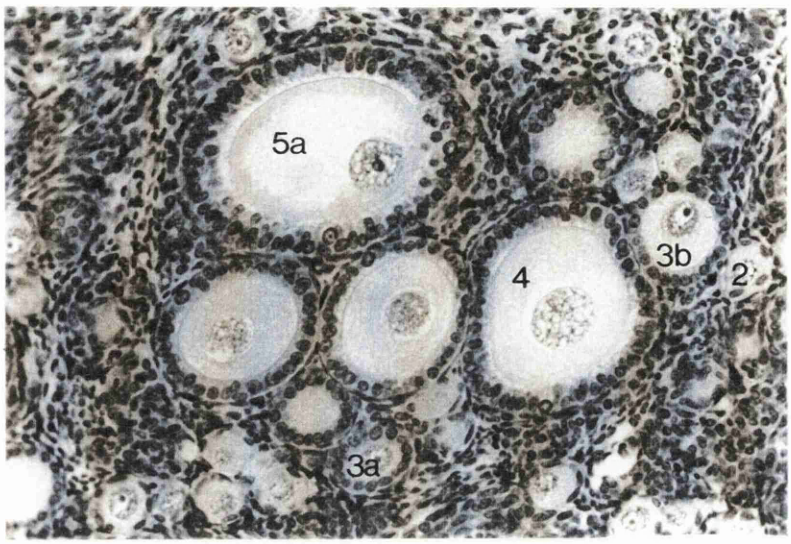


Fig. 3.2. Types of antral follicle (x30).

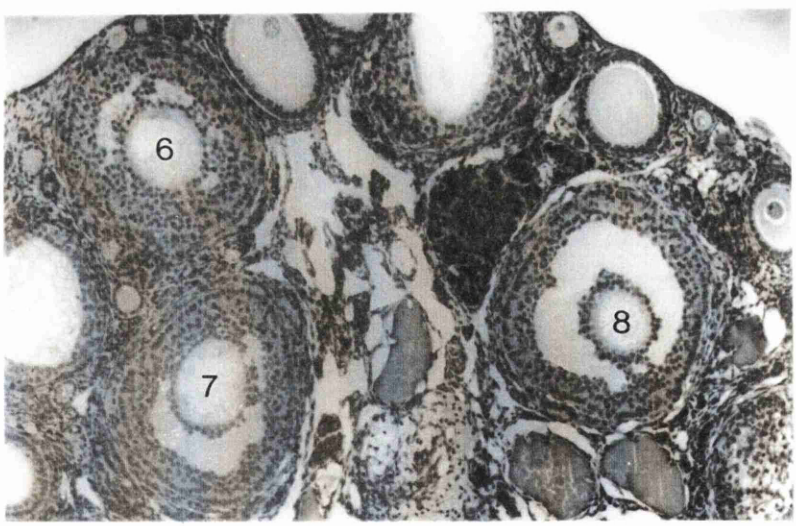


Fig. 3.3a. Graph of follicle and oocyte growth in unpaired and paired females.

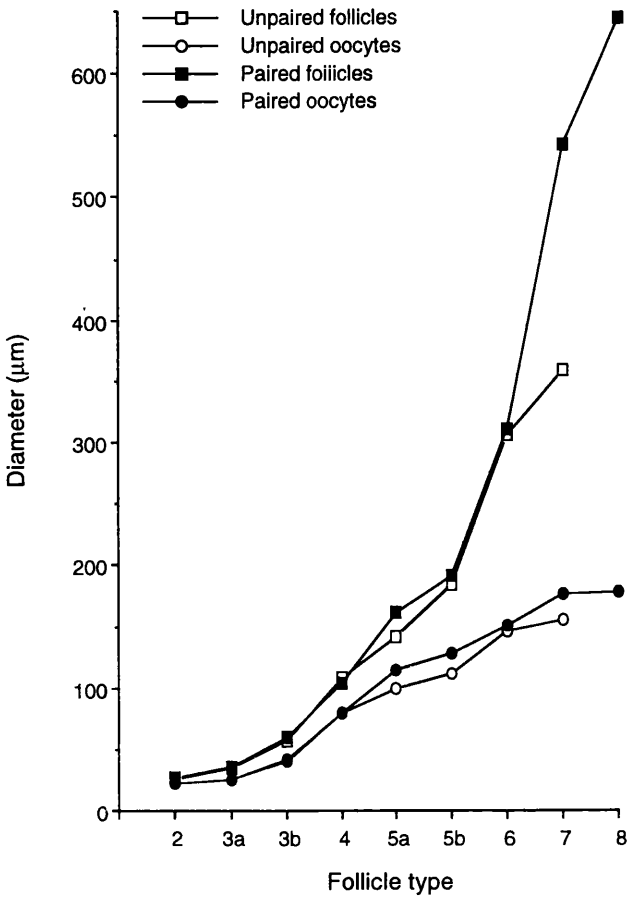


Fig. 3.3b. Graph of oocyte growth in relation to its follicle.

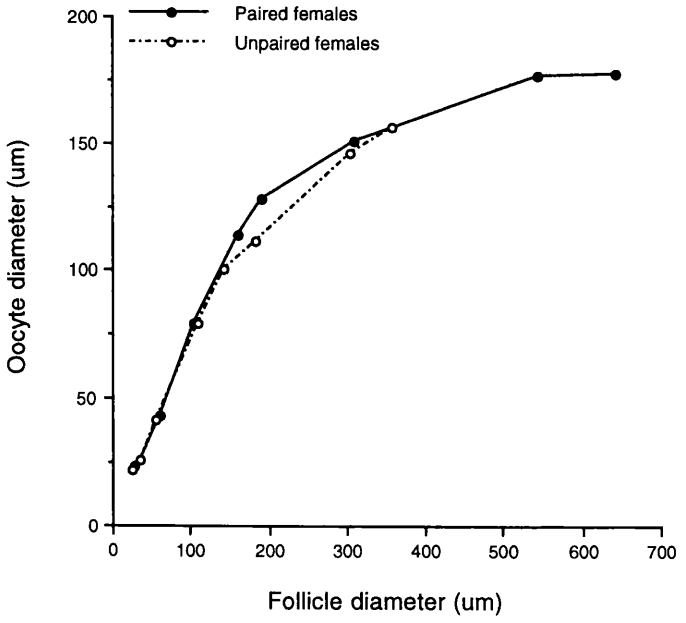


Fig. 3.4. Polyovular follicles (arrowed) (x30).

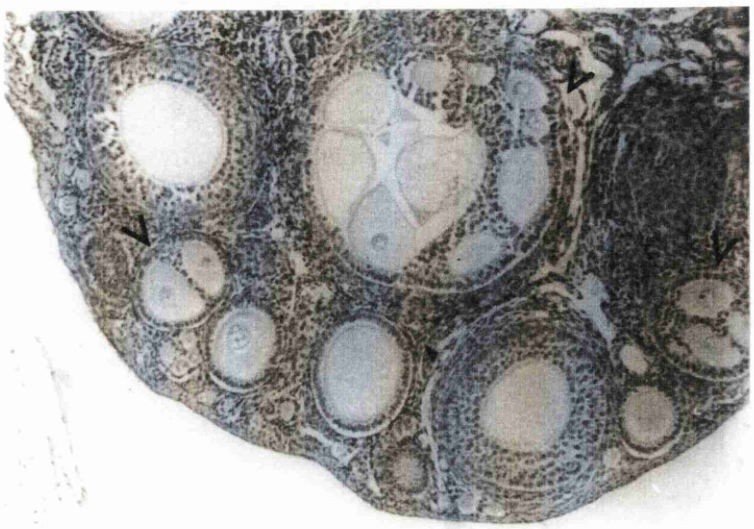


Fig. 3.5. Polyovular follicles containing similar sized oocytes (x 70)

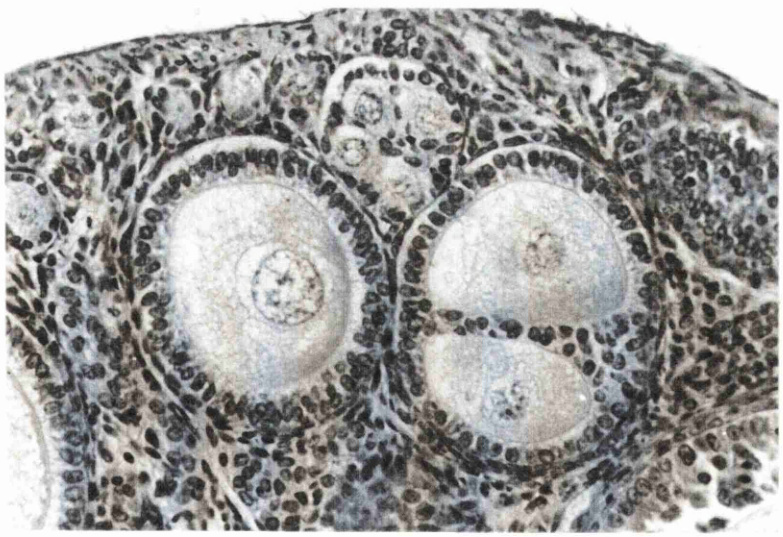


Fig. 3.6. Corpus luteum (CL). (x30).

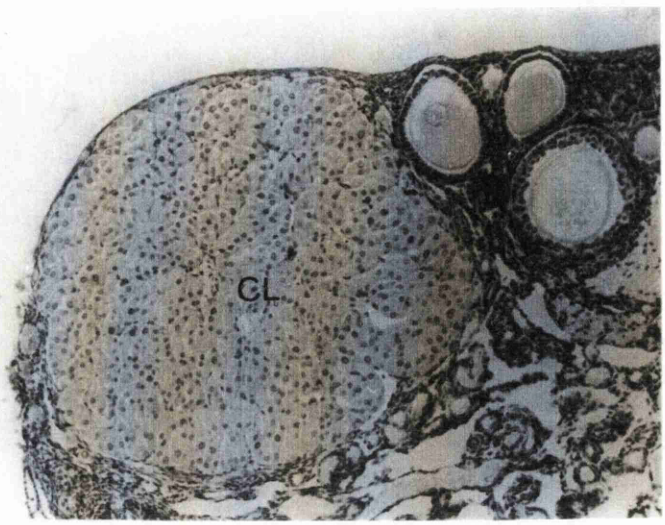


Fig. 3.7. Corpus luteum consisting of large, luteinized granulosa cells (gl) being infiltrated by theca interna cells (ti) (x75).

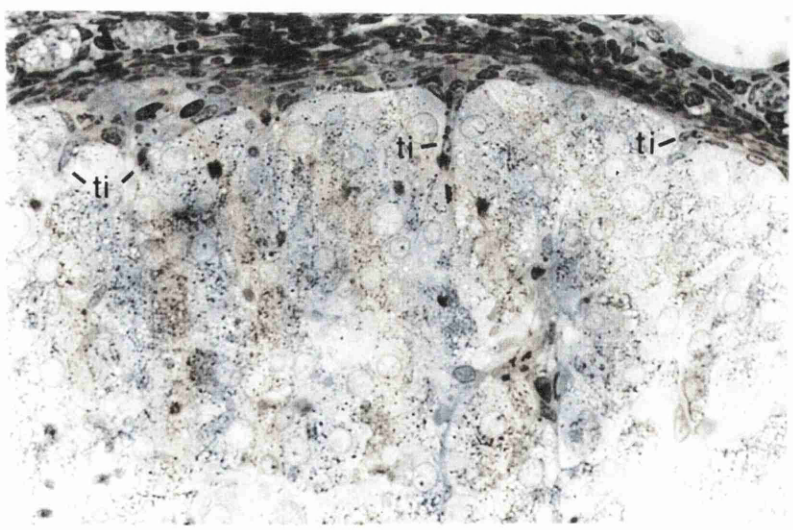


Fig. 3.8. Ovary containing vacuolated interstitial tissue (IT) and corpora lutea (CL). (x30).

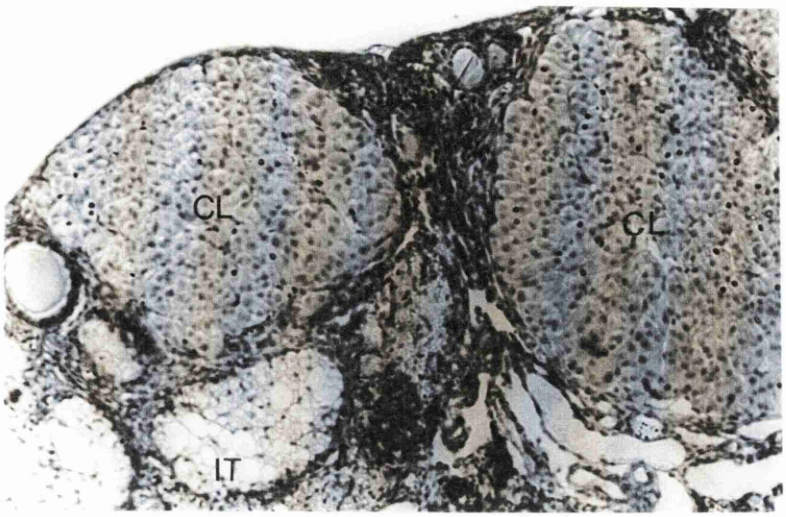
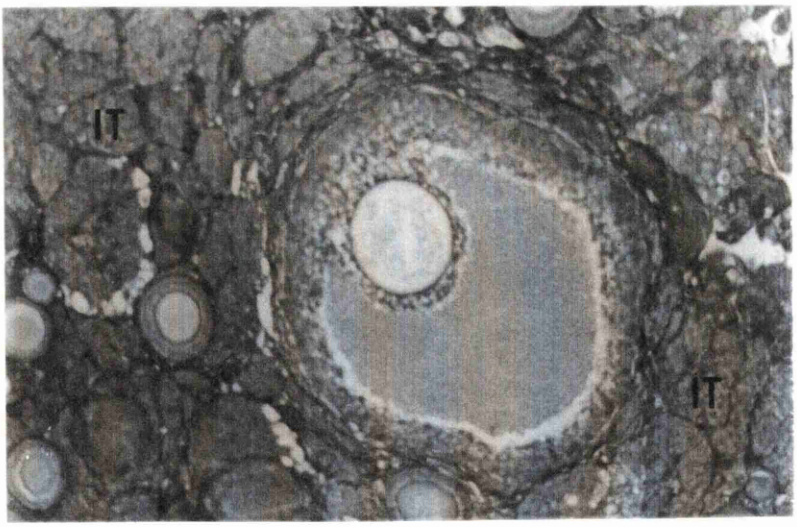


Fig. 3.9. Ovary with prominent corded interstitial tissue (IT). (x30).



3.4. DISCUSSION.

3.4.1. Ovarian morphology.

Morphologically, the adult *M. domestica* ovary appeared to be similar to that of the eutherian mammal and previous workers studying various other marsupial species have also indicated such a similarity (Martinez-Esteve, 1942; Woolley, 1966; Mossman & Duke, 1973). *M. domestica* resembles other marsupials such as *Dasyurus viverrinus* (Hill, 1910), *Didelphis virginiana* (Martinez-Esteve, 1942), *Pseudocheirus peregrinus* (Hughes *et al.*, 1965); *Isoodon macrourus* and *Perameles nasuta* (Lyne & Hollis, 1983) in that the cumulus cells do not form a corona radiata as seen in eutherians.

3.4.2. Follicle and oocyte growth.

As recently observed (Falconnier & Kress, 1992; Rodger *et al.*, 1992), a strictly biphasic growth pattern did not appear to hold entirely true for *M. domestica* since oocyte growth continued throughout the antral period. With respect to the actual diameters reached by the oocytes and follicles at different stages of development however, there were quite significant differences between this study and the previous two. In the Glasgow colony, *M. domestica* follicles had reached an average of 306 μm and their oocytes 148 μm in diameter when antral fluid began to accumulate. This was considerably larger than previously observed: Falconnier & Kress reported that antrum formation commenced in follicles of 201 μm with oocytes of 99.5 μm and Rodger *et al.* (1992) recorded it in follicles of 170 μm with oocytes of 128 μm.

There are several possibilties which may account for, or partly contribute to, both the variable size results obtained in *M. domestica* and the different biphasic growth pattern seen in other marsupial species. Not all of the groups used the same fixative, embedding medium or method of measuring. For example, Falconnier & Kress (1992) took their measurements from photographs of semithin sections whereas all of the other groups used a calibrated eyepiece to measure the diameters directly from Bouin's or formalin-fixed wax sections.

It is probably the decision of exactly where to measure the diameters of the follicles and oocytes which accounts for the greatest variation. Falconnier & Kress (1992) obtained their measurements from the mean value of two diameters, perpendicular to each other, passing through the oocyte nucleus. As the position of the germinal vesicle is not always central within the oocyte and the oocyte itself is not

always central within the follicle, using this as a marker cannot act as a standard in every follicle. This method does not guarantee that the maximum diameter is being recorded and would give an inaccurate picture of oocyte and follicle growth in relation to each other. To monitor accurately the absolute growth of both the follicle and its oocyte, it was decided in this study that the average maximum diameter reached by each of them should be measured. The largest size discrepancy would occur in antral follicles where, due to the eccentric position of the oocyte, the largest diameters for each would not appear in the same section - it was therefore necessary to compare their sizes in serial sections so that the maximum diameter could be found for each of them. It is not possible to comment on measurement variations that may have occurred in the study by Rodger *et al.* (1992), as they have not stated their criterion for measurement taking, whether they were maximum diameters or if they took the average of two recordings. However, females studied by this group had undergone a superovulation regime which may have resulted in the production of smaller follicles than normal. Analysis of preovulatory follicles from superovulated cows has revealed that such follicles are usually 30% smaller than preovulatory follicles in control cycles (Mariana *et al.*, 1991).

Another important factor is the actual number of follicles measured and how many animals were involved. Compared to the 768 follicles from nine animals in this study other groups have had fairly low numbers to work with. Falconnier & Kress (1992) measured a total of 228 follicles, from an unstated number of animals, while Rodger *et al.* (1992) only presented data from 56 follicles, with just two to six observations for each stage, obtained from six animals. Moreover, in the paper by Falconnier & Kress (1992) their photograph of a typical Graafian follicle would have been classed as atretic in this study and so would have been discarded. If their measurements included those taken from atretic follicles then this is likely to contribute to the different size recordings.

Apart from the study by Panyaniti *et al.* (1985) on *M. eugenii*, neither of the *M. domestica* studies or those on various other marsupial species (Alcorn, 1975; Lintern-Moore *et al.*, 1976) have monitored or considered the reproductive state of their females. As shown in this report, this is an important factor since the antral follicles, and their oocytes, of paired females reach a significantly larger size during oestrus than those of unpaired females.

Although not likely to be major factors when considered individually, when all of these possible sources of variation are taken into account they are likely to make a significant contribution towards the

differing results found between this study and previous ones. Finally, as far as *M. domestica* is concerned, it is possible that there is a significant variation between animals in different colonies. As reported by van Oorschot *et al.* (1992), animals caught in two separate regions of Brazil differed significantly both in size and several morphological traits. A distinct difference in allele frequency and heterozygosity was detected between the two groups which led these authors to the conclusion that the genetic differences between populations within the colony should be considered in the selection of experimental subjects. It is therefore feasible that such variations may be playing a part in the differing results obtained between colonies.

3.4.3. Polyovular follicles.

Although, polyovular follicles were not that uncommon in *M. domestica*, with 24% of females possessing them, their incidence was significantly lower than the 67% seen in *Didelphis virginiana* (Hartman, 1926). The various types of polyovular follicle described in Hartman's report were also identified in *M. domestica* and similarly most of them belonged to the type 1 category, with the oocytes separated by granulosa cells. Although Morgan (1943) made no mention of them, Hartman also found that the vast majority of *Didelphis virginiana* harboured polynuclear oocytes, but such structures were never observed in *M. domestica*.

As far as their origins are concerned, it would appear that Stöckel's theory of polynuclear oocyte division (see Hartman, 1926) can be discounted for *M. domestica* as despite the occurrence of polyovular follicles, such oocytes were never observed. Hartman (1926) was also doubtful about their role as he similarly found lots of polyovular follicles but no polynuclear oocytes in the cat and *vice versa* in a small number of *Didelphis virginiana* which had many polynuclear oocytes but no polyovular follicles. Loeb's suggestion (see Hartman, 1926) that polyovular follicles are formed by follicular concrescence was favoured by Papadaki (1978) following an ultrastructural study of the adult human ovary. However, considering that polyovular follicles have a fairly high incidence in young animals, which significantly decreases with age, it does seem an unlikely method of formation. It would appear most likely that polyovular follicles, at least in *M. domestica*, are formed when several oocytes become enclosed by a common layer of granulosa cells.

Surprisingly, Falconnier & Kress (1992) reported that polyovular follicles were extremely rare in *M. domestica* but as the total number of ovaries they examined was not stated it may be that they only had a small sample size and therefore less chance of finding them.

3.4.4. Atresia.

As in most adult mammals (Byskov, 1978, 1979; Hirshfield & Midgley, 1978), atresia in *M. domestica* was most common in large preantral and small antral follicles. Although it has been commonly believed that atresia can occur at any stage of follicular development, which is probably true, careful analysis of growth and atresia in a number of species has revealed that atresia is not equally distributed throughout follicle development. Studies in rats (Butcher & Kirkpatrick-Keller, 1984; Hirshfield, 1991) and cattle (Lussier *et al.*, 1987) have revealed that atresia is most prevalent near the end of follicle development.

3.4.5. The corpus luteum.

The *M. domestica* corpus luteum consisted of both granulosa and *theca interna* cells although only the former appeared to have undergone luteinization. The *theca interna* formed trabeculae which acted as a connective tissue skeleton and also carried the blood vessels into the gland. This observation is in agreement with others studying various other marsupial species (*Didelphis albiventris*: O'Donoghue, 1912, 1916; *Didelphis virginiana*: Martínez-Esteve, 1942; Guraya, 1968; *Sminthopsis larapinta*: Godfrey, 1969; *Isoodon macrourus* and *Perameles nasuta*: Lyne & Hollis, 1979). It is also very similar to that described in certain eutherian species such as

3.4.6. Interstitial tissue.

The two types of interstitial tissue identified probably correspond to the thecal and medullary type as classified by Mossman & Duke (1973). Although it was rarely possible to pinpoint its origin, the vacuolated form was most likely to be derived from the theca. When found as a large cluster, this tissue was not unlike a corpus luteum. Such a similarity has been noted in several eutherian species (water shrew: Price. 1953 and some New World monkeys: Dempsey, 1939) to the extent that it was sometimes difficult to distinguish between them. When fully differentiated, *M. domestica* thecal type interstitial

cells contained abundant vacuoles of lipoid material which usually dissolved in the histological processing, leaving lots of clear space in the cytoplasm, which enabled these cells to be distinguished from corpora lutea. It was not always possible to trace the origins of this interstitial tissue but it may be that it remains long after all the remnants of the follicle have disappeared and just breaks up into small groups of cells scattered throughout the ovary. The fact that there was no obvious interstitial tissue associated with every atretic follicle, either preantral or antral, seems a little surprising but as proposed by Mossman & Duke (1973) it may be the case that such follicles began to degenerate at a time when there was no stimulus for the differentiation of interstitial cells. The corded form of interstitial tissue probably corresponds to the medullary cord type and was very similar to that seen in the long-tailed weasel (Mossman & Duke, 1973).

Development of the vacuolated form of interstitial tissue was at a maximum in pro-oestrus and oestrus as has been noted in the woodchuck (Rasmussen, 1918), guinea pig (Stafford & Mossman, 1945), badger (Neal & Harrison, 1958) and wood mouse (Eriksson & Nyholm, 1983) and was present in smaller amounts during pregnancy. Corded interstitial tissue was also most apparent during pro-oestrus and oestrus but not observed during pregnancy which is similar to the pattern seen in Mustelids. As suggested by Deanesly (1970), it would appear that the development and proliferation of interstitial tissue at certain times may be in response to gonadotrophin surges.

Chapter 4

The ovary and follicle growth at the ultrastructural level

4.1. INTRODUCTION.

4.1.1. Follicle ultrastructure.

In the past thirty years, the fine structure of ovarian follicles from many eutherian species have been studied intensively. Ultrastructural examinations of oocytes and their follicles from various laboratory species (mouse: Yamada *et al.*, 1957; Wassarman & Josefowicz, 1978; rat: Sotelo & Porter, 1959; Odor, 1960; guinea pig: Anderson & Beams, 1960; Adams & Hertig, 1964; rabbit: Blanchette, 1961; Zamboni & Mastroianni, 1966 a,b, hamster: Weakley, 1966; 1967; 1969) have served as the foundation for comparative studies on those of domestic animals (cat: Liss, 1964; dog: Szabo, 1967; Tesoriero, 1981; cow: Fleming & Saacke, 1972; Kruip *et al.*, 1983; Hyttel *et al.*, 1986; de Loos *et al.*, 1989; sheep: Cran *et al.*, 1979; 1980; pig: Cran, 1985; horse: Vogelsang *et al.*, 1987): primates (monkey: Hope, 1965; human: Hertig & Adams, 1967; Baca & Zamboni, 1967; Sundström *et al.*, 1985) and some of the more unfamiliar species (bat: Wimsatt & Parks, 1966; Egyptian spiny mouse: Kang & Anderson, 1975; shrews: Kress, 1984a,b; blue fox: Hyttel *et al.*, 1990). For general reviews see Hadek (1965); Norrevang (1968) and Zamboni (1970).

In comparison relatively little attention has been paid to the ultrastructure of marsupial oocytes and such studies have only focussed on a particular stage of development. Ullmann (1978) examined the primordial oocyte of *Isoodon macrourus* and described various unusual structures including a conspicuous particulate paranuclear complex; a vesicle-microtubule complex and an aggregate of tubular cisternae. Lyne & Hollis (1983) studied the oocytes from Graafian follicles of this species and found crystalloid inclusions similar to those observed in the paranuclear complex of primordial follicles (Ullmann, 1978) and in yolk material of unilaminar blastocysts (Lyne & Hollis, 1976). Surprisingly, there appears to have been no work bridging the gap of oocyte development between these two stages. Breed & Leigh (1988, 1990, 1992) examined the morphological aspects of oocyte maturation and fertilization in *Sminthopsis crassicaudata*, and Moore and his colleagues have concentrated on fertilization and early embryonic development in *M. domestica* (Baggott & Moore, 1990; Taggart *et al.*, 1993; Moore & Taggart, 1993). The only study encompassing most of oocyte development appears to be that of Falconnier & Kress (1992), on *M. domestica*, and even this does not describe the final stages of maturation in great detail.

Other marsupial studies have been based on fertilized ova and blastocysts (Selwood & Sathananthan, 1988) or the structure of the oocyte investing coats (Hughes & Shorey, 1973; Krause & Cutts, 1983; Talbot & DiCarlantonio, 1984; Phillips & Fadem, 1987).

4.1.2. Oocyte organelles.

Apart from the commonly found cell organelles, mammalian oocytes have been observed to contain a number of special structures including a paranuclear complex, annulate lamellae, nuage and cortical granules. The paranuclear complex is the term given to the cytoplasmic region of the oocyte where, in some species, the majority of its organelles are concentrated (guinea-pig: Adams & Hertig, 1964; rabbit: Zamboni & Mastroianni, 1966a; human: Baca & Zamboni, 1967). Annulate lamellae are stacks of parallel, paired membranes which are found limiting flattened cisternae. At regular intervals the membranes of each unit are directly apposed to or fused with each other and these sites appear as electron dense areas. These structures are especially prominent in human oocytes (Adams & Hertig, 1965; Baca & Zamboni, 1967) and evidence has been provided that they form by the fusion of vesicles originating from the blebbing activity of the nuclear envelope (Kessel, 1963). The term nuage has been used to describe dense, fibrous material which exists either as small, discrete bodies in the cytoplasm or as "cementing material" situated inbetween clusters of mitochondria (Adams & Hertig, 1964; Eddy, 1974).

In contrast to the aforementioned structures which are not found in every species, cortical granules have been reported universally. They are small, spherical, membrane-bound organelles found in the cortex of unfertilized oocytes. At fertilization they fuse with the oolemma and release their contents into the perivitelline space altering the physical properties of the zona pellucida and thus preventing polyspermy (reviewed by Gulyas, 1980; Guraya, 1982; Cran, 1989). The close proximity of cortical granules and Golgi complexes was noted during early observations on various species (Adams & Hertig, 1964; Weakley, 1966; Baca & Zamboni, 1967; Zamboni, 1970) and it is generally thought that the Golgi are responsible for cortical granule synthesis. In bovine and porcine oocytes however, (Hyttel *et al.*, 1986; Cran, 1985) cortical granule formation is more closely associated with certain parts of SER than with Golgi. Once formed, cortical granules migrate towards the oocyte surface and this centrifugal movement appears to be a common feature during the later stages of mammalian oocyte

maturation (Zamboni & Thompson, 1972; Cran *et al.*, 1980; Kruip *et al.*, 1983). Two morphologically distinct cortical granule populations, based on electron density, have been observed in a number of species (Austin, 1961; Hadek, 1963; Szöllösi, 1967, 1971; Anderson, 1974; Selman & Anderson, 1975; Nicosia *et al.*, 1977; Cran & Cheng, 1985; Ducibella *et al.*, 1988) but it is unclear whether the two types are functionally different or merely represent varying maturational stages. The stages at which cortical granules are first observed also varies between species: in the mouse and rat they first appear in primary follicles (Szöllösi, 1967, 1976; Odor & Blandau, 1969) whilst in the monkey (Hope, 1965), human (Baca & Zamboni, 1967), hamster (Selman & Anderson, 1975; Hosoe *et al.*, 1993) and dog (Tesoriero, 1981) they are formed in the oocytes of multilayered follicles.

In the majority of marsupial species studied, cortical granule formation has been found to occur much later on in oocyte development than in eutherians and Mate *et al.* (1992) suggested that cortical granule formation may be a periovulatory event. In *Trichosurus vulpecula* and *Macropus eugenii*, Rodger (1991) found that preovulatory oocytes obtained for *in vitro* fertilization possessed few or no cortical granules and were unable to bind sperm. Cortical granules have been observed in secondary preovulatory oocytes from *Didelphis virginiana* (Rodger & Bedford, 1982) and *Sminthopsis crassicaudata* (Breed & Leigh, 1990) and their release was shown to accompany fertilization. However, Breed & Leigh (1992) do not support the "periovulatory event" proposed by Mate *et al.* (1992) as they observed cortical granules in primary follicles of *Sminthopsis crassicaudata*.

In their studies of the bovine oocyte, Senger & Saacke (1970) and Hyttel *et al.* (1986) discovered that an unusual form of mitochondrion, which they described as "hooded", was abundant in all mature oocytes. These forms possessed a hook-like extension which formed an extramitochondrial cavity that often appeared intramitochondrial when the plane of the section passed through the hood. They have also been seen in sheep (Fleming & Saacke, 1972; Russe, 1975; Cran *et al.*, 1980) and goat antral follicles (Fleming & Saacke, 1972) suggesting an association either with ruminants or metabolic characteristics peculiar to ruminants.

A great variety of different cytoplasmic inclusions have been observed within oocytes. In the mouse, a storage form of ribosomes has been described (Burkholder *et al.*, 1971) which exists as chains that are capable of cross-linking to form lattices. Similar arrangements of ribosomal fibrils, often associated with lamellar complexes, have been described in the Egyptian spiny mouse (Kang &

Anderson, 1975). Lamellar inclusions in the pocket gopher (King & Tibbitts, 1977) appear in a variety of unusual forms which were considered to be different planes of section through either a set of concentric cylindrical sheets of varying diameter or a single sheet wound in a spiral fashion. Nilsson (1980) reported that mature mouse, rat and hamster oocytes were packed with highly ordered arrays of parallel chains or lattice-like structures. Structures such as vesicles and multivesicular bodies have also been described in various species such as the cow (Fleming & Saacke, 1972; Kruip *et al.*, 1983; Hyttel *et al.*, 1986; de Loos *et al.*, 1989); sheep (Russe, 1975; Cran *et al.*, 1980); shrews (Kress, 1984a, b); human (Sundstrom et al., 1985); horse (Vogelsang *et al.*, 1987) and blue fox (Hyttel *et al.*, 1990). All of these assorted organelles and structures have been referred to as yolk and the composition of the various yolk forms shall be discussed further in chapter 6.

4.1.3. Oocyte and follicle communication.

Throughout follicular development the granulosa cells and oocyte can communicate with each other via gap junctions. These are channels through the plasma membrane which allow the passage of low molecular weight molecules between cells and such movement of metabolites from one cell directly into another cell is termed metabolic cooperativity or metabolic coupling (Gilula *et al.*, 1972). Utilizing lanthanum tracer and freeze fracture techniques it has been revealed that gap junctions are present between granulosa cells (Albertini & Anderson, 1974) and between granulosa cells and oocytes (Anderson & Albertini, 1976).

Data from several studies (Eppig, 1979; Moor *et al.*, 1980; Bachvarova *et al.*, 1980; Heller *et al.*, 1981; Brower & Schultz, 1982; Colonna & Mangia, 1983; Buccione *et al.*, 1987) support the hypothesis that granulosa cells supply nutrients to the oocytes via gap junctions and that these junctions are required for oocyte growth. It has been found that, *in vitro*, oocytes only grow normally when the usual association with their companion granulosa cells is maintained. Growth of naked oocytes has been achieved by culturing them on monolayers of granulosa cells (Bachvarova *et al.*, 1980; Herlands & Schultz, 1984) but at a much slower rate than that achieved with oocytes still enclosed in granulosa cells (Eppig, 1977). In contrast, when oocytes are cultured as oocyte-granulosa cell complexes, which have their normal cell-to-cell association then they will grow successfully (Eppig, 1979). In addition to this it has also been reported that the actual rate of oocyte growth *in vitro* is directly correlated to the

number of granulosa cells - the more granulosa cells, and therefore higher amount of metabolic coupling, the faster the rate of oocyte growth (Brower & Schultz, 1982). It has also been demonstrated that, via gap junctions, the oocyte plays an essential role in the development and function of the granulosa cells (Buccione *et al.*, 1990; Salustri *et al.*, 1990) as it secretes a soluble factor(s) which enables the granulosa cells to respond to gonadotrophic stimulation and undergo cumulus expansion.

4.1.4. The corpus luteum.

Observations on the fine structure of the corpus luteum have been reported on numerous eutherian species: rat (Lever, 1956); mouse (Yamada & Ishikawa, 1960); armadillo and mink (Enders, 1962); rabbit (Enders, 1962; Blanchette, 1966; Koering & Thor, 1978); hamster (Yates *et al.*, 1967); lemur (Sisson & Fahrenbach, 1967); cow (Priedkalns & Weber, 1968); pig (Cavazos *et al.*, 1968; 1969; Lemon & Loir, 1977); human (Adams & Hertig, 1969a, b; Crisp *et al.*, 1970); white-tailed deer (Sinha *et al.*, 1971); gerbil (Bagwell, 1977); guinea pig (Paavola, 1977); sheep (O'Shea *et al.*, 1979; 1980).

As is often the case, the information on marsupials is far less extensive and only a handful of species have been examined: *Isoodon macrourus, Perameles nasuta* (Gemmell, 1979; Hollis & Lyne, 1980); *Trichosurus vulpecula* (Shorey & Hughes, 1973); Philander opossum (Enders, 1973) and *Macropus rufogriseus* (Walker *et al.*, 1983)

In general, luteinized cells have been found to be typically characteristic of steroid-secreting cells with abundant SER, numerous mitochondria and lots of lipid droplets. Distinct electron-dense, membrane-bound secretory granules have been described within the the luteal cells of numerous species (human: Crisp *et al.*, 1970; pig: Belt *et al.*, 1971; Gemmell & Stacy, 1979; rat: Long, 1973; Anderson & Sherwood, 1984; gerbil: Bagwell, 1977 and sheep: Gemmell *et al.*, 1977). In many of these species the granules are believed to store relaxin while in cattle (Wathes *et al.*, 1983) and sheep (Rodgers *et al.*, 1983) they have been found to contain oxytocin. Recently Parry & Renfree (1994) have reported that the granules found in the luteal cells of *Macropus eugenii* contain relaxin.

4.2. MATERIALS AND METHODS.

4.2.1. Preparation of resin sections.

Twelve ovaries, from 9 animals, were sliced with a razor blade into pieces of less than $1mm^3$ and treated according to the following protocol.

1. 3% glutaraldehyde in 0.1M phosphate buffer	- 2 hours.
2. 0.1M phosphate buffer	- several rinses.
3. 1% Osmium tetroxide in 0.1M phosphate buffer	- 1½ hours.
4. 0.1M phosphate buffer	- several rinses.
5. 30%, 50%, 70%, 90% ethanol	- 15 minutes each.
6. Absolute ethanol	- 2 x 15 minutes.
7. Dried absolute ethanol	- 15 minutes.
8. Absolute ethanol / propylene oxide (75:25)	- 15 minutes.
9. Absolute ethanol / propylene oxide (50:50)	- 15 minutes.
10. Absolute ethanol / propylene oxide (25:75)	- 15 minutes.
11. Propylene oxide	- 3 x 15 minutes.
12. Propylene oxide / resin (50:50)	- 24 hours on rotator.
13. Araldite resin	- 48 hours at 60^oC.

For light microscopy:

Semithin 1 μm sections were cut, using freshly made glass knives, on a Reichert "OmU3" ultramicrotome and stretched with chloroform. They were then mounted on glass slides, dried on a hotplate and stained with either 1% Toluidine blue or Paragon. When sections of interest were found, a corresponding series of ultrathin sections were cut.

For transmission electron microscopy:

Ultrathin sections of 80 - 120 nm were cut and stretched, as before, and mounted on formvar-carbon coated copper grids. These were stained with 2% methanolic uranyl acetate and Reynold's lead citrate, for 5 minutes in each, and examined under a Zeiss "902" transmission electron microscope.

4.3. OBSERVATIONS.

4.3.1. Oocyte development.

In the quiescent oocyte, of the type 2 follicle, there were a few clusters of ovoid and elongate mitochondria, a small amount of SER and a juxtanuclear Golgi complex consisting of a few flattened cisternae and vesicles. There was no indication that these organelles were arranged to form a paranuclear complex. Gap junctions were occasionally observed between the oocyte and granulosa cell membranes. The granulosa cells had elongate nuclei, usually with highly irregular contours, a few rounded and elongate mitochondria which were smaller than those in the oocyte, some SER and Golgi bodies.

In the oocytes of 3a and 3b follicles there were many more mitochondria which were mostly elongate in shape and, like most of the organelles, found predominantly in the central region of the oocyte. Occasionally, unusual forms of mitochondria with a hook-like extension were observed (Fig.4.1). There was a small amount of SER which was usually associated with mitochondria, and several Golgi complexes. The Golgi consisted of flattened stacks of lamellae with a few, small vesicles and were scattered throughout the oocyte. A few lamellar structures usually consisting of two, although sometimes up to five, parallel cisternae with electron-dense material sandwiched between them were observed (Fig.4.2). In some cases the enclosed material appeared as a continuous, solid line while in others it was split into several, regular segments. The oocytes contained membrane-bound vesicles which were generally electron lucent and could be divided into two types - the smaller form existed as multivesicular bodies with membranous contents while the larger form contained a small amount of flocculent material and often appeared to be empty (Fig.4.3). In 3a follicles a few microvilli had developed in isolated areas on the oocyte surface and it was usually in these regions that small patches of electron-opaque and slightly granular zona pellucida material, were first observed. By the 3b stage, the discontinuous islands of zona pellucida had coalesced to form a complete layer, 0.2 - 1.2 μm thick, around the oocyte. The oolemma was covered with microvilli which were quite uniform in length and width and projected into the zona pellucida. Slender processes from the granulosa cells traversed the zona pellucida to meet the oocyte surface. Gap junctions were observed at the points of contact between the granulosa cells and oocyte as well as along the plasma membrane of adjacent granulosa cells. As seen in the oocyte, the granulosa cell organelles had increased in number and size and were distributed

randomly. Theca cells were first seen forming around the 3a follicle and by the 3b stage, a complete layer of theca cells, with irregular nuclei, a few mitochondria and some RER had developed.

In type 4 follicles, the oocyte mitochondria had increased in number but decreased in size from the previous stage. The SER was distributed throughout the oocyte and remained similar in relative abundance to that seen in primary follicles. A close association still existed between the mitochondria and SER and the Golgi complexes migrated to a more peripheral location and showed increased activity - the stacks of lamellae became larger, showed significant swelling and were associated with aggregates of microvesicles (Fig.4.4). The number of vesicles increased and the larger, flocculent form became more abundant than the smaller, multivesicular bodies. Pinocytotic vesicles, some of which were coated, were present in the peripheral cytoplasm of the oocyte and were concentrated around the areas of contact with the granulosa cell processes (Fig.4.5). SER and Golgi were often clustered around the vesicles and it appeared that they took part in the formation of the multivesicular bodies which then incorporated the pinocytotic vesicles. As they grew in size, the enclosed membranes disintegrated to produce the larger, flocculent form of vesicle. In this and subsequent stages of development, the nucleus was often irregular in areas with an undulating nuclear membrane (Fig.4.6). Mitochondria, RER and large Golgi complexes were the predominant granulosa cell organelles although small patches of SER and occasional lipid droplets were observed.

The oocytes of 5a and 5b follicles were dominated by vesicles, mostly the large, flocculent form, which continued to increase in size and number. There was lots of pinocytotic activity at the oocyte surface and active Golgi complexes were commonly found near the periphery associated with numerous vesicles. As the vesicles grew they coalesced with their neighbours (Fig.4.8) to form a large, irregular mass until the centre of the oocyte was occupied by these vesicular masses, which showed no obvious polarity or size gradation, and most of the organelles were displaced to the periphery. The perivitelline space, which appeared as a palely-stained band containing some flocculent material, had formed between the oocyte and the zona pellucida and many granulosa cell processes could be seen terminating in it before they reached the oocyte surface (Fig.4.9). The granulosa cells contained ovoid, vacuolated mitochondria, large amounts of RER arranged in large concentric rings, lipid droplets, SER and Golgi complexes (Fig.4.10). Unlike in earlier stages, the theca cells possessed some SER and lipid droplets.

In the type 6 and 7 follicle, the appearance of the oocyte was similar to the previous stage although slightly larger in size. It was packed full of vesicles and most of the organelles were confined to the peripheral zone of the oocyte which was relatively vesicle-free. Intercellular spaces developed between the granulosa cells of type 6 follicles as the antral fluid began to accumulate and by the type 7 stage a complete antrum had formed. The *cumulus oophorus* had developed around the oocyte, separating it from the antral fluid, and the granulosa cells appeared similar to those in the previous stage. The theca cells now contained abundant SER, lipid droplets and Golgi complexes.

In type 8 follicles the oocyte mitochondria had undergone a striking transformation whereby they were often very elongated and appeared to have microfilaments running longitudinally along their outer membrane (Fig.4.11). When sectioned transversely it was possible to see the individual microfilaments, of which there were usually a dozen or more, arranged regularly around the surface of the mitochondrion (Fig.4.12). The zona pellucida was now 2.5 - 4 μm thick and granulosa cell processes could no longer be seen passing through it. The cells of the *cumulus oophorus* contained numerous lipid droplets and as the follicle developed they became loosely arranged due to the formation of large spaces between them (Fig.4.13). By the preovulatory stage there was extensive degeneration of the cumulus cells and withdrawal of the ganulosa cell processes.

4.3.2. The corpus luteum.

This structure consisted mostly of luteinized granulosa cells (Fig.4.14) interspersed with theca cells and blood vessels (Figs.4.15. & 4.16). The luteinized granulosa cells were large and polyhedral with a large, pale, central nucleus. SER was very common, and in some cells occupied most of the cytoplasm. Generally there was less RER than SER but its abundance varied between cells - in those where it was found it tended to form stacks of cisternae which were localized in small patches (Fig.4.17). The mitochondria were large and varied in shape from spherical to cup-shape to elongate. Lipid droplets, showing varying degrees of preservation (Fig.4.18), were numerous in many of the cells although the amount and distribution varied considerably between them. Numerous, electron-dense structures, measuring 0.3 - 0.5 μm, were also present in varying abundance and with no obvious distribution.

Fig. 4.1. Oocyte from type 3b follicle with hooked mitochondrion (hm) and cup-shaped mitochonrion (m) (x24,000).

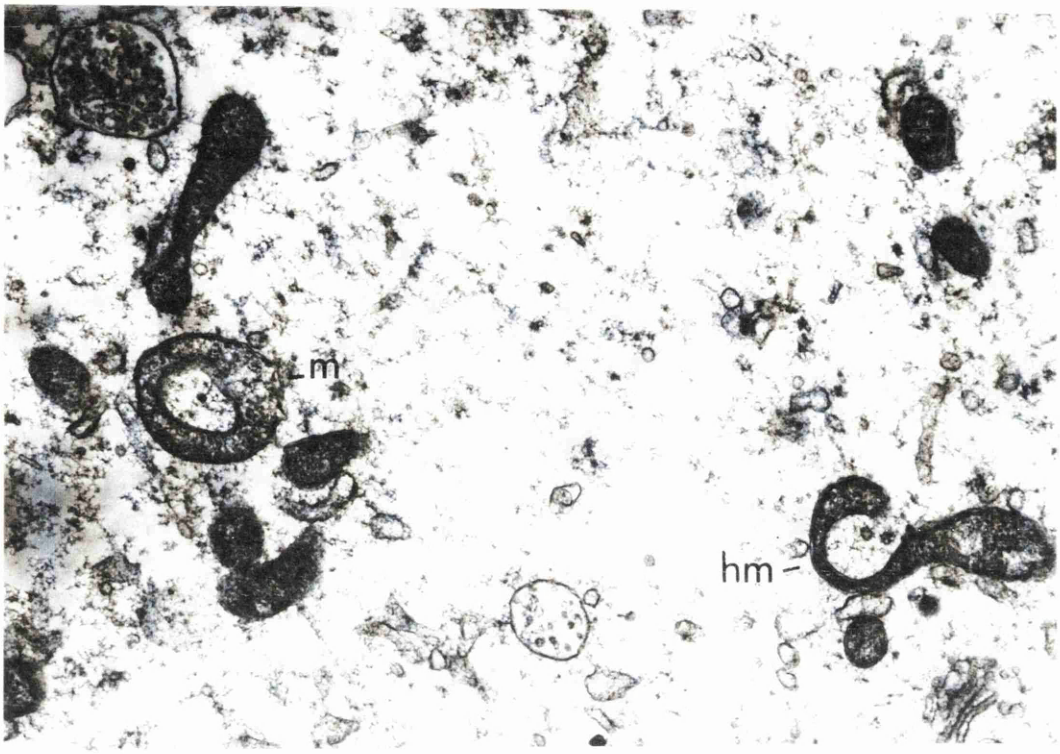


Fig. 4.2. Section of oocyte showing lamellar complex (lc), golgi (g) and multivesicular bodies (arrowed) (x24,000).

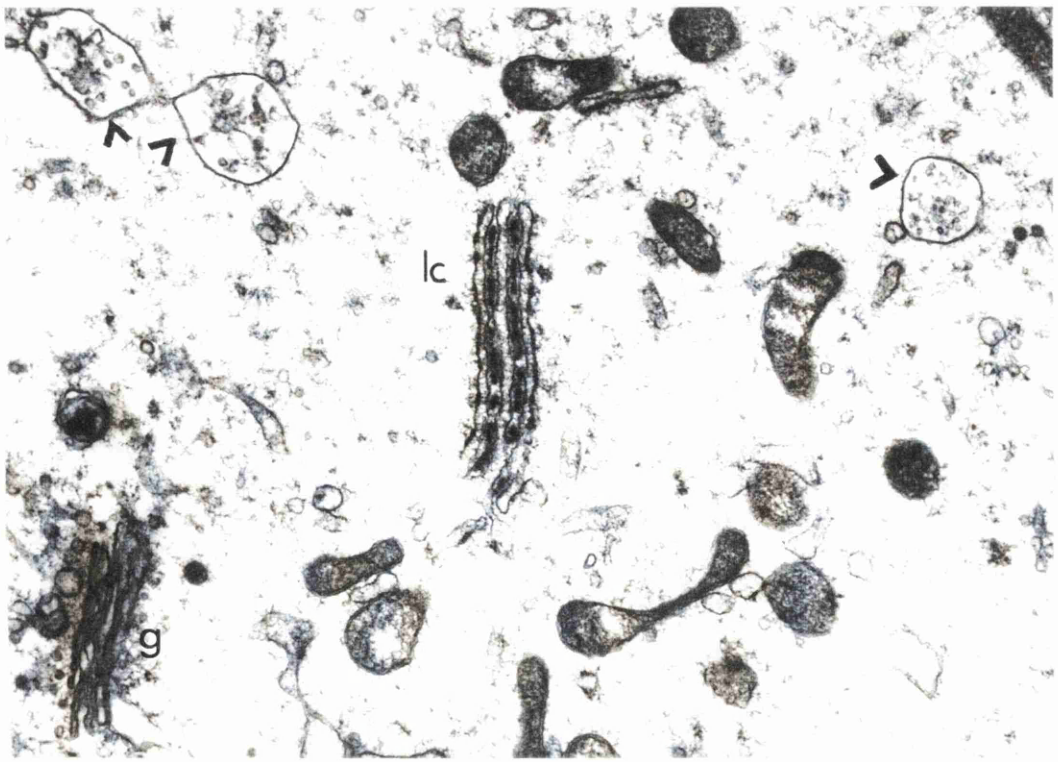


Fig. 4.3. Oocyte containing multivesicular bodies (mvb), large flocculent vesicles (v) and active golgi bodies (g) (x14,000).

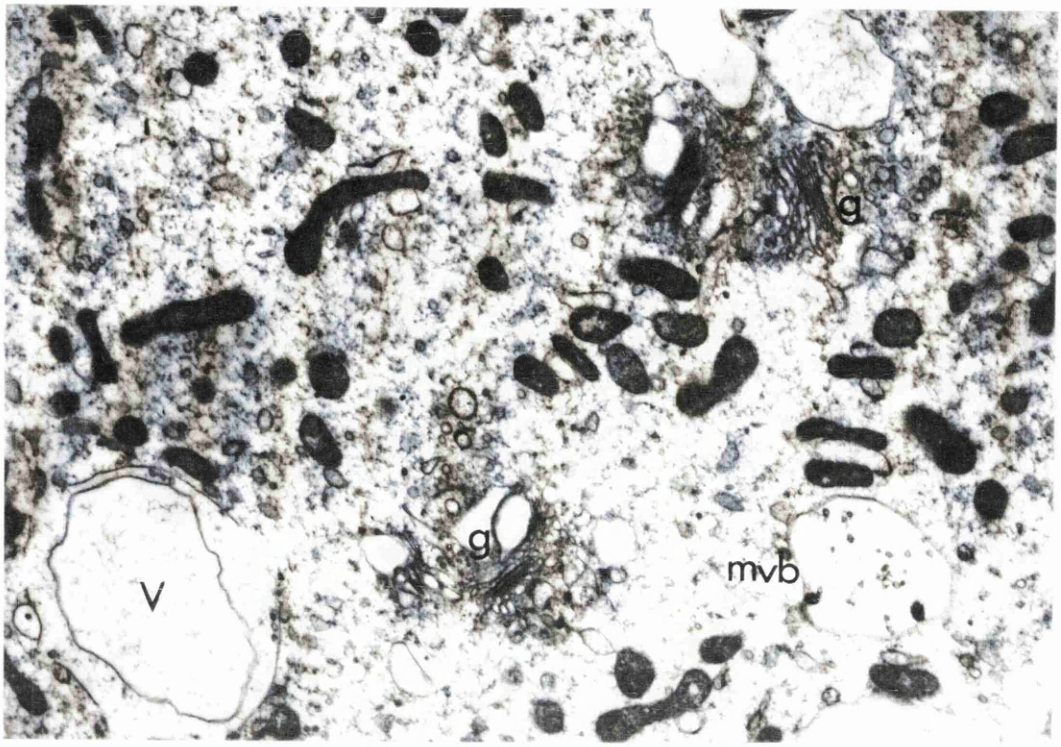


Fig. 4.4. Outer region of oocyte with microvilli (mv) projecting into the zona pellucida (zp), and mitochondria (m) and golgi (g) associated with vesicles (x11,000).

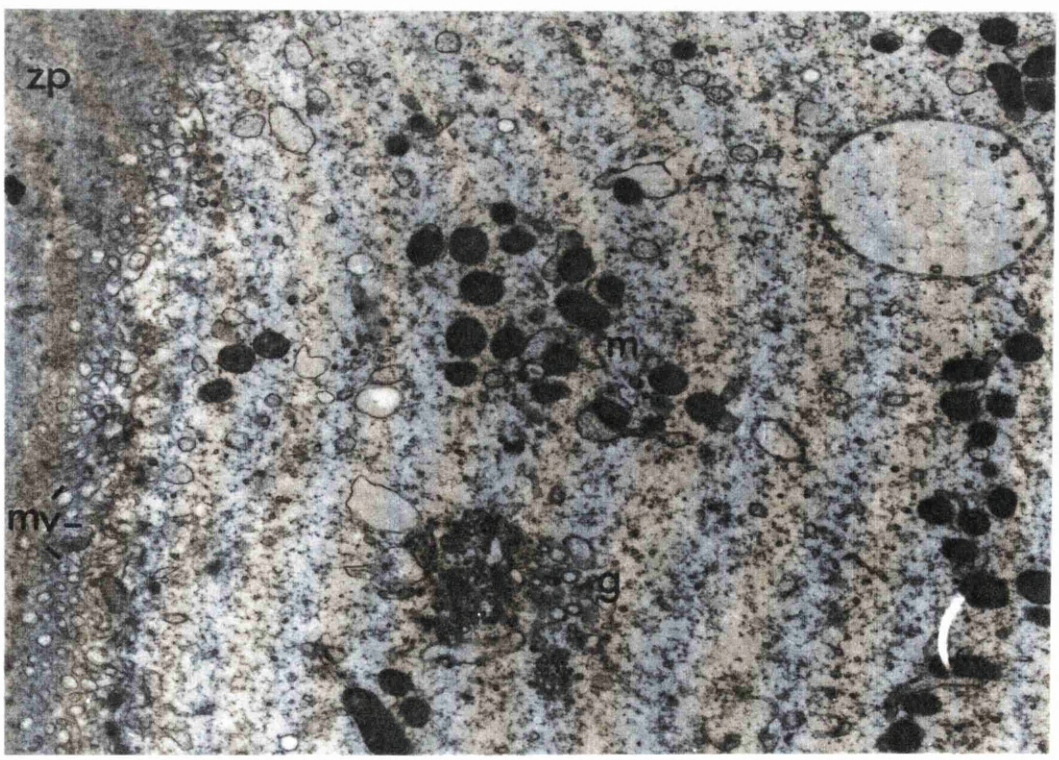


Fig. 4.5. Section of oocyte periphery showing zona pellucida (zp), microvilli and coated vesicles (arrowed) (x40,000).

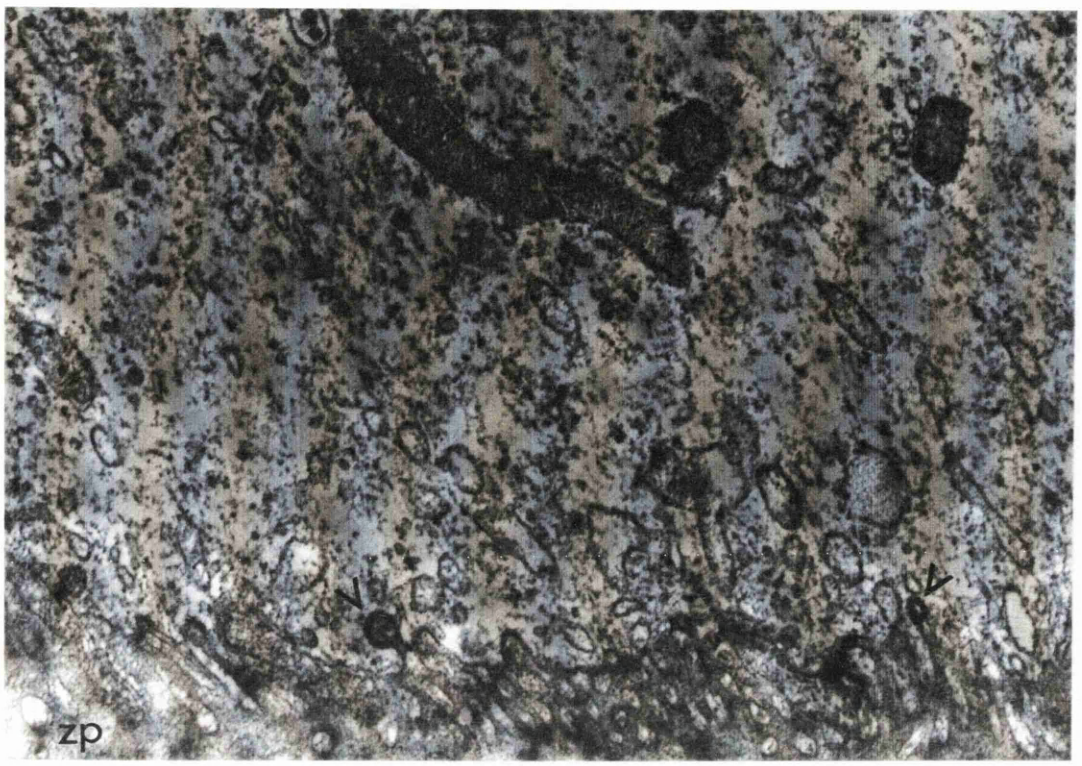


Fig. 4.6. Oocyte with large vesicles (v), mitochondria (m) and golgi body (g) adjacent to undulating nuclear membrane (arrowed) (x14,000).

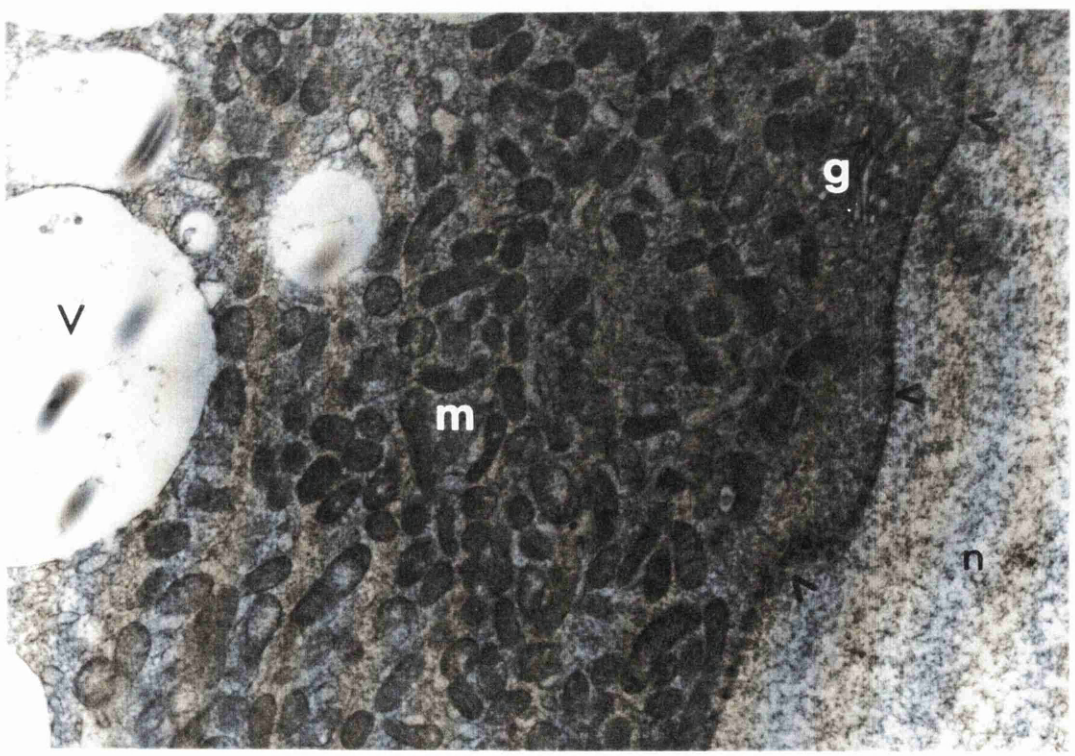


Fig. 4.7. Oocyte with pinocytotic vesicles (arrowed) entering the oocyte via the microvilli (mv) and migrating towards the multivesicular bodies (mvb) (x24,000).

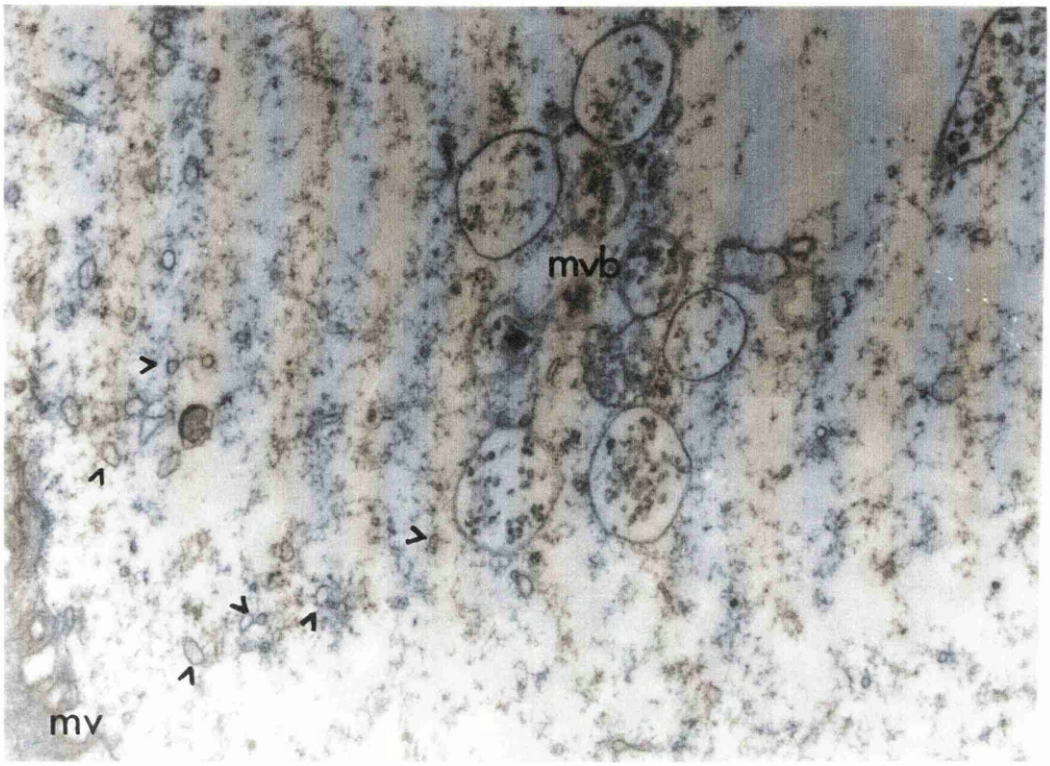


Fig. 4.8. Large vesicles coalescing (x24,000).

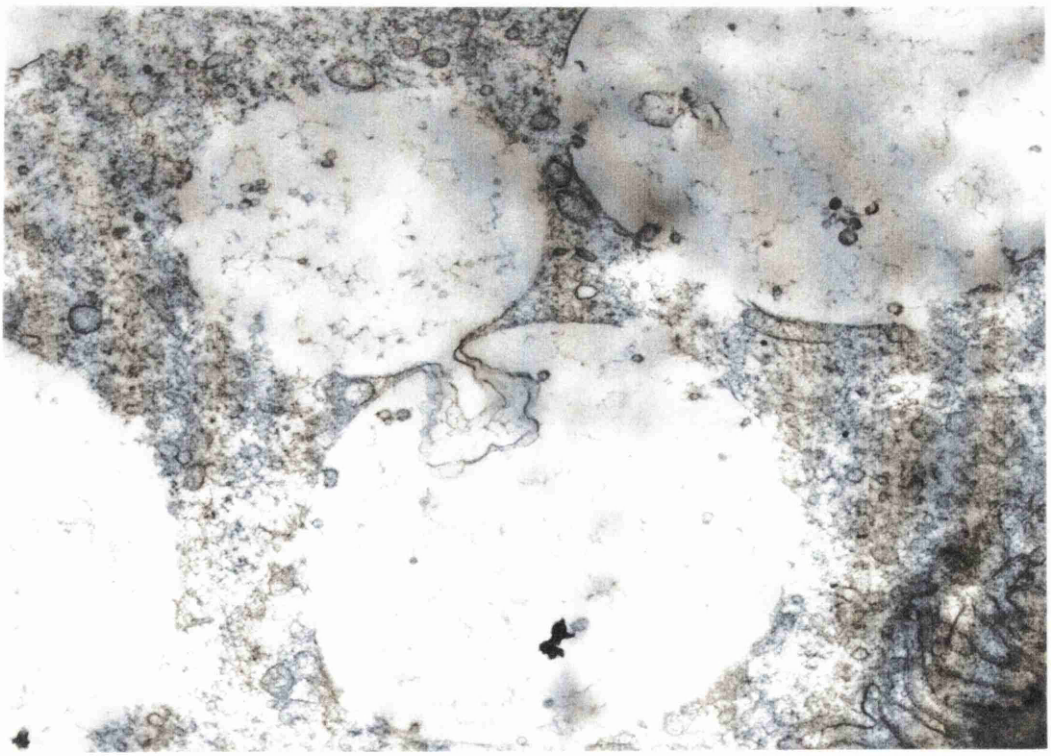


Fig. 4.9. Section of type 7 follicle showing oocyte periphery with microvilli projecting into the perivitelline space (pvs), zona pellucida (zp) and cells of cumulus oophorus (c) some of which contain lipid droplets (L) (x6,000).

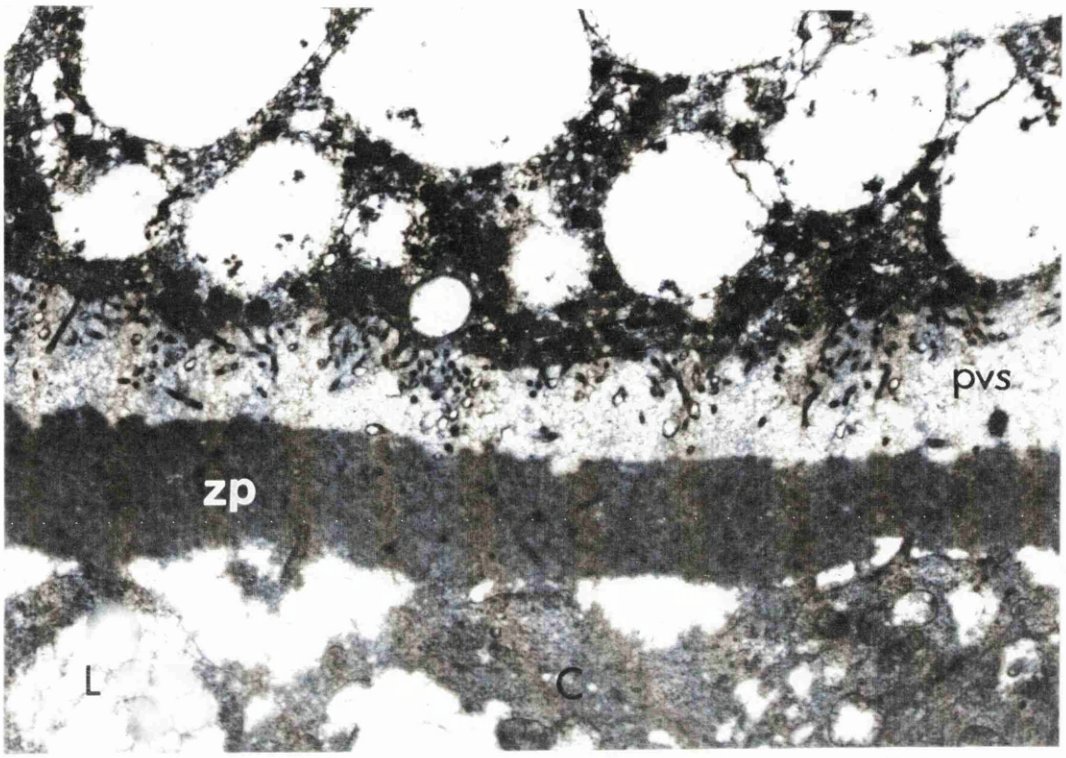


Fig. 4.10. Granulosa cells with smooth endoplasmic reticulum (ser), rough endoplasmic reticulum (rer) and lipid droplets (L) (x14,000).

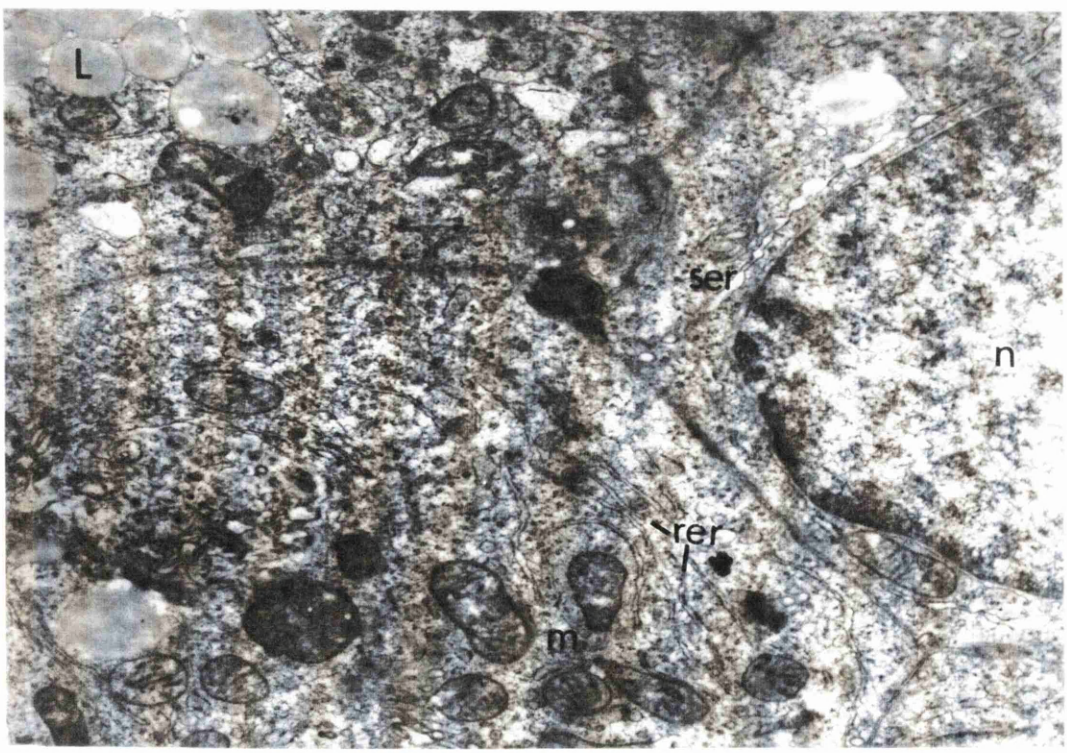


Fig. 4.11. Oocyte from type 8 follicle containing large vesicles (v) and elongated mitochondria (m) apparently bearing microfilaments. The perivitelline space (pvs) and zona pellucida (zp) are clearly visible (x14,000).

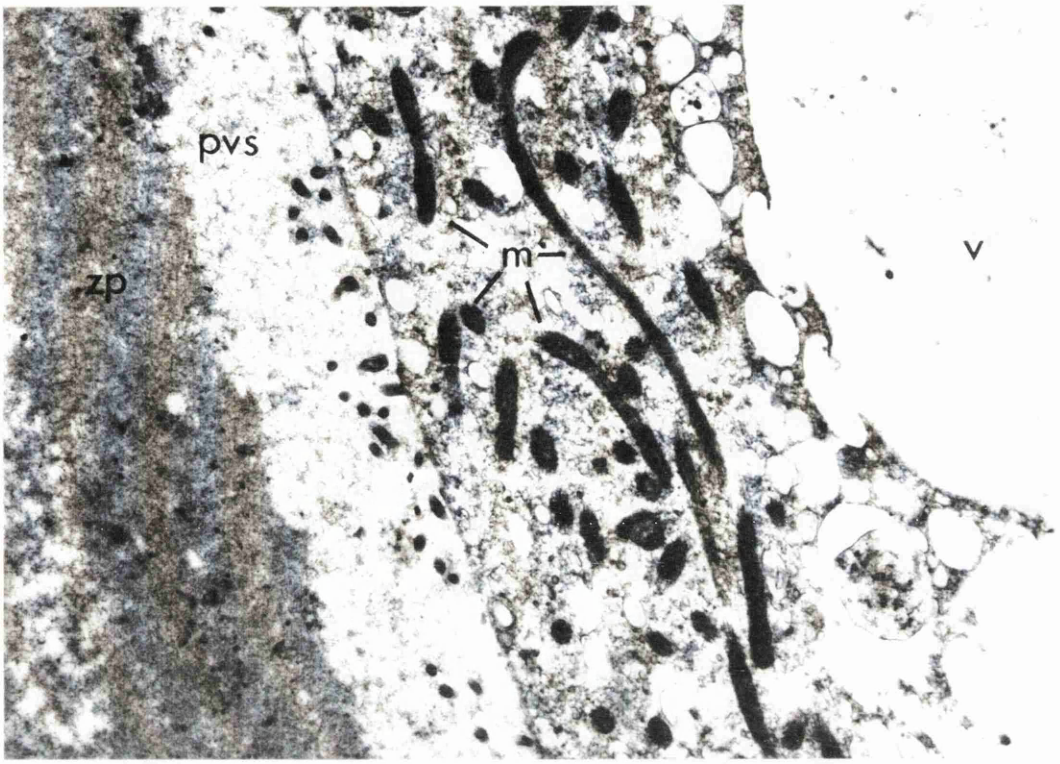


Fig. 4.12. Transversely sectioned mitochondria showing individual microfilaments arranged regularly around their outer surface (x60,000).

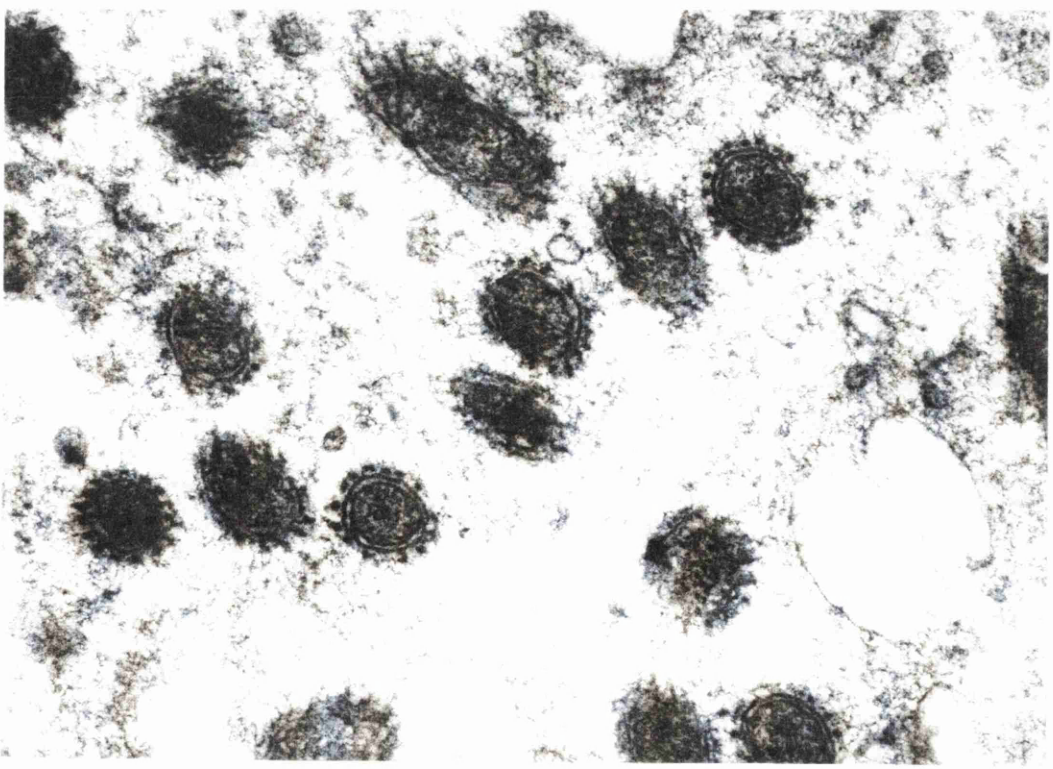


Fig. 4.13. Section of type 8 follicle showing oocyte packed full of vesicles, loosely arranged cumulus oophorus and antral fluid (a) (x800).

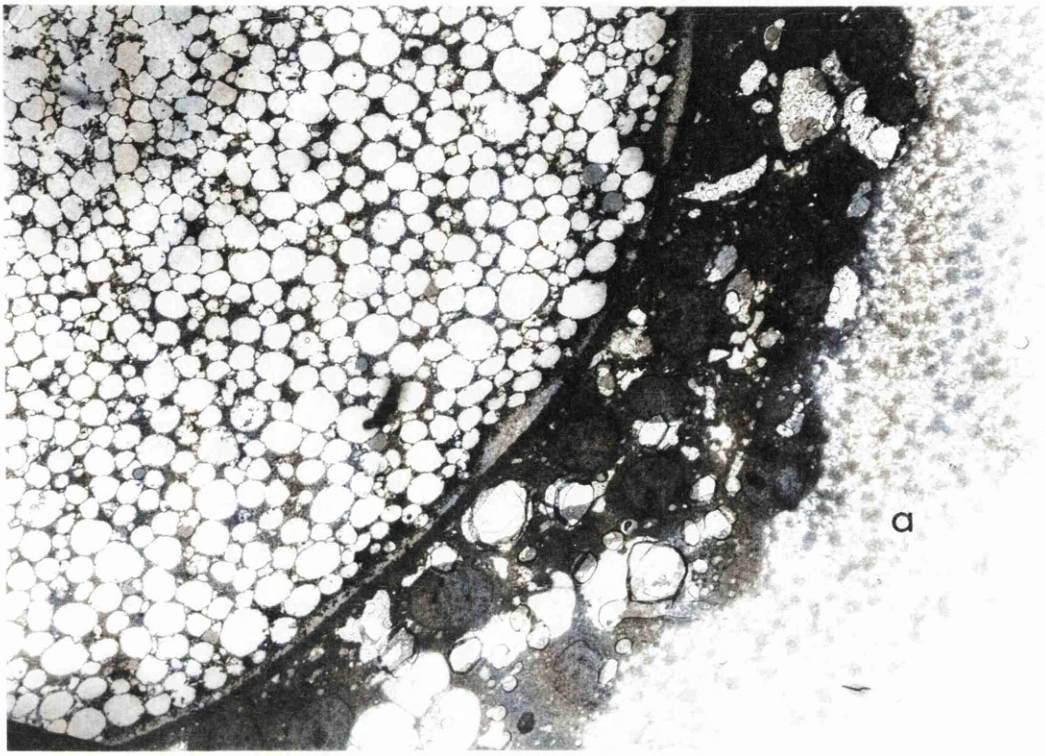


Fig. 4.14. Corpora lutea (CL) adjacent to type 4 follicle with two layers of granulosa cells (g) (x1400).

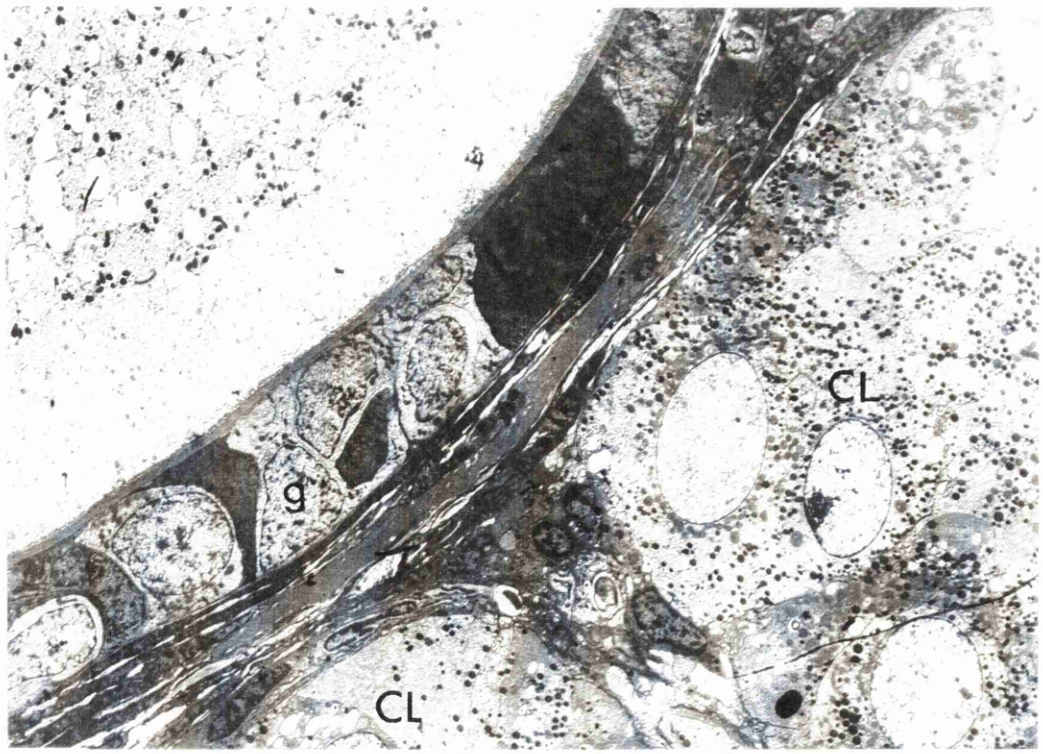


Fig. 4.15. Luteinized granulosa cells (gl), theca interna (ti) cell and blood vessel in a corpus luteum (x6000).

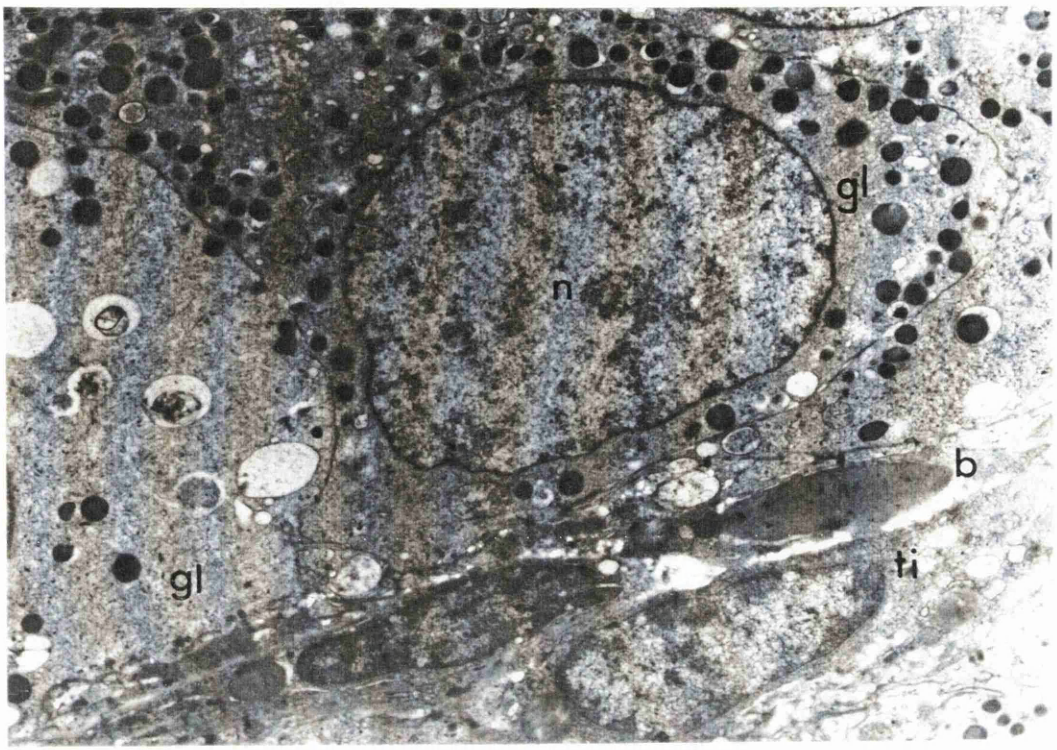


Fig. 4.16. Section of corpus luteum showing large luteinized granulosa cell with rough endoplasmic reticulum (rer) and mitochondria (m) (x6000).

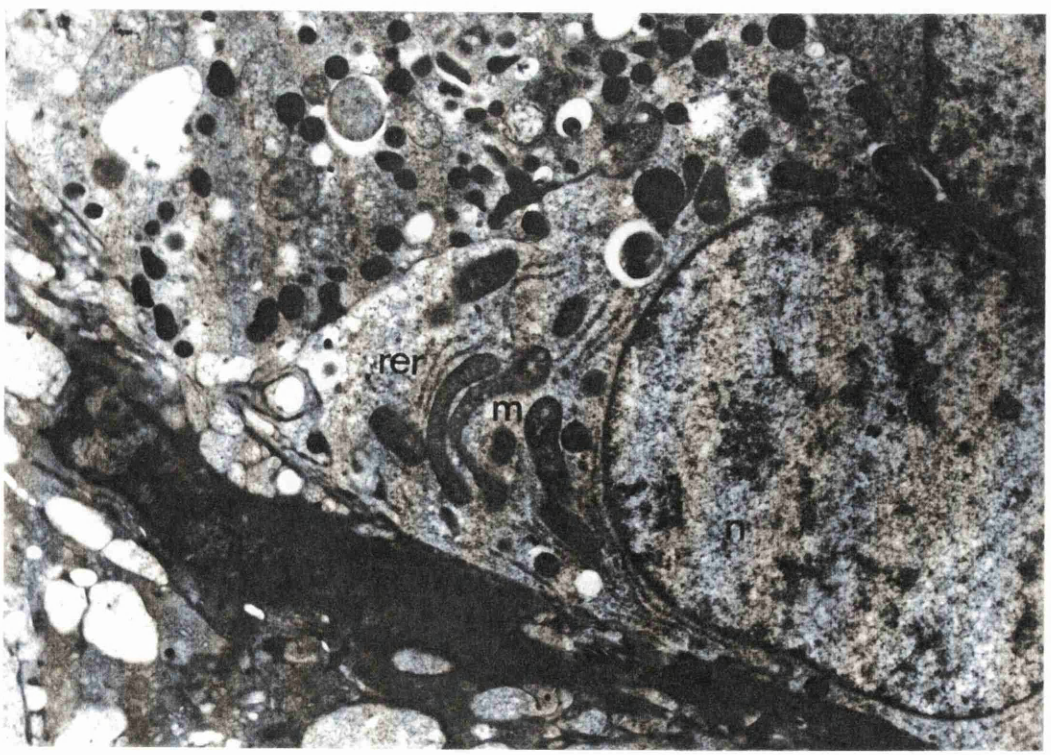


Fig. 4.17. Luteinized granulosa cell with abundant smooth endoplasmic reticulum (ser), stacks of rough endoplasmic reticulum (rer)and lipid vesicles (L) (x14,000).

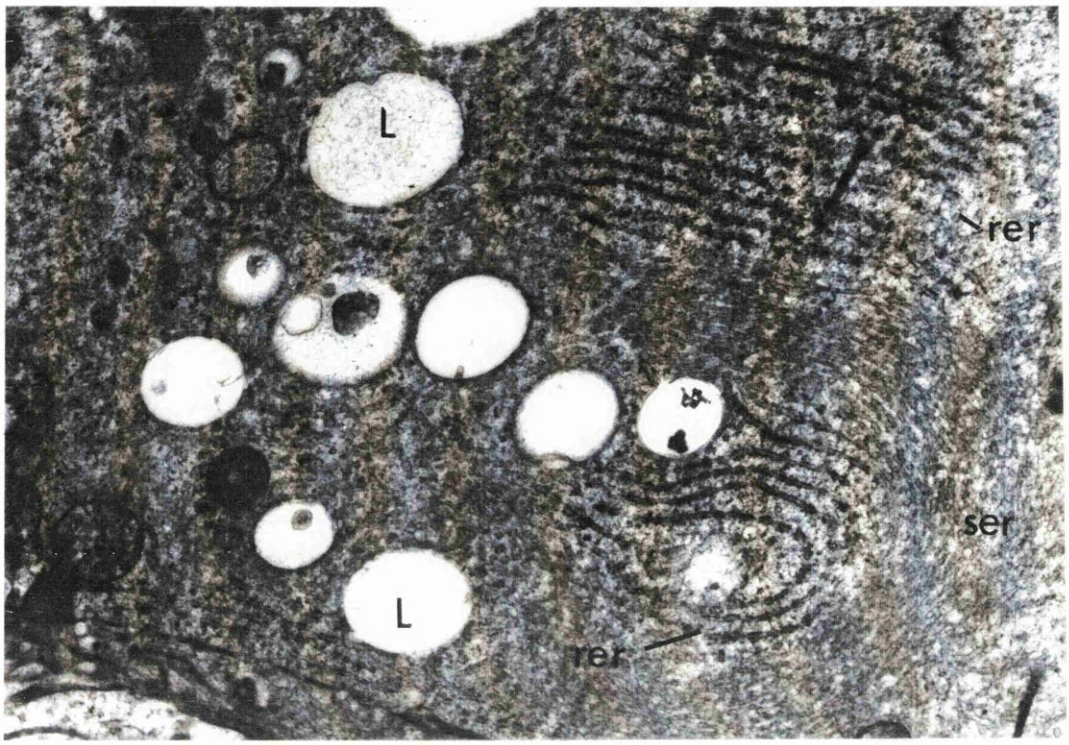
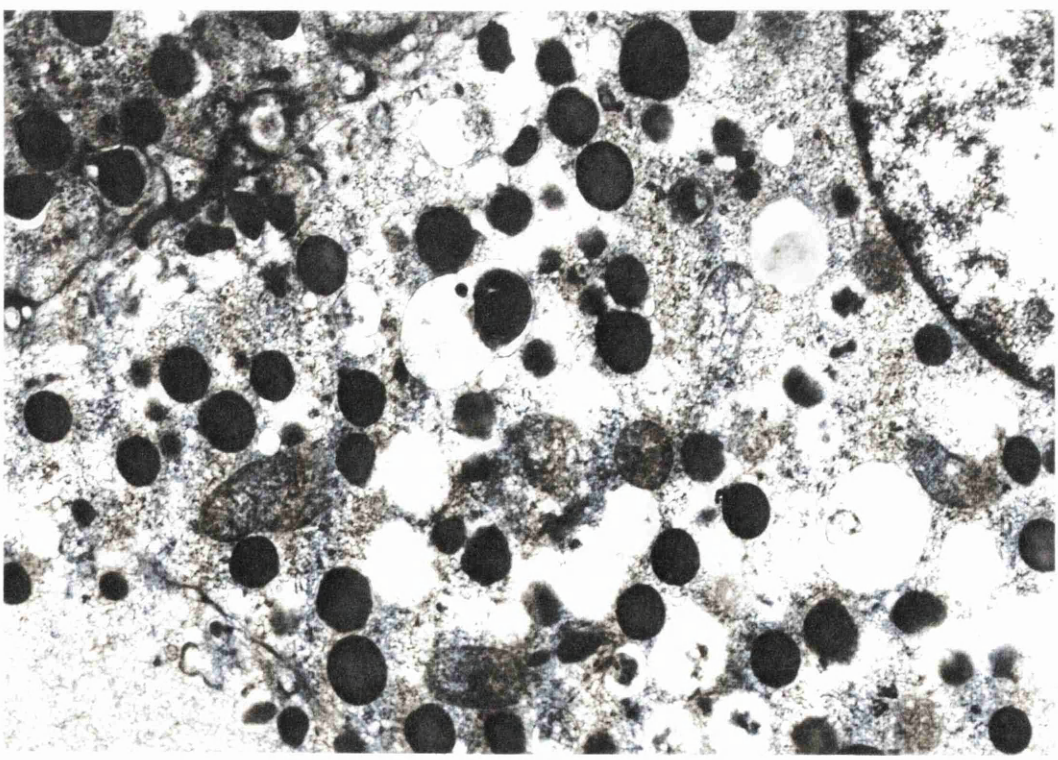


Fig. 4.18. Luteinized granulosa cell containing lipid showing varying degrees of preservation (x14,000).



4.4. DISCUSSION.

4.4.1. Oocyte development.

Many aspects of the maturational changes that occured within the *M. domestica* oocyte resembled those reported for other mammalian species (Anderson, 1974; Szöllösi, 1970; Zamboni, 1970). As described by Adams & Hertig (1964) the immature primordial oocyte was "morphologically a very simple cell which probably represents a state of maintenance metabolism". Growth and development of the oocyte was associated with an increase in the number of organelles and a change in their distribution which reflected the high degree of metabolic activity associated with such extensive growth.

In this species there was no indication of the paranuclear complex, vesicle-microtubule complexes or aggregates of tubular cisternae that Ullmann (1978) described in the oocyte of *Isoodon macrourus* primordial follicles. Oocytes appeared to lack annulate lamellae and nuage was never observed. The former finding is in contrast to Falconnier & Kress (1992) who reported the occurrence of occasional annulate lamellae but did not actually provide any photographic evidence of their existence. An undulating nuclear outline, similar to that in *M. domestica*, has previously been reported in the horse (Vogelsang *et al.*, 1987) and the bandicoots *I. macrourus* and *Perameles nasuta* (Lyne & Hollis, 1983). Although an irregular or wrinkled nuclear envelope can signify atresia, the follicles in this study, and those in the other two studies appeared overtly healthy with no indications that they were atretic. The lamellar complexes, with electron-dense centres, were unlike the bilaminar lamellae seen in the hamster (Green, 1985) which do not have a dark, inner region. In this study, they were only found occasionally in primary follicles whereas Falconnier & Kress (1992) described them as regular features of both primary and secondary follicles These structures have previously been described as confronting cisternae by Ghadially (1988) who proposed that they were formed from RER. However, due to the lack of RER in the *M. domestica* oocyte such an origin would have to be discounted. The presence of ribosomes and RER in oocytes has been found to vary dramatically between species. In her study of *Potorous tridactylus*, Dairi (1988) found that the oocytes contained ribosomes whereas, in *M. domestica* and the horse (Vogelsang *et al.*, 1987) endoplasmic reticulum has been found to be exclusively of the smooth variety. Similar to the report of Zamboni (1970), the SER disappeared as the

oocyte enlarged, giving rise to numerous vesicles found in association with the mitochondria (Ruby *et al.*, 1968).

It is acknowledged that a wide range of mitochondrial forms exist in different animal cells - cup-shaped mitochondria have been reported in the Leydig cells of neonatal rat testis (Christensen & Chapman, 1959), while mitochondria with cavities have been described in the rat liver (Stephens & Bils, 1965). The unusual mitochondria described as "hooded mitochondria" by Senger & Saacke (1970) which were previously thought to be unique to ungulate oocytes were occasionally observed in *M. domestica* oocytes. They were very similar to the bovine form, but less abundant and occurred only in the oocytes of preantral follicles rather than antral and mature stages. The functional significance, if any, of these forms is not clear especially as they were the exception rather than the rule in this species. It has been suggested that in the bovine oocyte, as well as increasing the surface area of the mitochondria, the inner surface of the hood may provide a specific microenvironment facilitating the exchange of metabolic intermediates between mitochondria and SER (Fleming & Saacke, 1972). The origins and functional significance of the transformed mitochondria in the preovulatory oocyte, which apparently had microfilaments attached to them, is at present unresolved. Although they have appeared in several papers (Mate *et al.*, 1992; Moore & Taggart, 1993; Taggart *et al.*, 1993) their unusual form was not referred to. More surprisingly, in their ultrastructural study of oocyte development, including this late stage, Falconnier & Kress (1992) made no mention of these striking organelles.

A relationship between mitochondria and other organelles has been described in many animals. In *M. domestica,* as in bovine (Fleming & Saacke, 1972; Kruip *et al.*, 1983; Hyttel *et al.*, 1986), and rabbit oocytes (Zamboni & Mastroianni, 1966), mitochondria were commonly found in close association with SER. In other species such as the mouse (Yamada *et al.*, 1957) mitochondria were observed to be clustered around the Golgi and in the rabbit they have also been described around lipid droplets (Blanchette, 1961). As seen in previous studies (Adams & Hertig, 1964; Zamboni & Mastroianni, 1966; Szöllösi, 1967; Baca & Zamboni, 1967;), the Golgi bodies underwent striking ultrastructural changes during oocyte development indicative of increasing activity. Initially the Golgi consisted solely of stacks of flattened lamellae with a few associated small vesicles. As they became more active, they migrated from a juxtanuclear position to a predominantly peripheral location and divided into multiple complexes. The lamellae increased in number, became swollen and were

associated with a large conglomerate of vesicles and granules. The relationship of the SER to the Golgi was similar to that observed in the horse (Vogelsang *et al.*, 1987) and human oocyte (Sathananthan *et al.*, 1985). The presence of well developed Golgi and SER was presumably correlated to lipid synthesis.

The development of the zona pellucida and theca cells at the 3a stage was much earlier than that reported by Falconnier & Kress (1992) in this species. They did not observe any zona development until the late primary follicle (equivalent to the 3b stage) and theca formation was first described around multilayered follicles. The timing of theca development in this study is similar to that described in the rat by Hirshfield (1991) who suggested that theca cells may be present at the very outset of follicle growth. It would appear that the timing of theca development is species-specific for in the hamster the theca is not formed until there are 7 or 8 layers of granulosa cells (Roy & Greenwald, 1985). The width of the fully formed zona pellucida was found to be 2.5 - 4.0 μm and this is within the range observed by Falconnier & Kress (1992). In other marsupials the zona pellucida varies in thickness from 1.5 μm in *Dasyurus viverrinus* (Hill, 1910) to 6 μm in *Trichosurus vulpecula* (Hughes & Hall, 1984) and *Macropus eugenii* (Renfree & Tyndale-Biscoe, 1978). This is considerably thinner than that seen in eutherians where the zona pellucida ranges from 10 - 30 μm in width (Austin, 1961).

The vesicles which accumulated in the *M. domestica* oocyte were similar to, although much more abundant, than those seen in the cow (Fleming & Saacke, 1972; Kruip *et al.*, 1983; Hyttel *et al.*, 1986; de Loos *et al.*, 1989); sheep (Cran *et al.*, 1980); pig (Cran, 1985); human (Sundstrom *et al.*, 1985); horse (Vogelsang *et al.*, 1987); and blue fox (Hyttel *et al.*, 1990). Tesoriero (1981a) studying canine oocytes observed osmiophilic lipid vesicles in the earlier stages of oocyte development but later on they became more transparent until they appeared similar to those of *M. domestica*. However, instead of the vesicles coalescing, as seen in *M. domestica*, those in the canine remained separate and showed no evidence of fusion or coalescence (Tesoriero, 1981b).

Such conspicuous vacuolation of the oocyte also resembled that described in other marsupials (Lyne & Hollis, 1983; Breed & Leigh, 1988). There was no polarity exhibited by these vesicles which is in agreement with previous observations on preovulatory follicles (Baggott & Moore, 1990). This changes later on in development as zygotes have been reported to have markedly polarized cytoplasm (Baggott & Moore, 1990; Selwood & VandeBerg, 1992). These vesicles and the vesicular mass they form are identical to the "yolk" vesicles and "yolk" mass previously described in *Dasyurus viverrinus*

(Hill, 1910); *Didelphis virginiana* (Hartman, 1916; 1919) and *Sminthopsis crassicaudata* (Breed & Leigh, 1988; 1990).

With respect to vesicle formation, it appeared that the SER and Golgi were responsible for the formation of the smaller multivesicular bodies which gradually became larger as they were added to by the incoming pinocytotic vesicles. As they increased in size, the enclosed vesicle membranes disintegrated to form the large, flocculent vesicles which coalesced with each other and eventually filled the oocyte. The passage of pinocytotic vesicles into the multivesicular bodies has recently been confirmed by Falconnier & Kress (1992) who were able to follow their movement by means of horseradish peroxidase tracer experiments.

The distribution of organelles in the oocyte periphery resembles that seen in the sheep (Cran *et al.*, 1980) and other marsupials (*Isoodon macroura* & *Perameles nasuta*: Lyne & Hollis, 1983; *Sminthopsis crassicaudata*: Breed & Leigh, 1988). Congregation of mitochondria at the oocyte periphery has also been observed in the rat (Sotelo & Porter, 1959), hamster (Hadek, 1969) and cow (Fleming & Saacke, 1972) but is not found in all species (Zamboni, 1972). Presumably, such positioning would represent an advantageous position for processing molecules entering the oocyte.

In this study, cortical granules were not observed at any stage of oocyte development which is in contrast to Falconnier & Kress (1992) who claim to have observed cortical granules in the outer regions of oocytes from Graafian follicles. However, as their report was not substantiated photographically it is not possible to comment on their identification of these organelles. An absence of cortical granules in *M. domestica* was also noted by Baggott & Moore (1990) examining preovulatory oocytes as well as Taggart *et al.* (1993) studying unfertilized and fertilized oocytes. Following fertilization Taggart *et al.*, (1993) observed clumps of membrane-bound vesicles containing fine membreanous material at regular intervals around the surface of the egg. These vesicles appeared to bud off from the oolemma, disintegrate in the perivitelline space and release their contents but it was unclear whether this process was a true cortical reaction. This apparent lack of cortical granules in *M. domestica* differs from observations made in eutherian species (Yanagimachi, 1988) and other marsupials (Selwood, 1982; Breed & Leigh, 1990; 1992). It has been suggested (Rodger & Bedford, 1982) that the cortical granule controlled block to polyspermy may be weak in marsupials. Instead, the mucoid coat, secreted by the oviduct, may play an important role (Rodger & Bedford, 1982; Baggott *et al.*, 1987; Breed & Leigh,

1990) by trapping supernumery sperm. Whatever the role of the mucoid coat in the block to polyspermy, it invariably traps a large number of sperm, the appearance of which is a good indicator of fertilization. Alternatively, marsupials may exhibit a unique system for preventing polyspermy. In *Didelphis virginiana* (Talbot & DiCarlantonio, 1984) and *Sminthopsis crassicaudata* (Breed & Leigh, 1988) the perivitelline space has been found to contain an extracellular material composed of protein granules and hyaluronic acid filaments (Talbot & DiCarlantonio, 1984). In the present study the perivitelline space was observed to contain some diffuse, flocculent material and Taggart *et al.*, (1993) reported that a fine electron-dense material presumed to have originated from the perivitelline space, formed a dome-shaped plug across the large hole in the zona pellucida produced during sperm penetration.

Formation of the perivitelline space in the 5b follicle was earlier than that in several eutherian species (cow: Fleming & Saacke, 1972; Kruip *et al.*, 1983; Hyttel *et al.*, 1986; blue fox: Hyttel *et al.*, 1990) where it is not observed until just before ovulation. There is also a difference of opinion concerning its formation in *M. domestica* - the timing in this study is similar to that of Falconnier & Kress (1992) but Selwood & VandeBerg (1992) claimed that, *in vitro,* zygotes could be distinguished from unfertilized eggs due to their possession of a perivitelline space. However, as the latter group examined their material solely with the aid of a dissection microscope their identification may not be as accurate as that achieved using electron microscopy.

The breakdown of the *cumulus oophorus* began in the late antral stage and in contrast to the eutherian situation where the oocyte is invested by a full cumulus the *M. domestica* oocyte is naked at ovulation (Baggott *et al.*, 1987; Baggott & Moore, 1990). This has also been found to be the case in other marsupials such as *Sminthopsis crassicaudata* (Breed & Leigh, 1988). Surprisingly, the conspicuous disintegration of the *cumulus oophorus* was not mentioned by Falconnier & Kress (1992) who described the formation of a corona radiata around the oocytes of antral follicles. This was probably a misnomer on their part for it is generally recognized that marsupials do not form the characteristic corona radiata observed around eutherian oocytes.

4.4.2. Corpus luteum.

Observations were similar to those of various eutherian and other marsupial species. The variation in mitochondrial shape, from spherical to elongate resembled that described in several species (Enders, 1973) and the localized stacks of RER cisternae were similar to those observed in humans (Adams & Hertig, 1969 a,b; Crisp *et al.*, 1970).

Lipid droplets, showing varying degrees of preservation, were numerous in many of the cells. The differences in vesicle osmiophilia may have represented a variation in either the composition of the lipid, the concentration of lipid components, or a combination of these two possibilities. In some cases the differences in morphology could be accounted for due to the extraction of material during processing as some of the osmiophilic droplets had a less electron dense centre which one would expect if there was a problem in preserving the inner regions. At first it was thought that the osmiophilic structures might be secretory granules but, measuring 0.3 - 0.5 μm, they were considerably larger than the 0.2 μm seen in sheep (Gemmell *et al.*, 1974; O'Shea *et al.*, 1979) and 0.15 μm granules seen in *Trichosurus vulpecula* (Shorey & Hughes, 1973) and *Isoodon macrourus* & *Perameles nasuta*., Hollis & Lyne, 1980). There was no evidence that these structures were being secreted, and they were usually observed in the central rather than the peripheral regions of cells, so it seems more probable that they represented some form of unsaturated lipid. In the rabbit corpus luteum several varieties of lipid, distinguished by size and osmiophilia, have been reported by Blanchette (1966). She found that the smaller droplets measuring 0.5 μm were intensely osmiophilic while the larger droplets varying in size from 1 - 2 μm were pale: the size and description of both of these are similar to those observed in *M. domestica*. One way to confirm whether these osmiophilic structures were in fact lipid would have been to examine tissue which had not been osmium-fixed and so would not have had any lipid preserved. Alternatively, one could have tried to immunocytochemically detect the presence of relaxin, or perhaps oxytocin, within these granules. Unfortunately, neither of these strategies were possible as the tissue was obtained from the only animal with corpora lutea in her ovaries, both of which had been fixed in osmium tetroxide.

The exact age of the corpora lutea from this animal was unknown but from the information available it is probable that they were beginning to cease functioning. The female was separated from the male 9 days before her ovaries were removed, so must have been a minimum of 9 days pregnant. In

Didelphis virginiana (Fleming & Harder, 1983) the corpora lutea start to decline on about day 8 of its 12 day gestation period and it was earlier observed (see chapter 2) that in *M. domestica* the corpora lutea have practically disappeared 6 days after parturition. Therefore, it seems likely that the corpora lutea examined were nearing the end of their active life but had not started to exhibit the characteristic changes seen in regressing corpora lutea (Sinha *et al.*, 1971; Shorey & Hughes, 1973; Hollis & Lyne, 1980) such as pycnotic nuclei, discontinuous cell membranes, disorganized cytoplasm, degenerating mitochondria with large electron-dense inclusions or hyaline bodies. There was also no evidence of the lancet-shaped spaces commonly found in the regressing luteal cells of the rabbit (Koering & Thor, 1978) and *Isoodon macrourus* & *Perameles nasuta* (Hollis & Lyne, 1980). Such spaces have also been observed in degenerating rabbit interstitial tissue (Davies & Broadus, 1968), so in some species these structures may be characteristic of regressing steroidogenic cells.

Chapter 5

Follicle growth *in vitro*

5.1. INTRODUCTION

5.1.1. Ovarian follicle culture.

Detailed investigations into the mechanisms of ovarian follicular development have been restricted due to the lack of a culture system that could support follicular growth through all stages of development from preantral right through to ovulation. Until recently, studies of follicle growth *in vitro* have therefore been limited to particular phases of follicular development.

Culture of antral follicles has mainly involved short-term incubation and studies on these stages have provided valuable information about steroid secretion (Carlsson *et al.*, 1985; Moor *et al.*, 1973) and oocyte maturation (Lindner *et al.*, 1974; Moor & Trounson, 1977; Fukui *et al.*, 1988). Although antral follicles have the advantage of only requiring a short incubation period for oocyte maturation, it is paradoxical to find that complete development of mouse follicles at the antral stage is more successful when grown *in vitro* from preantral stages rather than when explanted at the antral stage. It has been proposed that this is because smaller follicles have longer to adjust to culture conditions and are in a well controlled environment when they reach the critical preovulatory phase (Gosden *et al.*, 1993). Oocyte and follicle development have also been studied by culturing cumulus-oocyte complexes (Eppig, 1980; Schroeder & Eppig, 1984; Eppig & Schroeder, 1989) or granulosa-oocyte complexes (Eppig, 1977; Eppig & Downs, 1987; Daniel *et al.*, 1989; Eppig & Schroeder, 1989; Gore-Langton & Daniel, 1990). Cumulus-oocyte complexes are obtained by aspirating antral follicles whereas granulosa-oocyte complexes, which are denuded of any theca layers and much of the basement membrane, are produced by disaggregating ovaries with proteolytic enzymes such as collagenase, pronase or trypsin (Eppig, 1977; Torrance *et al.*, 1989). In the mouse both of these systems have resulted in oocyte growth and maturation (Eppig, 1977; Eppig & Downs, 1987; Vanderhyden *et al.*, 1992) and, following IVF, embryonic development producing live offspring (Schroeder & Eppig, 1984; Eppig & Schroeder, 1989). Similar results have also been achieved with varying success in sheep (Staigmiller & Moor, 1984), cattle (Moor & Trounson, 1977; Goto *et al.*, 1988), rats (Daniel *et al.*, 1989; Vanderhyden & Armstrong, 1989) and pigs (Yoshida *et al.*, 1990). These studies have proved to be useful methods for "farming" oocytes and can be used to examine many aspects of oocyte growth and maturation.

Preantral follicle growth *in vitro* has been studied in a few species such as the mouse (Eppig, 1977; Eppig & Schroeder, 1989; Vanderhyden *et al.*, 1992), hamster (Roy & Greenwald, 1989), rat

(Daniel *et al.*, 1989; Gore-Langton & Daniel, 1990), cat (Jewgenow & Pitra, 1993) and human (Roy & Treacy, 1993). A number of attempts have been made to produce antral follicles from preantral stages *in vitro* but with limited success. Explants of human ovarian cortex have been maintained for up to 12 days and although no degenerative changes were exhibited the follicles showed no growth (Baker & Neal, 1974). Using primary follicles which were obtained from collagenase-digested mouse ovaries and cultured in collagen gel, Torrance *et al.* (1989) achieved growth from the unilaminar up to the multilaminar follicle but without antral development. Roy & Greenwald (1989) were the first to achieve early antral development from preantral hamster follicles which they had dissected mechanically and cultured in agar-coated wells. This was followed by Qvist *et al.* (1990) who reported the development of antral and preovulatory follicles from clusters of mechanically dissected preantral mouse follicles.

The first successful culture system for the development of individual preantral follicles through antrum formation and ovulation was recently developed in the mouse by Nayudu & Osborn (1992). This method employed mechanical dissection of preantral follicles, which were cultured on a Millicell membrane, and introduced conditions which allowed quantitative studies of individual follicles from size and quality-controlled material. The ability to culture complete follicles *in vitro* is extremely valuable as it maintains the normal three-dimensional relationships of the follicle unit and provides the oocyte with a maturation environment as close as possible to the *in vivo* condition. It also has an important advantage over *in vitro* maturation of oocytes from antral follicles since the earlier follicles have had less time for negative selection to act and therefore a larger proportion may be expected to be healthy (Nayudu, 1994). The culture system has subsequently been modified and used to investigate follicle metabolism, steroid production and the effects of gonadotrophins and growth factors on follicle development (Nayudu *et al.*, 1992, 1993, 1994; Boland *et al.*, 1993, 1994; Hartshorne *et al.* 1994 a,b; Spears *et al.*, 1994).

Although ovulation *in vitro* is well established with this culture system not every follicle is capable of ovulation and in those that are, there is a variation in the degree of oocyte maturation and cumulus expansion. Of course the ultimate test of oocyte normality following culture is the ability to become fertilized and undergo normal embryonic development. The production of live young from preantral follicular oocytes has only been achieved in mice and the major work in this area has been conducted by Eppig and his colleagues using granulosa - oocyte complexes (Eppig & Schroeder, 1989;

Eppig, 1991; Eppig *et al.*, 1992). Recently however, Spears *et al.* (1994) reported the birth of a single live mouse pup from an oocyte cultured from an individual preantral follicle.

For species other than the mouse, only a few attempts have been made to culture individual preantral follicles and the success has been limited. Nayudu *et al.* (1992) were able to grow marmoset follicles *in vitro* but these only reached the early antral stages of development. Work with marsupial tissue *in vitro* has been limited and only conducted in a few species. Stages in *Didelphis virginiana* organogenesis have been studied *in vitro* (New & Mizell, 1972) as have *Macropus eugenii* bilaminar blastocysts (Tyndale-Biscoe & Renfree, 1987). Successful culture of marsupial embryos has been achieved during cleavage until formation of early unilaminar blastocyst stages in *Antechinus stuartii* (Selwood & Young, 1983), *Sminthopsis crassicaudata* and *S. macroura* (Selwood, 1987).

In vitro studies of early embryonic development in *M. domestica* have been performed (Baggott & Moore, 1990; Selwood & VandeBerg, 1992) and recently IVF has been achieved in this species (Moore & Taggart, 1993; Taggart *et al.*, 1993). The earliest follicle stages cultured have been cumulus-oocyte complexes (Selwood & VandeBerg, 1992) and to our knowledge the following study of preantral follicles *in vitro* is the first of its kind for a marsupial species (Butcher & Ullmann, 1993).

5.2. MATERIALS AND METHODS.

5.2.1. Culture system.

Individual ovarian follicles were cultured using the method of Boland *et al.* (1993) modified from Nayudu & Osborn (1992). All procedures were conducted using aseptic techniques and, excluding removal of the ovaries, were performed in a laminar flow hood.

Media and equipment were obtained from the following sources: Leibovitz L-15, Minimal Essential Medium α (MEMα), fetal calf serum, sodium pyruvate and non-treated 96 V-well microtitre plates from Gibco (Paisley, U.K.). Glutamine, bovine serum albumin (BSA), insulin, transferrin, mineral oil and tissue culture water from Sigma (Poole, Dorset, U.K.). Follicle stimulating hormone (hFSH) was a gift from the National Hormone and Pituitary Program (NIDDK, University of Maryland School of Medicine, U.S.A.), prepubertal mouse serum was kindly given by Dr. N. Boland from the Physiology Department, University of Edinburgh Medical School, Millex filters were obtained from Millipore (Edinburgh, U.K.) and Microfine insulin syringes from Becton Dickinson (Edinburgh).

Supplements for both media were prepared no more than 3 months in advance, aliquoted into the required volumes and stored at -75°C.

5.2.2. Preparation of opossum serum.

Blood was collected aseptically, by cardiac puncture, from newly killed females using a 23G needle. It was allowed to clot in the fridge, or on ice when being transported, and then centrifuged. The serum was separated from the clot, centrifuged again, aliquoted into Eppendorf tubes and either used immediately or stored at -75°C.

5.2.3. Media preparation.

Follicle isolation was carried out in a dissection medium of Leibovitz L-15, supplemented with glutamine (2mmol L^{-1}) and BSA (3 mg ml^{-1}). This was filter-sterilized through a Millex 0.22 μm filter and brought to either 33°C or 37°C in an oven. The follicle culture medium consisted of MEMα, supplemented with 5% serum (either fetal calf, prepubertal mouse or prepubertal opossum), glutamine (2mmol L^{-1}), insulin (5 μg ml^{-1}), sodium pyruvate (2mmol L^{-1}) and transferrin (10μg ml^{-1}). FSH (biological potency 5763 IU ml^{-1}) was added in concentrations of 0.5, 1.0 or 1.5 I.U. ml^{-1}. The

medium was filter-sterilized, 25 µl pipetted into each V-well and overlayed with 70 µl of filtered mineral oil. The culture plate was then transferred to a 33°C or 37°C incubator, gassed with 5% CO_2 in air, and allowed to equilibrate.

5.2.4. Follicle isolation and culturing.

Ovaries were excised aseptically, removing as much fat as possible, and placed in a sterile watchglass containing dissection medium at 33°C or 37°C. Under a dissection microscope the ovaries were teased apart into several clumps using two 28G needles attached to 1ml syringes. Individual follicles measuring 120 µm - 230 µm (types 4 - 5b), with a layer of theca cells and some attached stroma cells, were then manually dissected out. The follicles were measured with a calibrated ocular micrometer and transferred into V-wells, with a finely drawn BSA-coated Pasteur pipette, and returned to the incubator. They were measured and transferred to a new well with fresh medium every day until growth had ceased and then processed for transmission electron microscopy.

5.2.5. Experimental groups.

Ovaries (n=12) were obtained from 8 prepubertal and 1 adult female and follicles were cultured at different incubation temperatures with various FSH concentrations and types of serum to try and determine the optimum culture conditions for *M. domestica* follicles.

In experiment 1 follicles were incubated at 37°C in medium supplemented with 1 IU FSH ml^{-1} and either prepubertal mouse serum (n=12), fetal calf serum (n=12) or prepubertal opossum serum (n=12). The first culture run was conducted at Edinburgh University Medical School, where the techniques were shown to me by Dr. Niki Boland. In experiment 2 follicles were cultured at 33°C, with 1 IU FSH ml^{-1} and either prepubertal mouse serum (n=12) or prepubertal opossum serum (n=48). In experiment 3 follicles were cultured at 33°C, with prepubertal opossum serum using either 0 (n=12), 0.5 (n=12) or 1.5 (n=12) IU FSH ml^{-1}. In cultures conducted with opossum serum the follicles were incubated with serum from the donor animal.

For several months when the *M. domestica* colony ceased breeding (section 2.2.2) there were no prepubertal animals available so a culture run was conducted with follicles (n=12) from an adult female

at 33°C with her own serum and 1.0 IU FSH ml^{-1}. To try and minimize the concentration of endogenous gonadotrophins her smears were monitored to determine when she was in dioestrus.

5.2.6. Statistical analysis.

An unpaired Student's t-test was used to compare follicle growth between different experimental groups.

5.2.7. Follicle fixation and embedding.

1. 3% glutaraldehyde in 0.1M phosphate buffer	- 1 hour.
2 . 0.1 M phosphate buffer	- 3 x 5 minutes.
3. 1% Osmium tetroxide in 0.1M phosphate buffer	- 1 hour.
4. Distilled water	- 3 x 10 minutes.
5. 30%, 50%, 70%, 90% ethanol	- 15 minutes each.
6. Absolute ethanol	- 15 minutes.
7. Dried absolute ethanol	- 15 minutes.
8. Propylene oxide	- 3 x 5 minutes.
9. Propylene oxide / resin (50:50)	- 24 hours on rotator.
10. Araldite resin	- 48 hours at 60°C.

Semithin sections for light microscopy were cut and stained as described in section 4.2.1.

5.3. RESULTS

5.3.1. Follicle growth.

The effect of temperature, FSH concentration and serum type on follicle growth are shown in tables 5.1 and 5.2

5.3.2. Effect of temperature and serum on follicle growth.

In the culture run performed in Edinburgh, when the medium was supplemented with prepubertal mouse serum, 1.0 IU FSH and conducted at 37°C, all of the follicles grew with a mean increase in

Table 5.1. Follicle growth with 1.0 IU FSH ml^{-1} and various sera at different temperatures. Values are shown as means (± SEM).

Type of serum	Temperature (°C)	FSH concentration (IU/ml)	Follicles showing growth (%)	Increase in follicle diameter (µm)	Maximum diameter (µm)	Time taken to reach maximum size (days)
Calf	37	1.0	50	37.9 (±6.6)	221.5 (±17.4)	1.9 (±0.3)
Opossum	37	1.0	100	61.7 (±5.8)	227.5 (±13.5)	5.7 (±0.5)
Mouse	37	1.0	100	97.6 (±8.4)	285.8 (±7.4)	3.5 (±0.2)
Mouse	33	1.0	30	40.0 (±6.7)	185.7 (±6.8)	3.1 (±0.7)

Table 5.2. Follicle growth at 33°C with opossum serum and various FSH concentrations. Values are shown as means (± SEM).

Type of serum	Temperature (°C)	FSH concentration (IU/ml)	Follicles showing growth (%)	Increase in follicle diameter (µm)	Maximum diameter (µm)	Time taken to reach maximum size (days)
Opossum	33	0	100	77.5 (±7.8)	292.5 (±4.9)	4.6 (±0.5)
Opossum	33	0.5	100	80.9 (±9.8)	288.0 (±13.7)	6.1 (±6.4)
Opossum	33	1.0	100	118.1 (±9.5)	317.4 (±9.9)	6.4 (±0.4)
Opossum*	33	1.0	100	94.3 (±7.5)	282.5 (±7.6)	5.8 (±0.5)
Opossum	33	1.5	100	91.6 (±9.1)	291.7 (±6.2)	Growth interrupted ‡

* Adult female

‡ Growth interrupted due to incubator malfunction

diameter of 97.6 (± 8.4) μm. When the culture system was set up in Glasgow a source of prepubertal mouse serum was not available, so fetal calf serum was used on a trial basis - this time only 50% of the follicles showed any growth and those that did exhibited a significantly smaller (t=-6.08, df=22, p=0.0001) increase in diameter. When prepubertal opossum serum was added to the medium all of the follicles grew and showed a mean diameter increase 63% larger than those cultured with fetal calf serum.

In experiment 2, when the incubation temperature was lowered to 33°C, follicle growth with prepubertal opossum serum and 1.0 IU FSH was significantly higher (t=5.08, df=16, p=0.0001) and almost twice that observed at 37°C. With prepubertal mouse serum at this temperature follicle growth was poor - only 30% of the follicles grew and showed a mean diameter increase less than 50% of that observed at 37°C. On the occasion when the culture run was conducted with an adult female, follicle growth was not significantly different (t=1.92, df=17, p=0.072) from that observed in prepubertal animals.

5.3.3. Effect of FSH concentration on follicle growth.

When cultures were conducted at 33°C with prepubertal opossum serum there was no significant difference in growth (t=-0.26, df=18, p=0.80) between follicles cultured with no FSH and those cultured with 0.5 IU FSH ml^{-1}. With the addition of 1 IU FSH ml^{-1}, follicles grew significantly more (t=2.59, df=19, p=0.018) than those cultured with 0.5 IU ml^{-1} and 55% of them reached a diameter of 300 μm or more. Increasing the FSH concentration to 1.5 IU ml^{-1} did not produce any further significant growth (t=2.01, df=13, p=0.065) but it should be explained that on day 6 of the culture run the incubator malfunctioned, and the temperature rose to 40°C for a maximum period of 4 hours, which was clearly detrimental to the follicles. Until this temperature fluctuation occurred follicles cultured with 1.5 IU FSH showed more growth and had a faster growth rate than those at lower concentrations.

5.3.4. Follicle growth profiles.

Follicle growth is presented graphically in Figs.5.1 - 5.3 and, as can be seen, there was a range of growth profiles between experimental groups.

At 37°C, when fetal calf serum was used (Fig.5.1.a.) only half of the follicles grew and the remainder began to shrink after the first day of culture. The pattern was strikingly different when prepubertal mouse serum was added to the medium (Fig.5.1.b.) as every follicle increased in size and growth lasted for up to four days. Compared to mouse serum, follicles given opossum serum at 37°C exhibited a much slower rate of growth although they did continue to grow until up to day 5 of culture (Fig.5.1.c.). With mouse serum at 33°C (Fig.5.1.d.) there was a greatly reduced growth rate with most follicles remaining static in size. The mean growth rates for each of these treatments are compared in Fig.5.3.a.

The growth profiles of subsequent cultures, with opossum serum at 33°C, were quite similar to each other (Fig.5.2) with the actual rate of growth dependent on the FSH concentration. Without FSH (Fig.5.2.a) follicle growth was slow and had come to a standstill by day 6 of culture. In contrast, with 0.5 and 1.0 IU FSH ml^{-1} (Figs.5.2.b, 5.2.c and 5.2.d) follicle growth was faster and continued until at least day 7. As can be seen when the growth rates of each group are compared (Fig.5.3.b), the fastest growth occurred in follicles given 1.5 IU FSH ml^{-1}. The follicles cultured from the adult female, which are not illustrated, showed a similar growth profile to those from prepubertal animals under the same experimental conditions.

Overall, it appeared that the most favourable conditions for follicle growth were incubation at 33°C, with the addition of opossum serum and 1.0 to 1.5 IU FSH ml^{-1}.

5.3.5. Effect of follicle starting size.

The starting size of the follicles appeared to play a role in their subsequent growth and this also appeared to be related to the FSH concentration. The minimum starting size from which follicles grew was approximately 170 μm and those larger than 300 μm exhibited limited growth.

When cultured with 1.5 IU FSH, follicles with an initial diameter of 180 μm reached a maximum size of 300 μm whereas those cultured with 0.5 IU only showed a slight increase to 200 μm. Follicles with an initial diameter of 200 μm, did not respond differently to the two FSH concentrations and in both groups reached a maximum diameter of 300 μm.

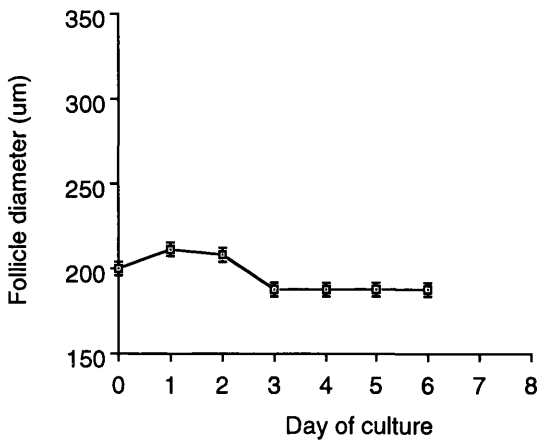
5.3.6. Follicle morphology.

Unfortunately, the follicles cultured in Edinburgh, with mouse serum at 37°C, were discarded and so could not be examined. When all of the remaining follicles were examined histologically, none of them showed antrum formation and their condition was dependent on the culture conditions they had been exposed to. Follicles cultured with mouse serum at 33°C and fetal calf serum at 37°C were clearly degenerating. Apart from those cultured without FSH, the majority of follicles cultured with opossum serum were in much earlier stages of atresia than those cultured with non-homologous serum. The actual degree of atresia appeared to be related to the growth pattern of the individual follicle and the concentration of FSH it was exposed to.

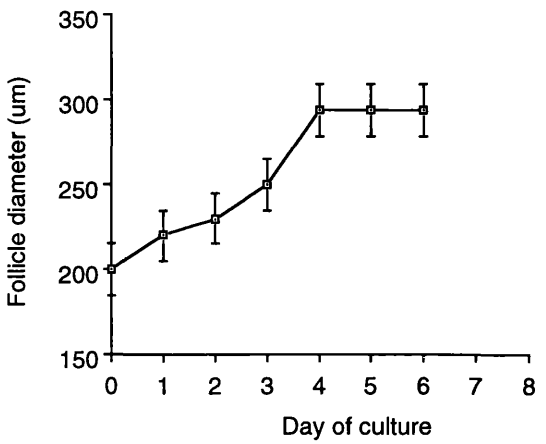
Follicles grown without FSH (Fig.5.4) were in an advanced stage of atresia: the oocyte had often ruptured, the basement membrane had broken down, there was little distinction between the granulosa and theca cells and the follicle had completely lost any semblance of structured arrangement. Follicles cultured with 0.5, 1.0 and 1.5 IU FSH ml^{-1} (Figs. 5.5, 5.6 & 5.7) looked healthier than those which received no FSH and although granulosa cells with pycnotic nuclei were present in varying amounts the overall structure of these follicles and their oocyte appeared to be normal. There was the suggestion of antral spaces forming in a few follicles but it is possible that this was an artefact of fixation. The appearance of the follicle was correlated to the growth it had shown during the culture period with those exhibiting continuous growth appearing the healthiest.

Fig.5.1. Growth rates (mean ± SEM) of follicles cultured with 1.0 IU FSH ml^{-1} and various sera at different temperatures.

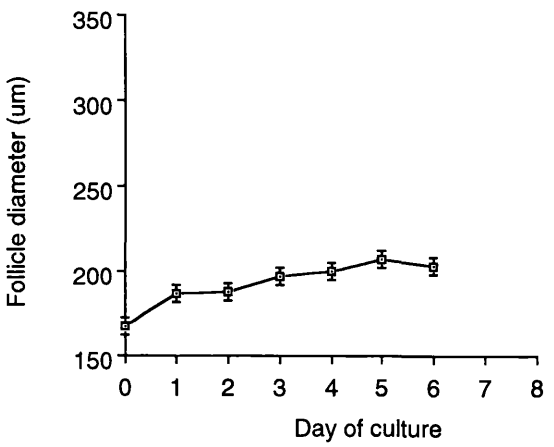
a. Fetal calf serum at 37°C



b. Mouse serum at 37°C



c. Opossum serum at 37°C



d. Mouse serum at 33°C

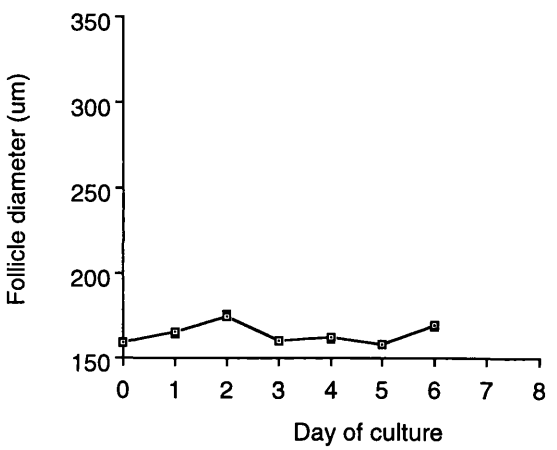
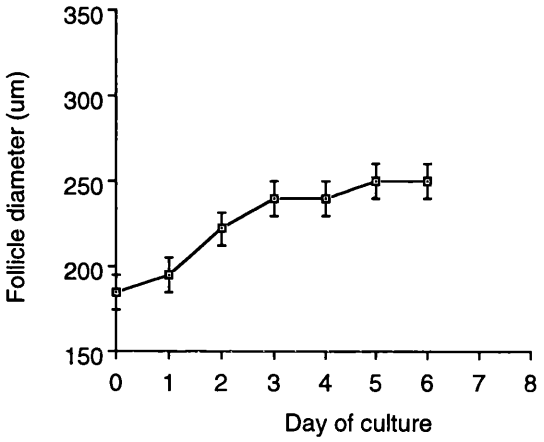
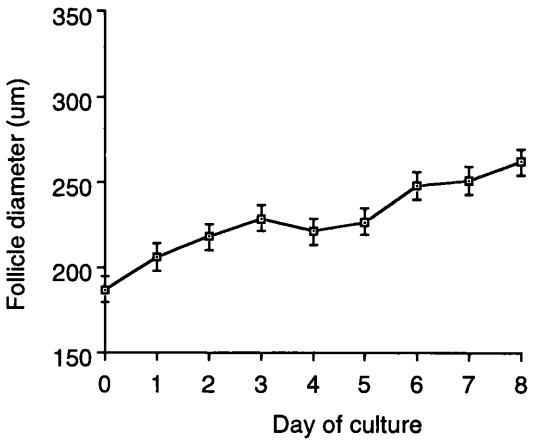


Fig.5.2. Growth rates (mean ± SEM) of follicles cultured at 33°C with prepubertal opossum serum and various FSH concentrations.

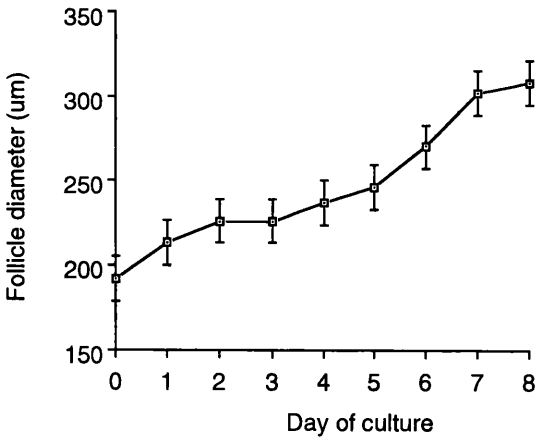
a. No FSH



b. 0.5 IU FSH ml^{-1}



c. 1.0 IU FSH ml^{-1}



d. 1.5 IU FSH ml^{-1}

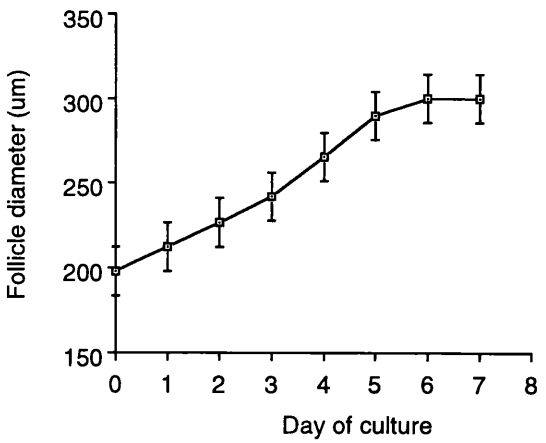


Fig.5.3.a. Comparison of mean growth with 1.0 IU FSH ml^{-1} and various sera at different temperatures

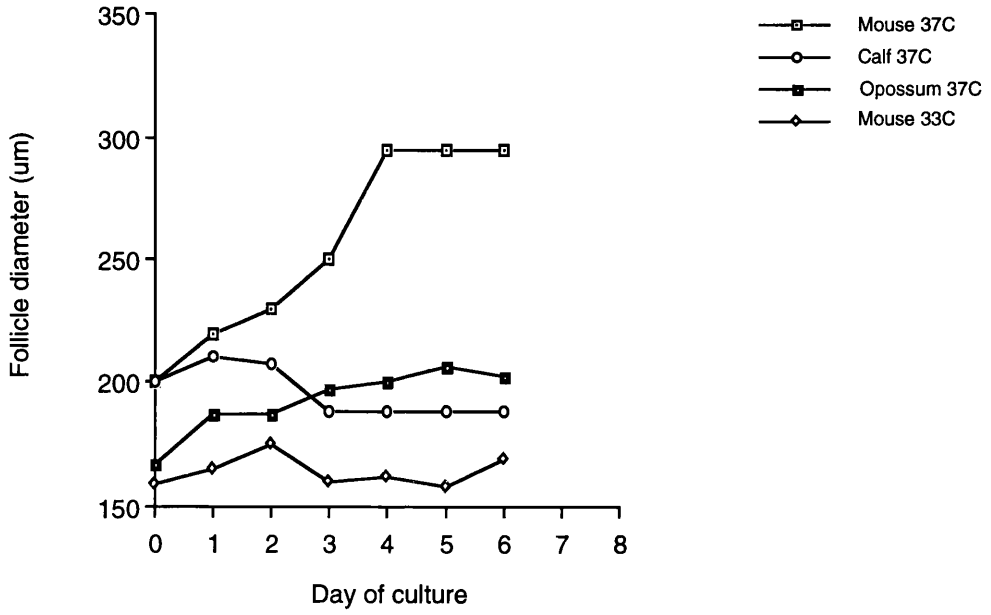


Fig.5.3.b. Comparison of mean growth at 33°C with prepubertal opossum serum and various FSH concentrations.

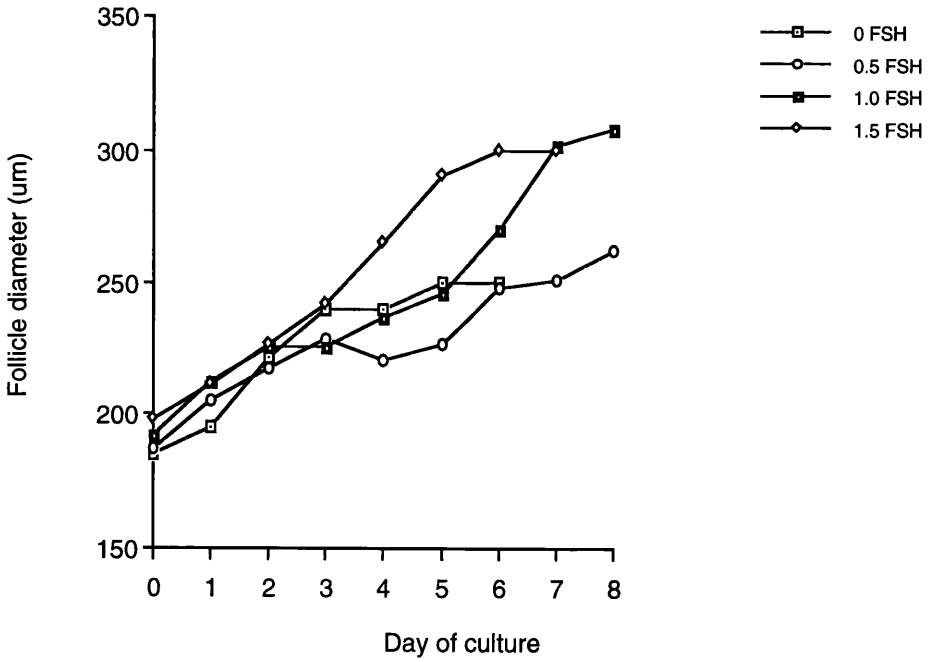


Fig. 5.4. Follicle cultured without FSH (x75)

Fig. 5.5. Follicle cultured with 0.5 IU FSH ml^{-1} (x75)

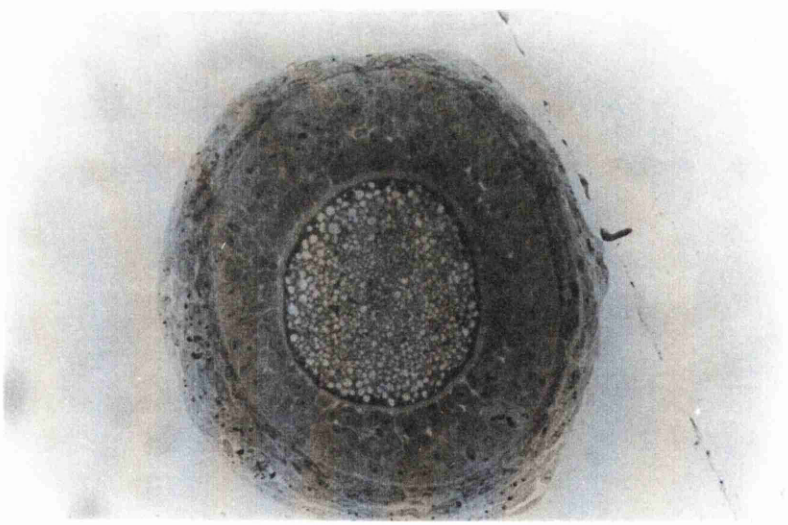


Fig. 5.6. Follicle cultured with 1.0 IU FSH ml^{-1}. Note the presence of pycnotic nuclei (p) and possible antral fluid forming in some areas (arrowed) (x75)

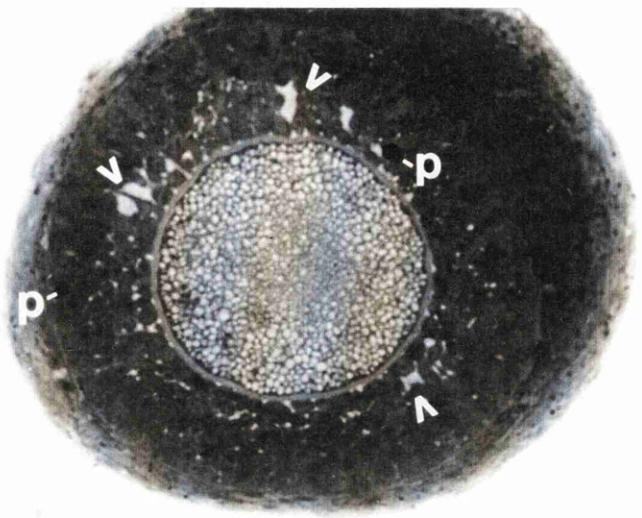
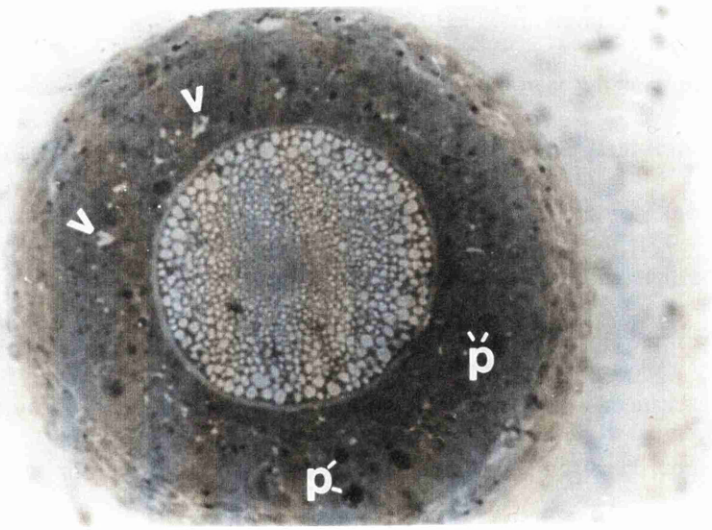


Fig. 5.7. Follicle cultured with 1.5 IU FSH ml^{-1} showing pycnotic nuclei (p) and possible antral fluid forming (arrowed) (x75)



5.4. DISCUSSION

5.4.1. Success of opossum follicle culture.

Although the culture system was able to support follicle growth for up to 8 days modifications are clearly necessary if it is to be suitable for antral development in this species (Butcher & Ullmann, 1993). In *M. domestica* antrum formation *in vivo* commences in follicles of approximately 300 μm diameter and although some cultured follicles reached up to 350 μm they remained preantral.

5.4.2. Effect of temperature and serum.

Studies into the effect of temperature on oocyte development have shown that bovine oocytes can be matured *in vitro* at various temperatures ranging from 35°C - 39°C (Lenz *et al.*, 1983; Katska & Smorag, 1985) with 35°C being the most favourable. The results in this study proved interesting as it appeared to be the combination of temperature and serum, rather than each one individually,which was most important.

As one might expect, the best follicle growth was achieved using opossum serum at the opossum body temperature of 33°C but surprisingly this was only slightly better, and not significantly different, ($p>0.05$) from supplementing with mouse serum and culturing at mouse body temperature. This would suggest that follicle growth will occur providing that the incubation temperature is compatible to that of the serum donor. The fact that incubation of follicles with opossum serum at a higher than natural temperature was more successful than mouse serum incubated at a lower temperature can probably be put down to a simple increase in the rate of enzyme reactions at the higher temperature and *vice versa*. The only apparent difference in follicle growth between these two groups was the longer period of growth observed with opossum serum so it would appear that for short periods mouse serum at 37°C is able to support follicle growth. If mouse serum is an acceptable substitute it is surprising that fetal calf serum should prove so unsuitable. Nayudu & Osborn (1992) also found that mouse serum was superior to fetal calf serum but as they were culturing mouse follicles it did not seem unusual that follicles would grow better in homologous serum. However, problems with ovine embryo culture have been traced to variations between batches of fetal calf serum although the critical factor (s) is not clear (K. Mycock, personal communication).It has been reported that fetal calf serum contains a relatively high level of the enzyme polyamine oxidase which, upon reaction with polyamines secreted from highly

proliferative cells, can form cytotoxic polyaminoaldehydes (Maurer, 1986). If certain batches contain this enzyme then it may explain reports on the suppressive activity of this serum.

Groups culturing *M. domestica* preovulatory oocytes and embryos have had contradictory results with respect to the optimum incubation temperature. Selwood & VandeBerg (1992) observed that oocyte maturation and embryo development was more successful at 32.6°C than 37°C. In contrast, Moore & Taggart (1993) found that 37°C was more suitable for *in vitro* fertilization than 33°C - at the higher temperature 62.5% of their oocytes were fertilized, 67% of which developed to the 2-cell stage or beyond, whilst at 33°C only 18% were fertilized. However, as the rate of embryo development achieved by Selwood & VandeBerg (1992) at 32.6°C was considerably lower than that of Moore & Taggart (1993) at 37°C it is possible that the composition of the incubation medium may have been a more critical factor than the temperature. With this in mind it is interesting to note that Selwood & VandeBerg (1992) supplemented their medium with fetal calf serum whereas Moore & Taggart (1993) used serum-free medium.

5.4.3. Effect of FSH concentration.

FSH was found to stimulate the growth of preantral follicles and this is in agreement with observations recorded by other groups. The presence of FSH receptors on preantral follicles suggested that this gonadotrophin may influence the development of preantral follicles (Roy *et al.*, 1987) and it was subsequently found that, in the hamster, FSH significantly lowered the incidence of atresia (Roy & Greenwald, 1989). Studies on the mouse revealed that the growth of primary follicles in culture depended on the presence of an adequate concentration of FSH (Qvist *et al.*, 1990) and that FSH was necessary for follicle growth and antrum formation *in vitro* (Nayudu & Osborn, 1992). It is thought that gonadotrophins may be beneficial for development of normal oocytes as those from hypogonadal mice have been found to undergo fertilization and embryogenesis at very low frequencies after *in vitro* maturation. Injecting these mice with pregnant mares' serum gonadotrophin (PMSG) greatly increased the number of oocytes that could be fertilized and complete preimplantation development after maturation *in vitro* (Schroeder & Eppig, 1989).

Although in this study the maximum growth was achieved with the addition of 1.0 IU FSH ml^{-1}, the rate of growth with 1.5 IU suggests that higher FSH concentrations may improve follicle growth. It

was during this experiment that the incubator thermostat malfunctioned and although the follicles were only exposed to a raised temperature for a maximum of 4 hours it was presumably long enough to cause irrecoverable damage. Until this temperature fluctuation occurred follicles cultured with 1.5 IU FSH showed more growth and had a faster growth rate than those in the other groups and from the appearance of the growth profile it does seem likely that, without this disruption, follicle growth would have continued. Further studies are obviously necessary to confirm or disprove this speculation but, unfortunately due to restrictions of time and equipment, it was not possible to repeat this experiment.

The original culture system was designed using prepubertal mice (Nayudu & Osborn, 1992) to ensure that there would be no complications caused by the degree and type of *in vivo* priming expected with adult animals. When I conducted a culture run using an adult animal there was no significant difference in either the growth or rate of growth between follicles from this animal and prepubertal animals. It would appear that by ensuring that the adult was in dioestrus, her levels of circulating gonadotrophins were sufficiently low so as not to interact with the exogenous FSH.

As FSH had a positive effect on follicles, both in terms of growth and morphology, it would appear that human FSH is compatible with *M. domestica* FSH receptors. Various marsupial species (*Trichosurus vulpecula*: Rodger & Mate, 1988; *Bettongia penicillata*: Rodger *et al.*, 1992; *Sminthopsis crassicaudata*: Rodger *et al.*, 1992; *Macropus eugenii*: Rodger *et al.*, 1993) have been superovulated using either PMSG + human luteinizing hormone releasing hormone (LHRH) or PMSG + synthetic gonadotrophin releasing hormone (GnRH) regimes. Rodger *et al.* (1992) have also superovulated *M. domestica* using PMSG + hLHRH although they did not comment on the efficacy of this regime. Therefore, it would appear that there is not a marsupial - eutherian barrier with regard to these gonadotrophins.

5.4.4. Follicle starting size and growth profiles.

When the profiles for individual follicle growth (using opossum serum at 33°C and various FSH concentrations) were examined the starting size of the follicles appeared to play a role in their subsequent growth. Those not between approximately 170 μm and 300 μm showed little growth and for follicles within these values, the larger the follicle the faster its growth and the greater its increase in size tended to be. This may have been because the enclosed oocyte had nearly completed its growth and

so was not as dependent on the culture conditions as one in the early stages of growth. With follicles larger than 300 μm, which were bordering on the antral stage of development, there is a greater likelihood that they had already been exposed to negative selection and were destined to become atretic. Follicle growth was dependent on the amount of FSH present and the higher the FSH concentration the smaller the size of follicles that could be recruited.

5.4.5. Additional factors to consider.

The actual kinetics of follicle growth are important factors to discuss as they vary greatly between species. In the mouse a period of approximately 19 days is required for a type 3b follicle to reach the ovulatory stage and 4 days for a type 5b to reach this final size (Pedersen, 1970) while in the rat, Hirshfield (1984) reported that follicle growth from the type 6 to 8 stage may require as little as 24 hours. The mean time for a sheep follicle to pass from a 5a to a type 6 follicle is 130 days, further development to the preovulatory stage takes approximately 45 days thus taking a total period of approximately 6 months (Cahill & Mauléon, 1980). Pedersen (1970) also reported that mouse follicles actually grow faster *in vitro* than *in vivo* which is possibly because they are released from the inhibiting influence of other growing follicles (Nayudu & Osborn, 1992) or a result of artificial culture conditions (Hartshorne *et al.*, 1993). The dynamics of *M. domestica* follicles *in vivo* have not been monitored so it is not known how long follicle development takes in this species. However, as *M. domestica* is an induced ovulator, presumably follicle growth would occur rapidly following stimulation.

The mechanisms regulating follicle growth and differentiation are very complex and in addition to FSH there are numerous growth factors such as fibroblast growth factor (Gospodarowicz & Bialecki, 1979), epidermal growth factor (Dekel & Sherizly, 1985; Downs *et al.*, 1988; Boland & Gosden, 1994), platelet-derived growth factor (May *et al.*, 1990), transforming growth factor (Skinner & Coffey, 1988) and insulin-like growth factor (Carson *et al.*, 1989) which participate in these processes.

As the *M. domestica* oocyte continues to grow during the antral period, oocyte and follicle development are coordinated differently to the pattern observed in eutherians and work by Nayudu and her colleagues (1991; 1992) has suggested that the growth pattern of the follicle may be correlated with the normality of the oocyte. It may be that the culture medium is deficient in one or more critical factors and perhaps a higher concentration of serum is needed to supply the oocyte with sufficient

growth factors, binding proteins, lipids and yolk precursors for normal development. In other culture systems higher doses of serum ranging from 10% (Eppig, 1977; Carroll *et al.*, 1991; Jewgenow & Pitra, 1993), 15% (Moor & Trounson, 1977), 20% (Baker *et al.*, 1975) and 50% (Qvist *et al.*, 1990) have been employed.

Although the culture medium was replaced every day it is possible that, in the small volume of medium, the proportion of metabolites produced by the oocyte and granulosa cells were high enough to have an inhibitory effect. In the mouse, Boland *et al.* (1993) observed that lactate was a major product of follicle metabolism and that, throughout preantral and antral growth, follicles adopted a predominantly glycolytic mode of energy production (Boland *et al.*, 1994). Increasing the volume of culture medium may help to prevent such metabolites becoming concentrated enough to negatively affect follicle growth.

Despite considerable progress being made with the mouse towards the ultimate goal of culturing the entire span of follicle stages continuously there are still problems during the early period of growth which remain unsolved (Gosden *et al.*, 1993). Even in this species, which is considered to be an ideal model for such culture studies (Spears *et al.*, 1994), only 60% of follicles reached the Graafian stage with 33% undergoing ovulation (Boland *et al.*, 1993). In fact, the Graafian follicle pictured in the paper by Boland *et al.* (1993) appears to be in the early stages of atresia with a few pycnotic granulosa cells floating freely in the antrum.

Culture technology is currently limited by the follicle stages which adapt well to conditions *in vitro*. Follicles denuded of theca cells have a much slower growth rate and reach smaller final sizes than intact follicles. Differentiated thecal cells have been found to be a prerequisite for antral development (Nayudu & Osborn, 1992) and follicles below a certain size, depending on the species, do not have a thecal layer to allow them to proceed through to antral development. McNatty *et al.* (1980) reported that proliferation of human granulosa cells *in vitro* could be stimulated by coculturing them with minced theca cells. Stimulation of granulosa cell mitosis by extracts of pig theca (Makris *et al.*, 1983) and culture media conditioned with rat (Lobb *et al.*, 1988) and bovine (Bendell *et al.*, 1988) theca cells confirmed the mitogenic action of theca cells derived from several species. Analysis has revealed that theca cells produce TGF-β (Skinner *et al.*, 1987a) and an EGF-like molecule (Skinner *et al.*, 1987b)

which, with opposing effects on the granulosa cells, probably modulate granulosa cell mitosis and differentiation.

When using animals which have large follicles there could be a problem in getting optimal nutrient/metabolic/gas exchange in the central regions of the oocyte. However, oocytes do appear to be remarkably tolerant to periods of hypoxia as oocytes from rare felid species have been stimulated to mature and undergo *in vitro* fertilization after being stored in medium for up to 36 hours (Johnston *et al.*, 1991). It is even more astonishing that oocytes retrieved from mice deceased for as long as 6 hours demonstrated full developmental capacity even after pronounced post mortem changes had occurred. Culturing the recovered oocytes reversed the degenerative changes and the eggs produced were capable of fertilization and development of live young after transfer to foster mothers (Schroeder *et al.*, 1991)

There are many prospective applications for the oocytes obtained from such follicles grown *in vitro*. Because there is currently an acute shortage of human donor oocytes, one of the major uses is to meet the needs of women with gonadal dysgenesis and premature ovarian failure. A greater abundance of oocytes would also be beneficial in reducing the number of egg collection cycles and possibly avoid the need to superstimulate patients undergoing IVF or GIFT treatments (Gosden *et al.*, 1993). Comparable techniques could also revolutionize the technology of animal breeding and play an important role in the conservation of endangered species. Results also suggest that isolated follicles in culture may be a sensitive system for assessing the effects of potential toxicants on oocyte development (Nayudu *et al.*, 1994) and could therefore replace some of the toxicological studies still performed in whole animals.

Chapter 6

Yolk formation and composition

6.1. INTRODUCTION

6.1.1. Yolk in eutherian oocytes.

It is a common misconception that mammalian oocytes do not contain yolk material and most of those investigated at the ultrastructural level exhibit a great variety of cytoplasmic inclusions which have been referred to as yolk (see section 4.1.2.). These inclusions have ranged from chains of ribosomes in the mouse (Burkholder *et al.*, 1971); parallel chains and lattice-like structures in the rat and hamster (Nilsson, 1980); lamellar complexes in the Egyptian spiny mouse (Kang & Anderson, 1975); lamellar inclusions in the pocket gopher (King & Tibbitts, 1977) and a variety of vesicles observed in various species. Vesicles are the most conspicuous organelles in bovine (Fleming & Saacke, 1972; Kruip *et al.*, 1983; Hyttel *et al.*, 1986; de Loos *et al.*, 1989) and ovine oocytes (Russe, 1975; Cran *et al.*, 1980) and it is thought that they contain storage products. From morphological examination Russe (1975) described the vesicles as yolk globules whereas, following histochemical analysis, Fleming & Saacke (1972) termed them granulated vesicles due to the PAS positive particles detected within them. Other workers have also observed vesicles in the rabbit (Zamboni & Mastroianni, 1966a,b), ferret (Hadek, 1969) and pig-tailed monkey (Szöllösi, 1972) and suggested that they represent a form of yolk.

6.1.2. Marsupial yolk.

In the pioneering studies on *Dasyurus viverrinus* and *Didelphis virginiana* by Hill (1910) and Hartman (1916; 1919) respectively, the accumulation of a "yolk mass" within the developing oocytes was reported. The presence of this yolk has subsequently been described in various species but very little work has actually been directed towards identifying this material.

Ultrastructural studies have shown that the yolk bodies of marsupials consist of lipid droplets and relatively simple protein yolk vesicles (Selwood & Sathananthan, 1988; Breed & Leigh, 1990). When in a yolk mass they are associated with various organelles including mitochondria, membranous structures and a fine granular cytoplasmic matrix (Selwood, 1992). Morphological reports, on various species at both the light and electron microscope levels, have frequently described the presence of lipid bodies (*Didelphis virginiana*: Hartman, 1916; McCrady, 1938; *Dasyurus viverrinus*: Hill, 1910; *Didelphis aurita*: Hill, 1918; *Isoodon macrourus* & *Perameles nasuta*: Lyne & Hollis: 1983; *Antechinus*

stuartii: Selwood & Sathananthan: 1988). Groups studying *M. domestica* oocytes have also stated that they contain lipid vesicles (Baggott & Moore, 1990; Selwood & VandeBerg, 1992; Moore & Taggart, 1993; Taggart *et al.*, 1993) but have not substantiated this with any form of chemical analysis.

6.1.3. Yolk polarity.

There are also several differences in the distribution of yolk between species. In the armadillo much of the yolk is situated at one pole (Newman, 1912) while in some bats, the cat, dog, ferret, fox and pig it is distributed fairly uniformly throughout the cytoplasm (Austin, 1961).

In the preovulatory oocytes of marsupials such as *Antechinus stuartii* (Selwood & Young, 1983) and *Sminthopsis crassicaudata* (Breed & Leigh, 1988; 1990), the yolk is centrally located. In *Dasyurus viverrinus* (Hill, 1910) the yolk is concentrated in one hemisphere whereas in *Didelphis virginiana* (Hartman, 1916, 1919; McCrady, 1938) and *Didelphis aurita* (Hill, 1918) the yolk is distributed in a sub-marginal zone. In those species which do not initially demonstrate polarity, the "yolk mass" becomes eccentric either during the formation of the first polar body (Breed & Leigh, 1988; 1990) or following ovulation (Selwood & Young, 1983).

6.1.4. Various yolk types.

The amount and histochemistry of yolk appears to differ greatly between species. For example, rat oocytes do not show conspicuous formation of either protein or lipid yolk whereas many species such as the rabbit, hare, guinea pig, goat and Indian water buffalo contain both types of yolk (Guraya, 1964).

Considerable evidence has been provided for the presence of lipid material in the oocytes of many eutherians, especially carnivores (Guraya, 1965; Szabo, 1967; Tesoriero, 1979; 1981a,b; 1982), some ungulates (Barker, 1951; Guraya, 1964; Homa *et al.*, 1986) and a few other species such as the nine-banded armadillo (Newman, 1912) and guinea pig (Adams *et al.*, 1966).

Employing various analytical techniques such as histochemistry, gas chromatography and thin-layer chromatography, various groups have shown that a wide range of lipids can exist within oocytes. In the pig (Barker, 1951; Homa *et al.*, 1986) triacylglycerols were reported to be the major constituent and this result was similar to the triglycerides and unsaturated cholesterol esters seen in the cat, dog

(Guraya, 1965) and guinea pig (Adams *et al.*, 1966). In his study of yolk formation in the dog, Tesoriero (1982) was able to detect saturated fats, diglycerides, triglycerides, cholesterol, phospholipids and glycolipid.

The only histochemical work conducted on marsupial oocytes appears to be that of Guraya (1965) on *Didelphis virginiana* in part of his extensive examination of bird, fish, mammal and reptile oocytes (see Guraya, 1964). He identified two types of yolk, which he described as compound and lipid yolk, but was unable to determine their mode of origin. The compound yolk which was in the form of small, spherical complexes of protein and carbohydrate was the more abundant of the two and was lightly interspersed with the lipid form which consisted of phospholipid. Concomitant with the growth of the oocyte, the compound yolk bodies increased in size and number but the intensity of their staining decreased from that observed during the early stages of their formation. The lipid yolk arose in the form of deeply Sudanophilic granules which made their appearance a little later than the compound yolk bodies and also accumulated during oocyte development.

6.1.5. Transfer of molecules into oocytes.

As described in section 4.1.3, oocytes are connected to their surrounding granulosa cells by gap junctions (Brower & Schultz, 1982) which represent a major route for the movement of low molecular weight metabolites from the granulosa cells to the oocyte (Moor *et al.*, 1980; Heller *et al.*, 1981; Colonna & Mangia, 1983). These metabolites include uridine, guanosine, choline, glucose (Moor *et al.*, 1980; Heller *et al.*, 1981) and the amino acids leucine (Cross & Brinster, 1974) and methionine (Heller *et al.*, 1981).

Autoradiography has made significant contributions to the understanding of cellular function in many organs including the ovary. By injecting animals with amino acids labelled with various radioactive isotopes it has been possible to record their incorporation, precise location and distribution within specific areas of interest. Utilizing autoradiography it has been demonstrated that systemically injected proteins labelled with ^{131}I (Glass, 1961; Mancini *et al.*, 1963; Glass & Cons, 1968), ^{14}C (Cross & Brinster, 1974) and ^{3}H (Colonna *et al.*, 1983; Colonna & Mangia, 1983; Haghighat & Van Winkle, 1990). are transferred from the blood into the ovary and then enter the oocytes. In the mouse this

transfer has been found to be stage dependent with the concentration of labelled molecules higher during periods of rapid oocyte growth (Glass & Cons, 1968).

Different strategies for nutrient uptake may be employed depending on the metabolites concerned. Whilst studying transport across the mouse oolemma, Colonna *et al.* (1983) found that amino acid transport was provided by the "L" and "ASC" types of transport system first described in Ehrlich ascites tumour cells and red blood cells (Oxender & Christensen, 1963). These both work mainly by exchanging internal and external amino acids but are slightly different in that the ASC-transport system is dependent on the concentration of Na+ ions present. Colonna *et al.* (1983) found that amino acids such as leucine, valine and phenylalanine with large aliphatic or aromatic side chains were carried by the so-called "L" system whereas amino acids with short linear or polar chains such as alanine, cysteine and serine were transported by the ASC system.

6.2. MATERIALS AND METHODS

6.2.1. Histochemical staining for carbohydrate.

Formalin-fixed sections were stained with periodic acid-Schiff (see Appendix A.2.1. for details of procedure). Negative controls were obtained by enzyme carbohydrate extraction using salivary amylase and positive controls were conducted on liver sections.

6.2.2. Histochemical staining for protein.

Formalin-fixed sections were treated with ninhydrin-Schiff or mercury bromophenol blue (Appendix A.2.2. & A.2.3.).

6.2.3. Histochemical staining for lipid.

Cryostat sections, post-fixed in formal calcium, were stained with standard Sudan black, bromine Sudan black, Oil Red O or ferric haematoxylin (Appendix A.2.4, 2.5, 2.6. & 2.7).

Negative controls were delipidised by treating sections for 1 hour with a solution of chloroform (66 ml), methanol (33 ml), distilled water (4 ml) and concentrated hydrochloric acid (1 ml) (Bayliss High, 1990).

6.2.4. Fluorescent probes for lipid.

Cryostat sections of ovaries were treated with a range of fluorescent lipid markers including acridine orange, merocyanine MC540, neutral red and nile red. The sections were incubated for 30 to 60 minutes with 10 µl stain ml^{-1} Leibovitz L-15 culture medium in tissue culture chambers (Lab-Tek, Miles Scientific). The slides were then washed thoroughly with medium to remove any excess stain and examined under a Leitz LaborLux S fluorescent microscope.

6.2.5. Autoradiography.

6.2.5a. Isotope administration.

Adult females were injected with 150 µCi or 300 µCi (2 - 4 µCi/g body weight) of ^{3}H-Leucine (specific activity > 110 Ci/mmol; ICN Biomedicals, Inc.). This was given intraperitoneally in 0.25 ml of sterile tissue culture water with a 26G needle. After a period of 2 - 2½ hours the animals were killed and their ovaries removed for processing.

6.2.5b. Tissue processing.

Ovaries were fixed in a solution of acetic acid: ethanol (1:3) for 1 hour, followed by formal saline for 24 hours (Appleton, 1972). They were then hand processed according to the following schedule:

1. 70%, 90%, 100% ethanol - 30 minutes each.
2. Histoclear - 15 minutes.
3. Histoclear: wax (1:1) - 15 minutes.
4. Paraffin wax - 2 x 1 hour.

They were blocked in fresh wax and serial sections, cut at 5 µm in the usual manner, mounted on chrome alum-gelatin subbed slides.

Negative control ovaries were treated identically but processed and cut separately.

6.2.5c. Preparation of autoradiographs.

Prior to dipping, sections were thoroughly dewaxed in either fresh histoclear or xylene and hydrated. Dipping was conducted in a dark room with an Ilford 902 light brown safelight filter. LM-1 autoradiographic emulsion (Amersham International plc.) was melted in a 43°C water bath, poured into a plastic dipping vessel and any surface bubbles displaced. Slides were dipped vertically into the vessel for 5 seconds, withdrawn and excess emulsion drained by standing on a pad of tissues. They were then placed on a chilled metal plate and left to gel for 10 minutes and when completely dry were sealed in a black light-tight box with a small tissue bag of activated silica gel. This was then placed in a refrigerator at 4°C for 5 - 35 days.

6.2.5d. Processing protocol.

Before processing, the slide box was removed from the refrigerator and allowed to equilibrate to room temperature for at least 2 hours. All processing was conducted in a dark room with a safelight as before. Slides were transferred into slide racks and placed in Ilford Phenisol developer (25% in deionized water) for 2 - 10 minutes. They were then placed in a stop solution (0.5% acetic acid in deionized water) for 1 minute followed by fixer (30% sodium thiosulphate in deionized water) for 8 minutes. Slides were then washed in gently running tap water (same temperature as processing chemicals) for 15 minutes followed by two 15 minute washes in distilled water.

Slides were immediately post-stained with Harris' haematoxylin and eosin, Giemsa or methyl green pyronin Y(Appendix A.1.7, 1.8. & 1.9).

6.3. RESULTS

6.3.1. Histochemical analyses.

Positive staining for protein was visible within oocytes of all stages (Figs.6.1 & 6.2). Staining was detected in the germinal vesicle but the cytoplasmic vesicles, thought to represent yolk, remained unstained. In type 2 follicles the oocyte was almost completely stained and this was also the case for type 3a and 3b follicles apart from a low number of small, unstained vesicles. In type 4 follicles, the oocyte had a more mottled appearance due to the formation of unstained vesicles scattered throughout the cytoplasm. In type 5a, 5b, 6 and 7 follicles the oocytes showed increasing vesicle accumulation and

there was progressively less staining for protein until it was detected only in the most peripheral region. The zona pellucida and all of the other ovarian cells also showed positive staining for proteins.

Carbohydrate staining in the oocytes showed an identical pattern to that described for proteins, with the vesicles remaining negative, except that the germinal vesicles remained negative. However, unlike the previous pattern of labelling there was only slight staining in the rest of the ovarian tissue.

Staining for lipid, with all of the lipid stains, proved completely negative within all sizes of oocyte (Fig.6.3). With the Sudan black stains there was strong, positive staining in the interstitial tissue indicating the presence of unsaturated cholesterol esters and triglycerides. Slight staining in the granulosa cells of antral follicles (Fig.6.4) suggested that some phospholipids were present. Staining with Oil Red O indicated the presence of unsaturated hydrophobic lipids in the granulosa cells of antral and atretic follicles.

All of the histochemical tests were repeated and in each case the same results were obtained.

6.3.2. Fluorescent lipid probes.

Preliminary results indicated that out of the various probes tested, Nile red and neutral red produced the most vibrant staining. Although these markers were taken up into most regions of the ovary, so that there was a background level of fluorescence throughout the tissue, the strongest staining was in the granulosa cells and theca cells. The unfixed oocytes did not section particularly well, and after staining often appeared incomplete, but from the material that was visible it appeared that the fluorescent markers had been incorporated into the oocytes (Fig.6.6).

6.3.3. Autoradiography.

The first batch of autoradiographs showed an excessive amount of background staining on both the experimental and negative control slides to the extent that they were almost completely black. It was initially thought that this was caused by over development but decreasing the development time to as little as 20 seconds did not rectify the problem. After testing for differences between various types of glass slides treated with different cleaning agents and subbing solutions the cause was found to be the histoclear used to dewax the sections prior to applying the autoradiographic emulsion. When the

histoclear was replaced with xylene the background staining was minimal even after several minutes of development.

Ovaries from females injected with 150 μCi of ^{3}H-Leucine (2 μCi/g body weight) and left for 2 hours showed very little incorporation and the grain density was only slightly higher than background levels. Doubling the dose of ^{3}H-Leucine produced an obvious increase in the amount of incorporation detectable in the ovary. The outer regions of the ovary showed a higher concentration of grains than the central areas and overall the heaviest labelling was visible in the granulosa cells and zona pellucida (Fig.6.5). There did not appear to be any connection between the size of the follicles and either the amount or distribution of labelling within them. All stages of oocyte showed light labelling which appeared to be evenly distributed throughout the cytoplasm.

Fig. 6.1. Section showing positive staining for protein in all areas other than oocyte vesicles (x120)

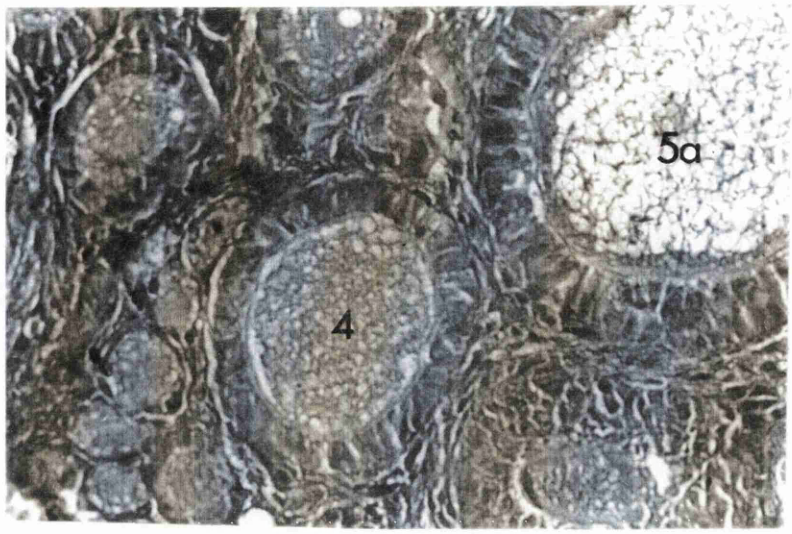


Fig. 6.2. Section showing positive staining for protein in all areas apart from oocyte vesicles (x75)

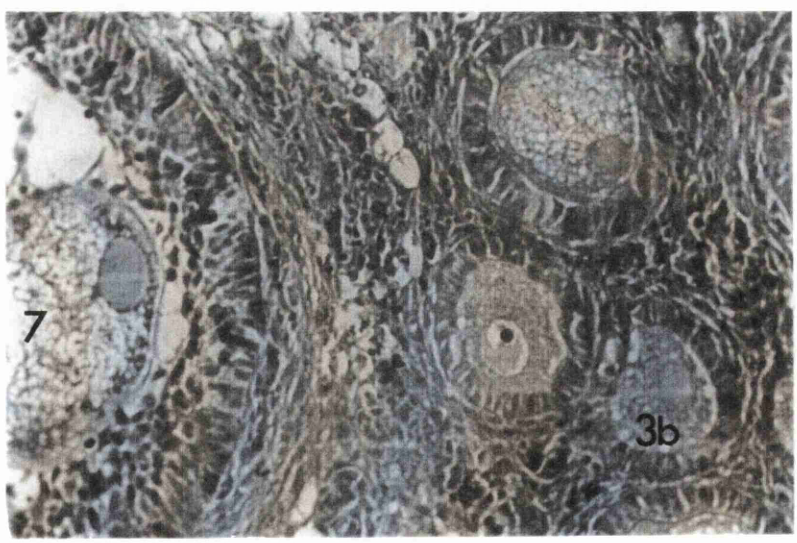


Fig. 6.3. Cryostat section stained for lipid showing no positive staining in oocytes. (x30)

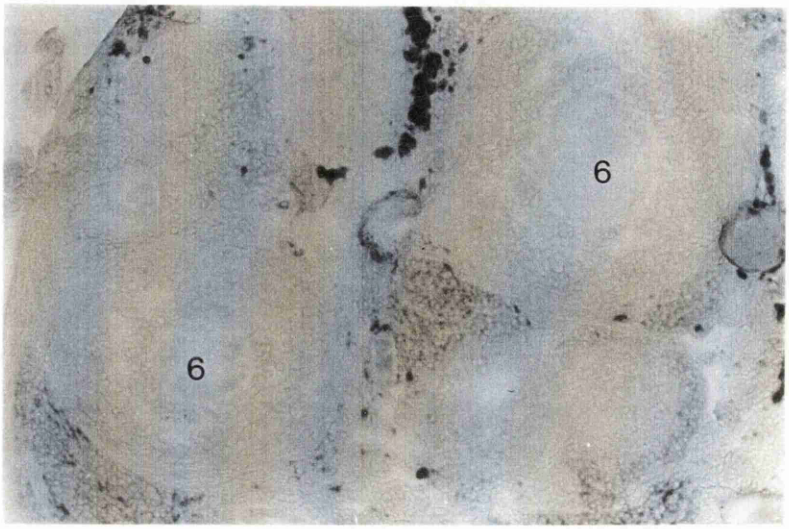


Fig. 6.4. Cryostat section stained for lipid showing positive staining in interstitial tissue but not in oocyte (x75)

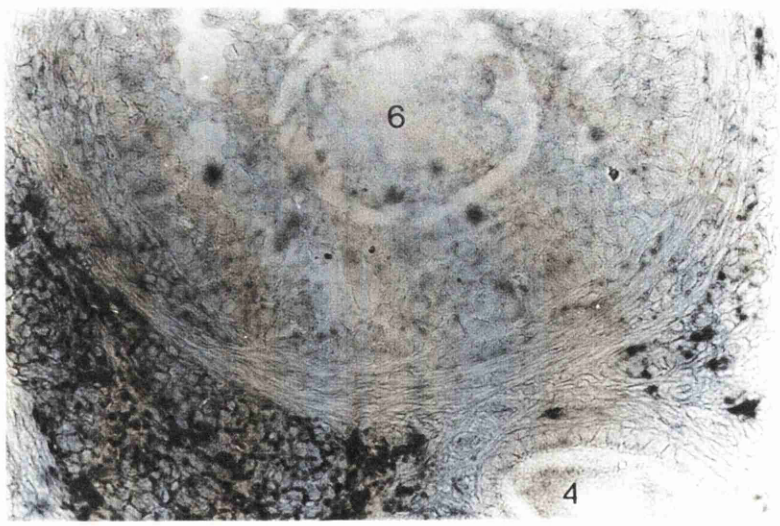


Fig. 6.5. Section of autoradiograph with strongest labelling in the granulosa cells and light, evenly distributed label within oocytes (x75)

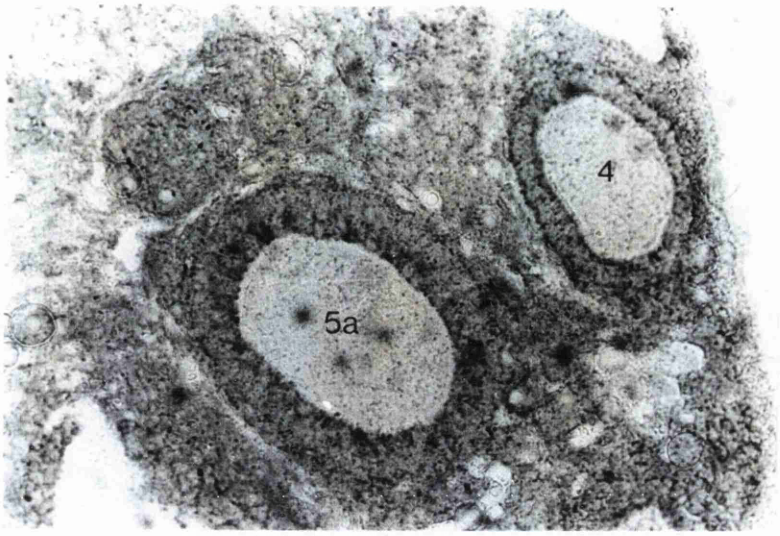
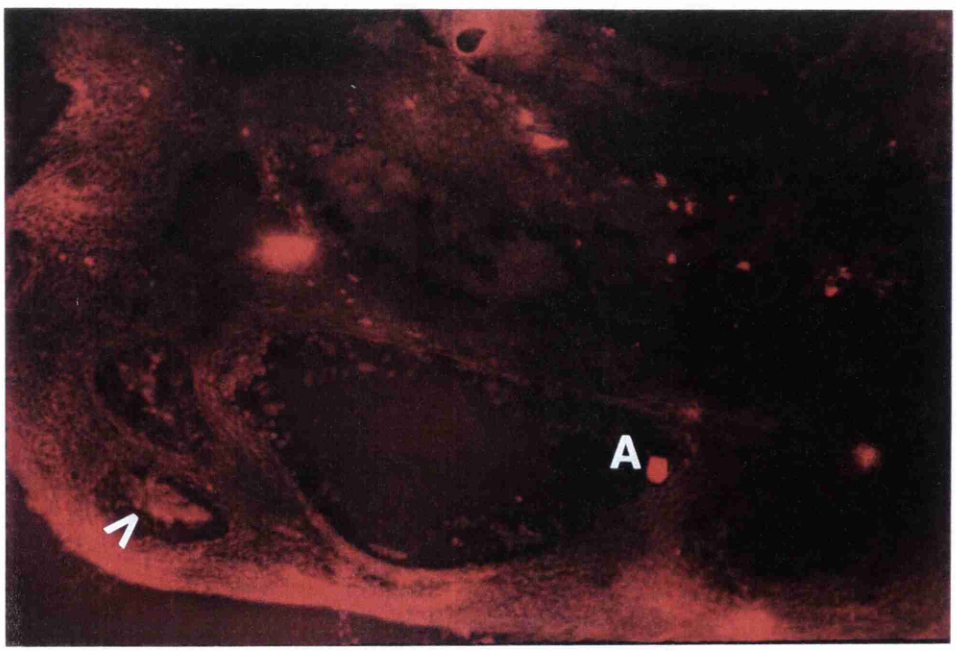


Fig. 6.6. Section of ovary treated with Nile Red showing staining in oocyte (arrowed). The oocyte of the large antral follicle (A) is not yet in view, but there is the suggestion of staining in this area (x40)



6.4. DISCUSSION

6.4.1. Histochemical analysis.

Histochemical staining failed to shed any light on the chemical nature of the conspicuous vesicles which develop in the *M. domestica* oocyte as they did not react with carbohydrate, protein or lipid stains. The oocyte cytoplasm did contain carbohydrate and protein but there was no evidence for the presence of lipid although, from the ultrastructural examination it appeared that the vesicles may have contained lipid which had been extracted during processing.

The negative reaction with all of the lipid stains was therefore surprising and the failure to identify their contents was even more perplexing. In his histochemical study of *Didelphis virginiana*, Guraya (1964) identified compound yolk and lipid yolk which arose in the central region of the oocytes. The droplets of lipid yolk stained homogeneously with various stains, including Sudan black, and indicated that they were phospholipid in nature. From his results the author believed that these Sudanophilic lipid droplets corresponded to the abundant vesicles previously described in various ultrastructural studies.

However, it is interesting to note that oocytes obtained from certain cats (Guraya, 1965), looked very similar to those of *M. domestica*. During pregnancy the central region of the cat oocyte was occupied by Sudanophilic vesicles but when cats were in oestrus these lipid bodies became Sudanophobic. This presumably indicated a biochemical change within the oocytes that was related to the reproductive state of the animal but the significance of this alteration remained undetermined. Thus there are obviously striking variations both between and within species which can be affected by the physiological conditionof the individual female.

In the earlier stages of oocyte development in the dog, Tesoriero (1981a) detected lipid vesicles which were osmiophilic but later on they became more transparent until they appeared similar to those of *M. domestica*. However, unlike that seen in *M. domestica*, treatment of these oocytes with Oil Red O revealed that they contained large amounts of neutral lipid yolk bodies (Tesoriero, 1981a). All developing dog oocytes contained glycoprotein material in the form of small, round to oval vesicles and these appeared to represent progressive stages in the formation of the lipid yolk bodies - as they enlarged they became less reactive until only the periphery was stained (Tesoriero, 1982). This decrease

in the staining activity appears to follow the same pattern described for the compound yolk vesicles in *D. virginiana* (Guraya, 1964).

Results of the histochemical staining in the other regions of the ovary were generally similar to those obtained in various other species. The *M. domestica* zona pellucida appeared to be composed of glycoprotein which has also been observed in the dog, mouse, rabbit (Tesoriero, 1984), *D. virginiana* (Guraya, 1968) and *T. vulpecula* (Hughes & Shorey, 1973). Similar to the studies by Guraya (1964, 1965, 1968) on various species including *D. virginiana*, some phospholipid was detected in the interstitial tissue and in the granulosa cells of antral follicles. In contrast to Guraya & Greenwald (1965) and Guraya(1968) there was no lipid identified in the granulosa cells of preantral follicles or in any of the theca cells in *M. domestica*.

6.4.2. Fluorescent probes.

It was only with the use of molecular probes that the presence of lipid within the oocytes was indicated. From this it would appear that the lipid vesicles were either resistant to the histochemical stains, consisted of a very "watery" lipid or were so soluble that they dissolved and could not be detected. Other workers studying the oocytes of the Australian species *Sminthopsis crassicaudata* have also failed to show the presence of lipid by means of histochemical analysis (W. G. Breed pers. comm.). The strongest staining was achieved with Nile red and previous groups have reported it to be an excellent vital stain for the detection of intracellular lipid droplets by fluorescence microscopy (Greenspan *et al.*, 1985).

Lipid analysis by means of lipid extraction with gas liquid chromatography or thin layer chromatography was considered but such a study would have required a very large quantity of oocytes. This may be practical when working with species where numerous ovaries are readily available, either from an abbatoir or routine veterinary surgery, but with our small colony of animals it was not a feasible option.

6.4.3. Autoradiography.

Despite initial methodological problems, autoradiographs showed that a metabolite such as leucine is transported from the circulation into the oocytes. Labelling appeared to be fairly uniform in

all stages of oocyte which is unlike the pattern seen in the mouse, where amino acid transfer has been described as stage dependent (Glass & Cons, 1968). However, Glass & Cons (1968) also reported an unusual fluctuation in labelling which was puzzling. In type 2 follicles the incorporation of radioactive human serum albumin into the oocyte was higher than that in oocytes from 3a follicles which in turn was higher than that in 3b follicles. For some reason it was only from this stage onwards that the concentration of label was related to the rate of oocyte growth.

6.4.4. Yolk formation.

As the *M. domestica* oocyte had a similar appearance to that of certain eutherian species, such as the dog (Guraya, 1965; Tesoriero, 1981) cow (Guraya, 1964) and pig it is posible that there is some underlying factor that these animals have in common.

One thing that marsupials share with these "yolky-egged" eutherian species is a relatively long period between fertilization and implantation. It has been reported that implantation occurs on day 10 of the 13 day gestation period in *D. virginiana* and on day 12 of the 15 day gestation period in *M. domestica* (Harder *et al.*, 1993). In the dog it is approximately 14 days before implantation occurs (Renton *et al.*, 1991) and in the larger ungulate species this time period varies from 2 - 3 weeks in the pig and sheep, nearly 3 weeks in the cow and up to 4 weeks in the horse (Catchpole, 1991). It therefore seems reasonable that such embryos would require substantial yolk reserves to support them during this length of time.

Chapter 7

General Discussion

7.1. General conclusions.

As individual chapters have included discussion on the results obtained, the purpose of this chapter is to bring together the major findings of this investigation and, where necessary, make recommendations for future research.

7.2. Oestrous and ovarian cycles.

Urogenital sinus smears were often difficult to decipher and this was probably due to the anatomy of the female marsupial whereby cells from the urinary tract and higher regions of the reproductive tract can contaminate smears. Despite this, the lengths for the duration of the oestrous cycle and oestrus period obtained in this study were in agreement with other groups which had managed to use urogenital sinus smears successfully. However, unlike previous observations, examination of unpaired females and females housed alone indicated that both groups of animals continued to exhibit oestrous cycles. All females appeared to exhibit repetitive cycles whereby the reproductive tract underwent preovulatory swelling and large antral follicles were formed in the ovaries. If the female was stimulated by a male, these large follicles completed their development and were ovulated - without male stimulation these follicles became atretic and were replaced by the next wave of follicles.

7.3. Ovarian morphology and follicle growth.

Morphologically the adult *M. domestica* ovary was found to be similar to that of the eutherian mammal and follicles in various stages of development, corpora lutea and interstitial tissue were observed. Folliculogenesis proceeded in an identical manner but contrary to eutherians, and most of the marsupial species studied, oocyte growth did not follow a strict biphasic pattern. The diameters reached by oocytes and follicles at the time of antrum formation were also considerably larger than those previously reported for this species. Unlike the eutherian situation, where the oocyte is invested by a full cumulus oophorus at ovulation, in *M. domestica* the cumulus cells had started to disintegrate and fall away from the oocyte by the preovulatory stage. As in some eutherian and marsupial species the corpus luteum consisted of granulosa cells, which had become luteinized, and theca interna cells which acted as a connective tissue skeleton and carried blood vessels into the gland. Two types of interstitial

tissue were identified and these were also similar in appearance to those reported for various mammalian species.

7.4. Oocyte ultrastructure and yolk formation.

Many of the maturational changes that occurred within the *M. domestica* oocyte resembled those seen in other mammalian species. Growth and development of the oocyte was associated with an increase in the number of organelles and a change in their distribution which reflected the high degree of metabolic activity associated with such extensive growth.

The most unusual finding was the presence of elongated mitochondria which appeared to have microfilaments running longitudinally along their outer membrane. The origins and functional sinificance of these mitochondria remains unclear and therefore warrants further investigation. As well as continuing with conventional electron microscopy, freeze-fracture analysis may provide some information about these extraordinary organelles.

The smooth endoplasmic reticulum and Golgi bodies appeared to be responsible for the formation of multivesicular bodies which then incorporated small vesicles which had pinocytotically entered the oocyte. As the multivesicular bodies increased in size, they became more flocculent in nature until they took on an empty appearance. They then coalesced with each other to form a large, vesicular mass which occupied most of the oocyte and was identical to the "yolk" mass previously described in other marsupial species.

Histochemical analyses failed to identify the nature of these vesicles and it was only from preliminary results with fluorescent molecular probes that the presence of lipid was indicated. From this, and the ultrastructural evidence available, it does seem highly probable that these vesicles contain lipid but further analysis should be conducted to try and obtain irrefutable evidence. Following treatment with the probes, the unfixed oocytes frequently disintegrated so that the fluorescence was only distributed sparsely in the remaining tissue. Repeating the fluorescent staining on thicker cryostat sections, so that the tissue is less fragile, may produce more conclusive results.

Autoradiography revealed that the amino acid leucine was transported from the circulation into the oocytes and, unlike the mouse, its uptake was fairly uniform in all developmental stages of oocyte.

Unfortunately, there was insufficient time to follow the pathway of such precursor uptake at the electron microscope level, so it would be of interest to look at this in future research.

7.5. Follicle culture.

Although the culture system was able to support follicle growth for up to 8 days, further modifications appear to be necessary for it to become suitable for antral development in this species. Until the incubator malfunctioned, the culture run conducted at body temperature with opossum serum and the highest concentration of FSH (1.5 IU FSH ml^{-1}) appeared to produce the largest amount of growth. In order to confirm this it would therefore be essential to repeat this experiment and also try supplementing the medium with higher doses of FSH as the optimum concentration may not have been found.

Plans are currently underway to continue and improve upon this follicle culture work with preantral *M. domestica* follicles as well as with various eutherian species. Although certain common principles may apply across species, success with such material will probably depend not only on developing the correct culture conditions but also on the follicle dynamics peculiar to the species. The culture of individual, preantral follicles through antral growth is a new achievment and, although it is still in its infancy, there is much optimism for its future. By adapting the culture system, for use with as many species as possible, it is anticipated that it will play an important role in the conservation breeding of the many eutherian and marsupial species now threatened with extinction.

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Appendix 1

Histological and cytological stains

A.1.1. Haematoxylin and Eosin.

Solutions:

Haematoxylin - 2.5 g Harris's haematoxylin in 25 ml absolute ethanol and 50 g aluminium potassium sulphate in 500 ml warm distilled water.Mix the two solutions and boil for 2 minutes. Add 1.25 g mercuric oxide and cool rapidly in cold water. When cold add 20 ml glacial acetic acid.

Putt's eosin - 10 g Eosin Y, 5 g potassium dichromate, 100 ml absolute ethanol, 100 ml saturated aqueous picric acid and 800 ml distilled water. Dilute 1:3 with water.

Scott's tap water substitute - 3.5 g sodium bicarbonate and 20 g magnesium sulphate in 1000 ml distilled water. Add a few crystals of thymol.

Method:

1. Histoclear	- 10 minutes.
2. Descending ethanols (100%, 90%, 70%, 50%, 30%)	- 3 minutes in each.
3. Distilled water	- 3 minutes.
4. Harris's haematoxylin	- 1 minute.
5. Rinse in tap water	
6. Scott's tap water substitute until blue.	
7. Rinse in tap water.	
8. Differentiate, if necessary, in acid alcohol.	
9. Putt's eosin	- 2 minutes.
10. Rinse in tap water.	
11. Ascending ethanols (30%, 50%, 70%, 90%, 100%)	- 30 seconds each.
12. Histoclear	- 5 minutes.
13. Mount in DPX.	

Results: Nuclei stain blue/purple. Cytoplasm stains pink.

A.1.2. Mallory's Triple Stain.

Solutions:

Saturated mercuric chloride in 5% glacial acetic acid.

1% aqueous acid fuchsin.

1% phosphomolybdic acid.

Mallory's stain - 2.5 g aniline blue (w.s.), 10 g orange G, 10 g oxalic acid, 500 ml distilled water.

Method:

1. Histoclear	- 10 minutes.
2. Descending ethanols (100%, 90%, 70%, 50%, 30%)	- 3 minutes in each.
3. Distilled water	- 3 minutes.
4. Saturated HgCl	- 10 minutes.
5. Rinse in distilled water.	
6. 1% acid fuchsin	- 4 minutes.
7. Differentiate in distilled water.	
8. Fresh 1% phosphomolybdic acid	- 1 minute.
9. Rinse in distilled water.	
10. Mallory's stain	- 75 seconds.
11. Distilled water	- 10 seconds.
12. 90% ethanol	- 10 seconds.
13. Absolute ethanol	- 2 x 10 seconds.
14. Histoclear	- 5 minutes.
15. Mount in DPX.	

Results: Nuclei stain red; muscle and various cytoplasmic constituents stain red to orange and connective tissue stains blue.

A.1.3. Toluidine Blue.

Sodium tetraborate (borax)	1 g.
Toluidine blue	1 g.
Distilled water	100 ml.

Dissolve borax in water, stir in toluidine blue and then filter.

Method:

1. Stain 1μm resin sections on 80°C hotplate for 5 to 10 seconds.
2. Rinse with water and dry on hotplate.
3. Mount in DPX.

Results:

Various shades of blue.

A.1.4. Paragon.

Borax buffer -	Boric acid	6 g.
	Sodium tetraborate	2 g.
	Distilled water	500 ml.
Paragon stain -	Basic fuchsin	0.625 g.
	Toluidine blue	1.875 g.
	30% ethanol	250 ml.

Method:

1. Cover sections with borax buffer and add 2 to 3 drops of Paragon stain.
2. Leave on 80°C hotplate for 5 to 10 seconds.
3. Rinse with water and dry on hotplate.
4. Mount in DPX.

Results:

Nuclei stain blue/purple. Cytoplasm stains pink.

A.1.5. Papanicolaou technique.

Stain preparation:

OG6 - Orange G stock solution (0.5% in 95% ethanol)	100 ml.
Phosphotungstic acid	0.015 g.
EA50 -Light green SF (0.1% in 95% ethanol)	45 ml.
Bismark brown (0.5% in ethanol)	10 ml.
Eosin yellowish (0.5% in 95% ethanol)	45 ml.
Phosphotungstic acid	0.2 g.
Lithium carbonate (saturated aqueous solution)	1 drop.

Method:

1. Rinse in 95% ethanol.
2. Rinse in 70% ethanol.
3. Rinse in distilled water.
4. Stain in Harris's haematoxylin for 5 - 30 seconds.
5. Rinse in distilled water.
6. Rinse in 95% ethanol.
7. Treat with 0.1% ammonia in 95% ethanol for 1 minute.
8. Rinse in 95% ethanol.
9. Stain in OG6 for 90 seconds.
10. Rinse in 95% ethanol twice.
11. Stain in EA50 for 90 seconds.
12. Rinse in 95% ethanol twice.
13. Rinse in absolute ethanol twice.
14. Rinse in histoclear twice and mount in DPX.

Results: Nuclei stain blue, superficial cell cytoplasm stains pink. Intermediate and parabasal cell cytoplasm stains blue/green.

A.1.6. Haematoxylin and eosin. (For cytology)

Method:

1. Rinse in 95% ethanol.
2. Rinse in 70% ethanol.
3. Rinse in distilled water.
4. Harris's haematoxylin for 30 seconds.
5. Rinse in tap water.
6. Stain in Putt's eosin for 40 seconds.
7. Rinse in tap water.
8. Rinse in 95% ethanol twice.
9. Rinse in absolute ethanol twice.
10. Rinse in histoclear twice and mount in DPX.

Results:

Nuclei stain blue/purple. Cytoplasm stains pink.

A.1.7. Haematoxylin and eosin. (For autoradiography)

1. Harris's haematoxylin - 30 minutes.
2. Differentiate in 0.2% HCl - 2 minutes.
3. Rinse in running water - 1 hour.
4. 0.5% Putt's eosin - 3 minutes.
5. Differentiate in cold, boiled, distilled water.
6. Allow to air dry.
7. Mount in histoclear.

A.1.8. Giemsa. (For autoradiography)

Method:

1. 5% Giemsa in 0.1M phosphate buffer - 10 - 15 minutes.
2. Displace stain with running tap water - 10 - 15 minutes.
3. Wash in diluent buffer.
4. 70% and 90% ethanol - 2 minutes in each.
5. Absolute ethanol - 2 x 2 minutes.
6. Histoclear - 2 x 2 minutes.
7. Mount in DPX.

Results:

Nuclei stain blue to purple; cytoplasm stains lighter blue.

A.1.9. Methyl green pyronin Y. (For autoradiography)

Method:

1. Methyl green pyronin Y solution - 5 minutes.
2. Rinse in deionized water.
3. 70% ethanol - 1 minute.
4. 90% ethanol - 2 minutes.
5. Absolute ethanol - 2 x 2 minutes.
6. Histoclear - 2 x 2 minutes.
7. Mount in DPX.

Results:

Cytoplasm stains pink and most nucleoli red; nuclei stain green.

Appendix 2

Histochemical stains

A.2.1. Periodic acid-Schiff (PAS)

Fixation & sections:

Bouin's solution or 10% formalin fixed paraffin wax sections.

Negative controls obtained by extraction of carbohydrate using salivary amylase.

Positive controls conducted on liver sections.

Solutions:

a. 0.5% aqueous Periodic acid solution.

b. Schiff's reagent.

Method:

1. Histoclear	- 10 minutes.
2. Descending ethanols (100%, 90%, 70%, 50%, 30%)	- 3 minutes in each.
3. Distilled water	- 3 minutes.
4. Treat with periodic acid	- 5 minutes.
5. Wash well with several changes of distilled water.	
6. Schiff's solution	- 15 minutes.
7. Wash in running tap water	- 5 to 10 minutes.
8. Counterstain with haematoxylin.	
9. Differentiate, if necessary, in acid alcohol.	
10. Blue in Scott's tap water substitute.	
11. Wash in water.	
12. Rinse in absolute alcohol.	
13. Clear in histoclear and mount in DPX.	

Results: Glycogen and other periodate reactive carbohydrates stain magenta.

A.2.2. Ninhydrin-Schiff

Fixation:

Neutral formal saline; formaldehyde vapour (for freeze-dried).

Sections:

Paraffin, cryostat or freeze-dried.

Solutions:

a. 0.5% ninhydrin in absolute ethanol.

b. Schiff's reagent.

Method:

1.	Histoclear	- 10 minutes.
2.	Descending ethanol (100%, 90%, 70%)	- 3 minutes each.
3.	Ninhydrin solution	- 18 hours at 37^{o}C.
4.	Wash in running tap water.	
5.	Schiff's reagent	- 45 minutes.
6.	Wash in running tap water.	
7.	Counterstain with an alum haematoxylin	± 30 seconds.
8.	Wash in tap water.	
9.	Ascending ethanol (30%, 50%, 70%, 90%, 100%)	- 30 seconds each.
10.	Clear in histoclear	- 5 minutes.
11.	Mount in DPX.	

Results: Amino groups - pinkish purple.

A.2.3. Mercury Bromophenol Blue

Fixation and sections :

Formalin -fixed wax sections.

Solution:

1% mercuric chloride and bromophenol blue in 2 % acetic acid.

Method:

1. Histoclear _ 10 minutes.
2. Descending ethanols (100%, 90%, 70%, 50%, 30%) - 3 minutes each.
3. Water - 3 minutes.
4. Stain for 2 hours at room temperature.
5. Rinse for 5 minutes in 0.5% acetic acid.
6. Butyl alcohol until blue dye is formed.
7. Clear in histoclear. - 5 minutes.
8. Mount in DPX.

Results: Proteins are stained deep clear blue.

A.2.4. Standard Sudan Black

Fixation and sections:

Cryostat sections

Method:

1. Rinse in 70% ethanol.
2. Saturated Sudan Black B in 70% ethanol, filtered just before use, for 15 minutes.
3. 70% ethanol until a delipidised control section appears colourless
4. Counterstain with 2% carmalum for 10 minutes.
5. Wash well and mount in glycerine jelly.

Results:

Standard Sudan Black stains unsaturated cholesterol esters and triglycerides blue-black; some phospholipids appear grey.

A.2.5. Bromine Sudan Black

Fixation and sections:

Cryostat sections post-fixed for one hour in formal calcium.

Method:

1. 2.5% aqueous bromine for 30 minutes.
2. Wash in water.
3. 0.5% sodium metabisulphite for one minute.
4. Wash thoroughly in distilled water.
5. Treat, together with an unbrominated section, with Standard Sudan Black.

Results:

Bromination enhances the reaction and also stains free fatty acids and free cholesterol.

A.2.6. Oil Red O

Fixation & Sections:

Cryostat sections post-fixed in formal calcium.

Preparation of stain:

Working solution prepared 1 hour in advance by mixing three parts of a stock solution of Oil Red O (saturated in 99% isopropanol) with two parts of 1% dextrin (to eliminate dye precipitation) and filtered just before use.

Method:

1. Rinse in 60% isopropanol.
2. Oil Red O for 15 minutes.
3. 60% isopropanol until a delipidised control section appears colourless.
4. Wash in water.
5. Counterstain nuclei with Mayer's haemalum for 3 minutes.
6. Wash well in tap water.
7. Rinse in distilled water and mount in glycerine jelly.

Results:

Unsaturated hydrophobic lipids, insoluble in dye bath and mineral oils, are stained red. Some phospholipids appear pink.

A.2.7. Ferric Haematoxylin

Fixation & sections:

Ideally unfixed cryostat sections, otherwise short fixed frozen sections.

Working Solution:

Mix three parts of solution a with one part of solution b and use within the hour.

Solution a.	Distilled water	298 ml.
	Conc. HCl	2 ml.
	$FeCl_3.6H_2O$	2.5 g.
	$FeSO_4.7H_2O$	4.5 g.
Solution b.	Distilled water	100 ml.
	Haematoxylin	1 g.
	This solution must be fresh.	

Method:

1. Treat duplicate sections (a & b) as follows:
   a. Extract in chloroform : methanol (2:1) for one hour.
   b. Extract with acetone for 15 minutes at $4^{o}C$.
2. Fix both sections in formal calcium for 30 minutes.
3. Rinse in distilled water.
4. Ferric haematoxylin for 7 minutes.
5. Wash in distilled water.
6. Dip several times in 0.2% HCl.
7. Wash in tap water.
8. Dehydrate in acetone, clear in histoclear and mount in DPX.

Results: Phospholipids stain blue.