BREEDING, GONADAL DEVELOPMENT AND STEROIDOGENESIS IN THE OVARY OF THE GREY SHORT-TAILED OPOSSUM, *Monodelphis domestica.*

Thesis submitted for the degree of

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DECLARATION.

I hereby declare that this thesis is my own composition and that the experimental work was performed by me alone.

None of the material in this thesis has been submitted for any other degree.

Philip Maitland.

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CONTENTS.

Subject	Page No.
Abstract	1
Chapter 1: General Introduction.	3
1.1: Steroidogenic Pathways in the Ovary.	4
1.2: Steroidogenic Cells of the Ovary.	4
1.3: Histological and Ultrastructural Characteristics of Steroidogenic Cells.	7
1.4: Origins of Eutherian Steroidogenic Cells.	
1.4.1: Interstitial Cells.	8
1.4.2: Granulosa Cells.	9
1.4.3: Theca Interna.	10
1.4.4: Corpora Lutea.	10
1.5: Origins of the Eutherian *Rete Ovarii.*	11
1.6: Marsupial studies.	12
1.7: Origins of Marsupial Steroidogenic Cells.	
1.7.1: Interstitial Cells.	13
1.7.2: Granulosa Cells.	13
1.8: Origins of the Marsupial *Rete Ovarii.*	14
1.9: Gonadal Sex Differentiation in Marsupials.	15
1.10: Meiosis in Mammals.	16
1.11: Description and History of *Monodelphis domestica.*	17
1.12: Aims.	19
Chapter 2: Materials and Methods.	20
2.1: Source of Animals.	21
2.2: Housing.	21
2.3: Diet.	21
2.4: Breeding.	22

2.5: Chromosome Analysis. 23

2.6: Histology.

2.6.1: Follicular Staging. 23

2.6.2: Paraffin Wax Histology. 24

2.6.3: Resin Histology. 25

2.7: Δ^5, 3β-Hydroxysteroid Dehydrogenase Staining.

2.7.1: Enzyme Histochemistry. 26

2.7.1.1: Method. 27

2.7.1.1: Results Obtained. 27

2.7.2: Immunocytochemistry. 27

2.7.2.1: Method. 28

2.7.2.2: Results Obtained. 29

Chapter 3: Behavioural and Statistical Analyses. 30

3.1: Introduction. 31

3.2: Breeding. 32

3.3: Litter Sizes and Sex Ratios. 34

3.4: Neonate Size and Growth. 34

3.5: Animal Sexing. 35

Figures from Chapter 3. 37

3.6: Discussion.

3.6.1: Breeding. 44

3.6.2: Litter Sizes and Sex Ratios. 46

3.6.3: Neonate Size and Growth. 47

3.6.4: Sexing of Young. 48

Chapter 4: Histological Study of Gonadal Development. 50

4.1: Introduction. 51

4.2: Prenatal Gonadal Development. 53

4.3: Testicular Development. 54

4.4: Ovarian Development.

4.4.1: General Development and Folliculogenesis. 55

4.4.2: Development of the *Rete Ovarii.* 56

Figures from Chapter 4. 58

4.6: Discussion.

4.6.1: Prenatal Gonadal Development. 70

4.6.2: Testicular Development. 70

4.6.3: Ovarian Development. 71

Chapter 5: Study of Δ^5, 3β-Hydroxysteroid Dehydrogenase. 73

5.1: Introduction. 74

5.2: Histochemical Staining for 3β-HSD.

5.2.1: Positive Controls. 77

5.2.2: Negative Controls. 77

5.2.3: Experimental Sections. 78

5.3: Immunocytochemical Staining for 3β-HSD. 78

5.3.1: Positive Controls. 79

5.3.2: Negative Controls. 79

5.3.3: Experimental Sections. 79

Figures from Chapter 5. 82

5.4: Discussion.

5.4.1: Comparison of Techniques. 93

5.4.2: Enzyme Histochemistry. 94

5.4.3: Immunocytochemistry. 95

General Conclusions. 97

References. 101

Appendix 1: Staining Procedures. 118

Appendix 2: Actual Data from *Monodelphis domestica.* 122

ABSTRACT.

The Grey, short-tailed opossum, *Monodelphis domestica*, is a relatively recent acquisition to the laboratory. Possessing the desirable characteristics of all marsupials, such as quite early sexual maturity, few chromosomes and birth at an early developmental stage, as well as a short *post partum* dependence period and being more easily laboratory bred and tractable compared to other Didelphids, *Monodelphis* makes a good laboratory animal for developmental studies.

This study takes advantage of the above facts, to add one more marsupial to the list of mammals in which physical development, gonadal development and the development of steroidogenesis in the ovary have been described.

It was found that by introducing the female opossum to the male for mating, instead of *vice versa*, her natural aggression was curbed which, in turn, increased the numbers of successful pairings by 27% and cut neonatal losses due to cannibalism by 58%.

The mating behaviour observed, average litter sizes attained of 7.5, the chronology of neonatal events and the calculation of a sex-ratio at weaning of 1 : 1 all confirmed previous studies by various authors.

Monodelphis pups' head-length increased in direct proportion to their age, whereas their weight increased exponentially. It was also found that pups obtained before attachment to the nipple weighed around 20 mg less than their siblings which had, presumably, been fed. Large weight variations lead to the suggestion that head-length would make a better ageing criterion.

The sexing of young by visual means proved impossible until day 3 (contrary to previous studies) and unreliable until day 8 when skin pigmentation, especially on the scrotum, is present. Gonads could be sexed at birth as the testis had already differentiated by then. Chromosomal analyses proved to be the best method of sexing pups, and confirmed the chromosome number of $2n = 18$.

Gonadal size sex differences were observed at birth. Testes, complete

with testicular cords and a *tunica albuginea*, were consistently larger than ovaries of the same age. The ovary differentiated between days 5 and 6, no germ cell cords were formed and meiosis commenced around day 14.

The ovarian blastema differentiated into stromal tissue and, between days 14 and 26, medullary cords. These cords, when they contacted oogonia, around day 29, provided the latter with their first follicle cells. Interstitial tissue was apparently formed from degenerated corpora lutea and possibly cells from atretic follicles and the stroma.

The *rete ovarii* developed from a condensation of cells in the hilar region on day 5 and infiltrated the gonad. The *rete* abutted onto the medullary cords after follicle formation had begun and connections to mesonephric structures were not observed.

Histochemical demonstration of the steroidogenic enzyme, 3β-HSD, produced similar results to those found in most mammals. Immunocytochemical staining for the same enzyme produced different results to those found in the human.

Combining the immunocytochemical and histological studies it was shown that *Monodelphis* ovaries are of the "immediate meiosis" type.

CHAPTER 1.

General Introduction.

1.1: Steroidogenic Pathways in the Ovary.

The ovary can synthesize steroid hormones from two sources; firstly directly from acetate, through mevalonate, squalene and cholesterol and secondly directly from blood-borne cholesterol. Pregnenolone, the immediate steroid precursor, is produced by carbon side-chain cleavage from cholesterol and can then be utilised in either the Δ^5 pathway (directly from pregnenolone) or via progesterone in the Δ^4 pathway. The Δ^4 route seems to be most commonly used (Brodie, 1983). The conversion from Δ^5 to Δ^4 steroids is, in all cases, mediated by the enzyme Δ^5, 3β-hydroxysteroid dehydrogenase (3β-HSD) (Figure 1). Therefore, the progestagens and androgens are directly dependent on the enzyme and oestrogens, being synthesized from androgens, are indirectly dependent on it. This means that if there is no 3β-HSD present, no steroids can be produced.

1.2: Steroidogenic Cells of the Ovary.

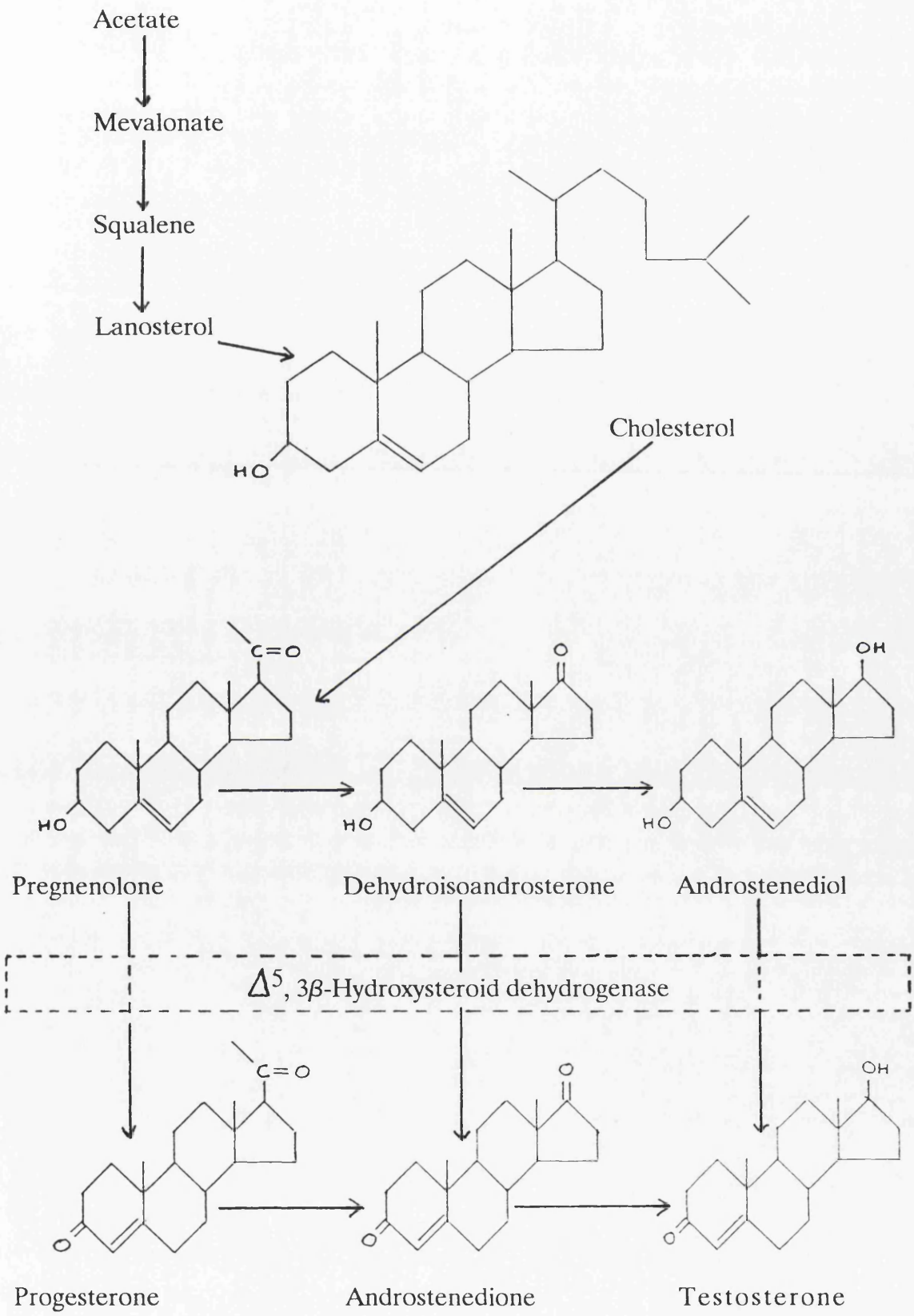
It is generally accepted that the steroid producing cells of the ovary are located in three structures, the stroma, the follicle and the corpus luteum (Baird, 1977; Peters & McNatty, 1980; Brodie, 1983). Peters & McNatty (1980) define the stroma as "all ovarian tissue lying between the follicles, corpora lutea and the vascular systems". Invariably, several of the cell types included in this definition of the stroma will not be steroidogenic so another term, the "interstitial tissue" or "interstitial cells", is used by several authors (Mossman & Duke, 1973; Harrison & Weir, 1977; Brook & Clarke, 1989) and in this study. This term is used to describe only the steroid synthesising cells in the ovary outwith the follicles and corpora lutea.

The pre-ovulatory follicles of the ovary, comprised of granulosa and theca cells, synthesise all three classes of steroids (Brodie, 1983). Progestagens are produced in both the theca and the granulosa cells. Some of the theca produced

Figure 1:

Diagram showing the steroidogenic pathways from acetate to progestagens and androgens. Δ^5, 3β-hydroxysteroid dehydrogenase is the essential final step to these Δ^4 steroids.

Figure 1.


Acetate
Mevalonate
Squalene
Lanosterol
HO
Cholesterol
C=O
HO
O
HO
OH
HO
Pregnenolone
Dehydroisoandrosterone
Androstenediol
Δ5, 3β-Hydroxysteroid dehydrogenase
C=O
O
O
O
OH
O
Progesterone
Androstenedione
Testosterone

progesterone is converted to the androgens testosterone and androstenedione, some of which is aromatized to oestrogens in the theca and some is passed into the follicular fluid or the granulosa cells. These androgens are then converted to oestrogens by the granulosa cells (Figure 2).

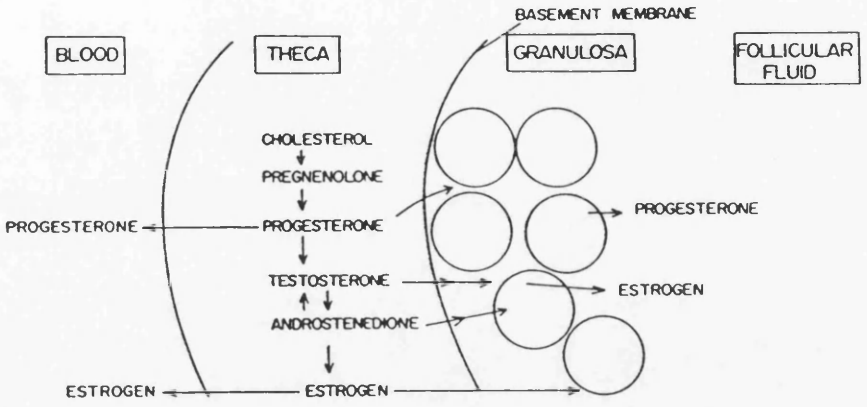


Figure 2:

Steroidogenic pathways within follicles. (From Brodie, 1983)

These steroidogenic pathways are, of course, dependent on the presence of a theca layer around the follicle which, in most mammals studied, does not occur until the late primary or early secondary follicle stage is reached and from then on oestrogen production by the follicle increases until just prior to ovulation.

Atretic follicles produce progressively less oestrogen and more androstenedione (Brodie, 1983), although Byskov *et al.* (1985) state that some of the atretic primary and early secondary follicles may luteinize. This means that progestagens will be produced, even though no corpora lutea are present in the ovary.

Corpora lutea in the majority of mammals produce mainly progestagens although it has been shown that in the human, several androgens and oestrogens are also produced (Peters & McNatty, 1980).

Steroid synthesis by the interstitial tissue of the ovary is difficult to

evaluate due to problems of isolating the interstitial tissue from the other ovarian constituents. From the studies so far undertaken, it appears that the interstitial tissue is mainly progestagen producing with some androgens also formed (Peters & McNatty, 1980).

The intracellular sites of steroid production in the ovary have been shown to be the smooth endoplasmic reticulum (Brodie, 1983; Nagai, 1985) and the cristae and inner membrane of the mitochondria (Nagai, 1985).

1.3: Histological and Ultrastructural Characteristics of Steroidogenic Cells.

Histological and ultrastructural examinations have been used to describe and locate steroidogenic cells in the ovary. Histologically, this usually entails staining tissue sections for the presence of lipid droplets containing cholesterol and its esters - one of the common cell markers of steroid biosynthesis (Guraya, 1964, 1968a, 1968b, 1972; Guraya & Greenwald, 1965; Mori & Matsumoto, 1970; Archibald *et al.*, 1971; Quattropani, 1973; Clegg & Clegg, 1975; Bjersing, 1977; Peters & McNatty, 1980; Troyer, 1980; Funkenstein & Nimrod, 1981; Johnson & Everitt, 1983; Brook & Clarke, 1989). This method is easy to use, but only for those cells which contain cholesterol. These cells are not necessarily steroidogenic although the basic "raw material" is present.

The observation of common ultrastructural characteristics, such as plentiful smooth endoplasmic reticulum, rounded mitochondria with tubular or vesicular cristae and abundant lipid droplets, makes the identification of steroid secreting cells relatively easy at the electron microscope level (Hart *et al.*, 1966; Mori & Matsumoto, 1970; Guraya, 1972; Quattropani, 1973; Pehleman & Lombard, 1978; Nagai, 1985; Yoshinaga *et al.*, 1988; Brook & Clarke, 1989).

1.4: Origins of Eutherian Steroidogenic Cells.

1.4.1: Interstitial Cells.

The interstitial cells of the ovary (sometimes referred to as the "interstitial gland" and also the "stroma") are either small groups or large aggregations of frequently very active steroid synthesising cells (Mossman & Duke, 1973; Harrison & Weir, 1977; Peters & McNatty, 1980; Brook & Clarke, 1989). These authors all agree that there are two origins of the interstitial cells, which are almost indistinguishable. One type is derived from the undifferentiated blastema of the ovary and the second arises through follicular atresia, especially from the theca interna. Many earlier authors did not realise this double origin, and stated only one of the two derivations. Allen (1904), Guraya & Greenwald (1965), Pupkin *et al.* (1966) and Guraya (1972) derived the interstitial cells from the atrophied theca interna, whilst Deanesly (1970) derived them from the undifferentiated blastema. Other reported sources of interstitial cells are the medullary cords (literally cellular cords in the medulla of the gonad), which according to Byskov (1978) are synonymous with the *rete ovarii*, and atretic primordial follicles (Guraya, 1968a).

Quattropani (1973), put forward a contrary view to those authors who derived the interstitial cells from the theca. He suggested that, due to the ultrastructural similarities, the theca was descended from the interstitial cells. This may be due to the confusing and often contradictory terminology used by many authors in this field of work. It is uncertain whether Quattropani (1973) used "interstitial cells" to describe the glandular "interstitial cells", as referred to in this study, or merely the undifferentiated blastema. If the latter is the case, then he is actually in agreement with the majority of authors on the origin of the interstitial cells. The amount of interstitial tissue varies widely between species, from only one or two noticeable cells in the mole (*Talpa europea*, Mossman & Duke, 1973) to the condition in the rabbit (*Oryctolagus cunniculus*, Mossman &

Duke, 1973) and the wood mouse (*Apodemus sylvaticus*, Brook & Clarke, 1989) where it almost fills the ovary.

The dictionary definition of "stroma" is that it is tissue composed of differentiated fibroblast cells but, as mentioned previously, confusion abounds as different authors apparently have their own definitions. The origin of the stroma itself is uncertain and much debated. Some of the stromal cells were derived from "regressed" interstitial cells and atrophied theca and granulosa cells (Allen, 1904; Guraya, 1968b; Quattropani, 1973; Peters & McNatty, 1980) but, as the stroma is evident early in ovarian development, this cannot be wholly correct. Johnson & Everitt (1983) and Pelliniemi (1975) respectively derived the stroma from the mesenchyme and the mesenchyme and the coelomic mesothelium. The most recent, and apparently most accepted view in most mammalian cases is that the stroma is derived from the *rete ovarii* (Fajer, 1981; Grinsted, 1981; Hoyer & Byskov, 1981; Wartenberg, 1981; Byskov, 1986).

1.4.2: Granulosa Cells.

The origin of the granulosa cells is still controversial (Deanesly, 1975; Byskov, 1975; Upadhyay *et al.*, 1979; Lintern-Moore *et al.*, 1981; Ullmann, 1981b). Several origins have been postulated as follows: the *rete ovarii* (Byskov & Lintern-Moore, 1973; Byskov, 1975, 1978, 1986; Byskov & Saxén, 1976; Byskov *et al.*, 1977; Stein & Anderson, 1979; Fajer, 1981); the ovarian mesothelium (Franchi & Mandl, 1962; Gondos, 1969; Motta & Makabe, 1982); the mesonephric tubules (Upadhyay *et al.*, 1979; Zamboni *et al.*, 1979); the ovarian somatic blastema (Odor & Blandau, 1969) which is equivalent to the "stroma" referred to by other authors (Peters & Pedersen, 1967; Peters, 1969; Deanesly, 1970). Byskov & Lintern-Moore (1973), Stein & Anderson (1979) and Fajer (1981) noted ultrastructural similarities between *rete* cells and granulosa cells, although these were not detailed, and that they appeared to be continuous, sharing a common

basement membrane. Byskov (1975), working on the cat, mink and ferret, derived the granulosa from both the *rete ovarii* and the ovarian mesothelium, as the follicles appeared to be in open connection with both structures. Peters & Pedersen (1967) injected neonatal female mice intraperitoneally with tritiated thymidine. One hour after injection it was found that some of the central "stromal" cells were labelled, along with strands of "stromal" cells reaching from the mesothelium to the centre of the gonad. Within three days of injection many of the "stromal" cells were less intensely labelled, which suggested dilution by cell divisions. A similar situation appeared to prevail in the occasional follicle cells attached to oocytes at the periphery of the cortex. Many granulosa cells in follicles of all sizes were labelled by day 7 after injection, suggesting that the "stroma" was the origin of granulosa cells.

1.4.3: Theca Interna Cells.

The theca interna cells of the follicle are unanimously agreed upon as having the undifferentiated blastema cells as their progenitors (Peters & Pedersen, 1967; Guraya, 1968b; Deanesly, 1970; Mossman & Duke, 1973; Quattropani, 1973; Harrison & Weir, 1977; Peters & McNatty, 1980; Baker, 1982; Johnson & Everitt, 1983). Peters & McNatty (1980) found cells which were ultrastructurally intermediate between the blastema and the theca interna.

1.4.4: Corpora Lutea.

There is no doubt at all that the corpora lutea present in an adult ovary are the remains of ovulated follicles, both the granulosa cells and the theca interna cells having luteinized. Luteal bodies may also, however, arise without ovulation occurring. The "accessory" corpus luteum consists of luteinized granulosa cells with an entrapped ovum (Peters & McNatty, 1980). This type of

structure, either with or without an ovum, can sometimes be found in pre-pubescent animals where atretic follicles sometimes luteinize (Byskov *et al.*, 1985).

1.5: Origins of the Eutherian *Rete Ovarii.*

The *rete ovarii* was first described by Waldeyer in 1870 as cords and tubules extending between the ovaries and the mesonephroi (Upadhyay *et al.*, 1979) and has several suggested roles throughout ovarian development. In eutherian mammals, two putative origins of the *rete* have been suggested: firstly from the coelomic mesothelium (Allen, 1904), and secondly from mesonephric cells (Byskov & Lintern-Moore, 1973; Stein & Anderson, 1979). The *rete* is described as a mesonephric derivative, which invades the ovarian hilum and then ramifies throughout the ovary (Byskov & Lintern-Moore, 1973; Byskov, 1975, 1978, 1986; Byskov & Saxén, 1976; Byskov *et al.*, 1977; O, 1978; Stein & Anderson, 1979; Fajer, 1981; Grinsted, 1981; Nikitin & Byskov, 1981; Wartenberg, 1981). A nomenclature for the constituent parts of the *rete ovarii* was introduced by Byskov & Lintern-Moore (1973), and is now widely accepted; this is: the intra-ovarian *rete*, which appears as cords and tubules ramifying through the ovary; the extra-ovarian *rete*, which comprises of convoluted tubules in the periovarian tissue; and the connecting *rete*, which joins both of the previously described parts.

Yoshinaga *et al.* (1988), studying the Galago (*Galago crassicaudatis crassicaudatis*), gave the mesonephros no role in gonadal formation, stating that connections between the gonadal *rete* cords and regressing glomeruli only occurred after oocytes had begun to aquire granulosa cells. Therefore the *rete ovarii*, which in the Galago appears not to be derived from the mesonephros, had no role in follicle formation, the granulosa cells for which were derived from the coelomic mesothelium.

The *rete ovarii* has also been described as necessary for the initiation of

meiosis (Byskov, 1975, 1978, 1981; Byskov & Saxén, 1976; Byskov *et al.*, 1977; O, 1978; Andersen *et al.*, 1981; Fajer *et al.*, 1979; Fajer, 1981; Grinsted, 1981; Nikitin & Byskov, 1981), with Byskov (1978), O (1978) and Andersen *et al.* (1981) suggesting that there is a "meiosis inducing substance" secreted by the *rete*.

1.6: Marsupial Studies.

All of the work referred to above was carried out on eutherian mammals and, until recently, comparatively little research had been carried out on marsupials. Marsupials are relatively undeveloped at birth, usually have short *post-partum* dependence periods and reach sexual maturity relatively early. Therefore, they make useful models for developmental studies (Burns, 1941; Alcorn, 1975; Catling & Vinson, 1976; Alcorn & Robinson, 1983; George *et al.*, 1985; Renfree *et al.*, 1987; Tyndale-Biscoe & Renfree, 1987; Dairi, 1988; Hutson *et al.*, 1988; Moore & Thurstan, 1988, 1990; Shaw *et al.*, 1988; Short *et al.*, 1988; Tyndale-Biscoe & Janssens,1988).

Gonadal differentiation has been studied on few of the 249 extant species of marsupials (*Bettongia gaimardi*: Dairi, 1988; *Dasyurus viverrinus*: Ullmann, 1984; *Didelphis virginiana*: Hartman, 1926; McCrady, 1938; Burns, 1941; Morgan, 1943; Renfree *et al.*, 1987; *Isoodon macrourus*, *Perameles nasuta*: Ullmann, 1978, 1981b, 1989; *Macropus eugenii*: Alcorn, 1975; Renfree *et al.*, 1987; Dairi, 1988; Hutson *et al.*, 1988; Shaw *et al.*, 1988; *Monodelphis domestica*: Fadem & Tesoriero, 1986; Moore & Thurstan, 1988, 1990; Baker *et al.*, 1990; *Potorous tridactylus*: Dairi, 1988). The study of the origins of the ovarian constituents has also been limited.

Steroidogenesis in marsupials has been studied on a limited number of species by only a few research groups (*Macropus eugenii*: Catling & Vinson, 1975; Hutson *et al.*, 1988; Shaw *et al.*, 1988; *Potorous tridactylus*: Dairi, 1988; *Didelphis virginiana*: Guraya, 1968b; George *et al.*, 1985; *Monodelphis domestica*: Fadem &

Tesoriero, 1986). Three techniques were used to study steroidogenesis: electron microscopy, histochemical staining and radiological assaying. Dairi (1988) used enzyme histochemistry, staining for 3β-HSD, in her study of *Potorous tridactylus* (neonates and day 86 pouch young) and *Macropus rufogriseus* (days 42 to 140) ovaries but did not detect any enzyme activity in these species. Guraya (1968b) used adult ovaries of *Didelphis virginiana* to examine histochemically which cells were steroidogenically active. The other steroidogenic studies were more concerned with the temporal origins of the steroids, using either embryonic or neonatal gonads.

1.7: Origins of Marsupial Steroidogenic Cells.

1.7.1: Interstitial Cells.

Only two studies appear to have considered the origins of marsupial interstitial cells. The interstitial tissue of the tammar ovary (*Macropus eugenii*) was derived from the medullary and hilar *rete ovarii* cords (Alcorn, 1975). He also states that these two cord structures are derived from the blastema. In the potoroo (*Potorous tridactylus*), Dairi (1988) derived the interstitial cells from the medullary cords which in this species also differentiated from the gonadal blastema. Therefore, in both marsupials studied, the interstitial tissue has, albeit indirectly, a blastemal origin.

1.7.2: Granulosa Cells.

Alcorn (1975), Ullmann (1981a) and Dairi (1988) derived the granulosa cells from the central somatic blastema of the ovary in the tammar (*Macropus eugenii*), the bandicoots (*Isoodon macrourus & Perameles nasuta*) and the potoroo (*Potorous tridactylus*) and bettong (*Bettongia gaimardi*) respectively. Apparently, no examinations of the origins of the theca interna cells have been undertaken in

marsupials.

These authors have also examined the origin of the marsupial ovarian blastema. In the tammar wallaby (Alcorn, 1975), the bandicoot (Ullmann, 1981a) and the native cat (Ullmann, 1984b), the blastema was derived from mesonephric mesenchymal cells, whereas Dairi (1988) gave a dual origin for that of the tammar, from both the mesothelium and the mesonephric mesenchymal cells.

1.8: Origins of the Marsupial *Rete Ovarii.*

The *rete ovarii* has been described in a variety of marsupial genera: *Bettongia* (Dairi, 1988), *Dasyurus* (Fraser, 1919; Ullmann, 1984), *Didelphis* (Fraser, 1919; Burns, 1941; Morgan, 1943), *Isoodon* (Fraser, 1919; Ullmann, 1981a), *Macropus* (Alcorn, 1975; Dairi, 1988), *Perameles* (Ullmann, 1981a), *Potorous* (Dairi, 1988) and *Trichosurus* (Fraser, 1919). Fraser (1919) described the *rete* as originating in an anterior continuation of the gonadal ridge and later becomes recognisable, as irregular cords and strands of cells, within the ovary. Mesothelial invaginations produced solid cell cords which contributed to the *rete* later in development. Morgan (1943) gave another account, in which the *rete* was derived solely from the mesothelium of the gonad, and extended into the ovarian hilus. Alcorn (1975), Ullmann (1981b) and Dairi (1988) described the *rete ovarii* as a condensation of mesenchymal cells which branch into the gonad. In the bandicoot, however, the *rete* does not penetrate far into the ovary, but makes contact with the medullary cords which branch throughout the gonad and may contact the follicles (Ullmann, 1981a, 1989).

Burns (1941) gave a lucid account of the origins of the *rete testis* in the Virginia opossum (*Didelphis virginiana*). The *rete* first appeared as cords or canals arising from the *rete* ridge mesothelium and later connected with the nephrostomial canals from the glomeruli in the vicinity of the *rete* ridge. These canals, or tubules, entered the gonad and appeared to end blindly. In the male,

these mesothelial canals closed soon after *rete testis* formation but remained open even in the adult female. Thus Burns (1941) described the *rete ovarii* as partly being derived from pre-existing mesonephric structures and partly from the mesothelial canals. The mesothelial *rete* invaginations and the funnel of the Mullerian duct (fimbria of the oviduct) are described by Burns (1941) as "morphologically members of a homologous series".

1.9: Gonadal Sex Differentiation in Marsupials.

It is often argued that birth, in mammals, takes place at a relatively arbitrary point during development. This is supported by the fact that in several marsupials gonadal differentiation occurs post-natally (*Dasyurus viverrinus*: Ullmann, 1984; *Isoodon macrourus*, *Perameles nasuta*: Ullmann, 1981a, 1981b, 1989; *Macropus eugenii*: Alcorn, 1975; Renfree *et al.*, 1987; Dairi, 1988) and in others at least male gonads differentiate *in utero*: *Bettongia gaimardi* (Dairi, 1988), *Didelphis virginiana* (McCrady, 1938; George *et al.*, 1985; Renfree *et al.*, 1987), *Monodelphis domestica* (Baker *et al.*, 1990) and *Potorous tridactylus* (Dairi, 1988). In all marsupials so far studied the ovary developmentally lags behind the testis by at least two days - similar to the standard developmental pattern of eutherians (Dairi,1988).

It should also be mentioned that in several cases actual sex differentiation takes place before that of the gonads: quite often a scrotum or pouch rudiment can be distinguished prior to obvious differentiation of the ovary or testis (*Macropus eugenii*: Alcorn, 1975; Renfree *et al.*, 1987; *Monodelphis domestica*: Fadem *et al.*, 1982; Moore & Thurstan, 1988).

1.10: Meiosis in Mammals.

Byskov (1979) introduced the idea that mammalian ovaries were of two distinct types depending on the timing of the onset of meiosis relative to their gonadal differentiation. These types were described as having either "immediate" or "delayed" meiosis.

Those which have delayed meiosis have their germ cells enclosed in "germ cell cords", sometimes resembling testicular cords (Byskov *et al.*, 1986). These "germ cell cords" are thought to perform similarly to the testicular cords in which the germ cells do not enter meiosis until sexual maturity. In these species (rabbit, cat, pig, sheep, cow), meiosis is therefore delayed in respect to the gonadal differentiation.

Immediate meiosis, on the other hand, takes place in the ovaries of those species in which there is no restriction of the germ cells by germ cell cords (mouse, rat, guinea pig, human), thus gonadal differentiation and the initiation of meiosis occur simultaneously.

Steroidogenesis in the two groups has been found to differ: those with delayed meiosis produce steroids throughout this time period, whereas those of the immediate meiosis group have a transient steroid production which is low or unmeasurable until the first follicles are formed (Byskov, 1979; Grinsted, 1981).

Only two marsupial species have been divided into these groups so far, both of these with immediate meiosis: *Potorous tridactylus* and *Bettongia gaimardi* (Dairi, 1988). The bandicoots *Isoodon macrourus & Perameles nasuta*, according to Ullmann (1981b, 1989), add confusion to the issue as their ovaries appear not to contain "germ cell cords" although the oocytes enter meiosis several weeks after gonadal differentiation.

"Meiosis inducing substance" (MIS) and "meiosis preventing substance" (MPS) have been shown to be produced by ovarian and testicular cells respectively (Byskov & Saxén, 1976; Byskov, 1979, 1986; Grinsted *et al.*, 1979;

Andersen *et al.*, 1981). In the ovary, MIS has been shown to be produced by the mesonephric cells of the *rete ovarii* (Byskov & Saxén, 1976), in that, if an ovary is removed from the mesonephros before the *rete* penetrates the gonad meiosis will be prevented (Byskov, 1974a, 1979). When an undifferentiated testis is cultured with a meiotic ovary, the germ cells in the testis will begin their meiotic prophase (Byskov & Saxén, 1976). Conversely, if a meiotic ovary was cultured with a testis in which the testicular cords had formed (producing MPS), the ovarian germ cells would be arrested in their meiotic prophase (Byskov & Saxén, 1976).

1.11: Description and History of *Monodelphis domestica.*

The grey, short-tailed opossum, *Monodelphis domestica*, is a small member of the family Didelphidae, or American opossums. Other members of this group include: the Virginia opossum (*Didelphis virginiana*), the Southern opossum (*D. marsupialis*), the mouse opossum (*Marmosa robinsoni*) and the grey, four-eyed opossum (*Philander opossum*). These animals are considered the most ancient of marsupials, and an estimated date of separation of these from the Australian marsupials is around 70 million years ago (VandeBerg, 1983).

Monodelphis adults weigh between 60 - 150 g and are intermediate in size between rats and mice. In captivity they show much lower aggression than other Didelphids, breed readily and can be kept with minimal husbandry. They have a short gestaton period of around 14 days, a large average litter size (7 per litter in laboratory animals, 8.5 per litter in wild populations) and the females do not have a pouch but a roughly circular mammary area on their abdomen. Moreover, they also have all the benefits of marsupials for study: quite early sexual maturity (4 to 5 months), few chromosomes (2n = 18) and young are born at an early developmental stage (VandeBerg, 1983, 1990).

The animals' specific name is derived from its readiness to occupy human habitation, often reducing the numbers of both invertebrate and vertebrate pests.

The generic name, however, poses more of a problem in its derivation. *Didelphis* was coined by Linneaus in his *Systema Naturae* - *Di-delphys* from the Greek means "with two uteri", meaning the internal uterus and the pouch which was looked upon as an external uterus. This was used by de Blainville in 1816 for a classification of mammals: Ornithodelphia = Monotremata, Didelphia = Metatheria and Monodelphia = Eutheria (Tyndale-Biscoe, 1973). *Monodelphis* was first used as a generic name by Wagner in 1842, probably to distinguish this animal from *Didelphis* due to the lack of a pouch.

Monodelphis were introduced to captivity in 1978 in the National Zoological Park in Washington D.C. from four male and five female specimens caught in the Pernam Buco region of Brazil. The original nine opossums bred prolifically and, in 1979, 20 were donated to the Southwest Foundation for Biomedical Research in Texas. From the animals at this establishment, almost all of the *Monodelphis* in captivity have been derived.

Before the appearance of *Monodelphis* in the laboratory attempts had been made to use several of the Didelphids for biomedical research. *D. virginiana* has been utilized for many studies, although its large size, aggressiveness and poor reproductive ability in laboratory conditions has precluded its use anywhere but in the United States (Jurgelski, 1974; Jurgelski & Porter, 1974; Jurgelski *et al.*, 1975; VandeBerg, 1983, 1990). Other Didelphids used have been *Marmosa elegans, M. robinsoni, Caluromys derbianus* and *Philander opossum*, with only the mouse opossum having limited success. None of these animals, however, appear to be well adapted to captivity (VandeBerg, 1990).

In the twelve years of its captivity, *Monodelphis* has been intensively studied: topics include physiology, anatomy, biochemistry, behaviour, genetics, communication and endocrinology, some of these having direct medical significance (VandeBerg, 1990).

1.12: Aims.

There were several aims to this study, all concerned with *Monodelphis domestica*. They were:

1 - To study the breeding behaviour of adults and growth of pups in comparison to previous studies.

2 - To study and describe gonadal development from new-born to adult animals, with particural reference to the *rete ovarii*.

3 - To ascertain the temporal and spatial origins of steroidogenic cells in the ovary.

CHAPTER 2.

Materials and Methods.

2.1: Source of Animals.

The mice (*Mus musculus*) used in this study were part of an already established breeding colony in the Developmental Biology Unit of Glasgow University. The grey, short-tailed opossums (*Monodelphis domestica*) were established in Glasgow for the purpose of this and other studies.

19 *M. domestica* were obtained from Manchester University (03/05/89), comprising 4 adult females, 4 adult males, 2 half-litters of females (n=2 and 3) and 2 half-litters of males (n=2 and 4). The colony at present (August 1991) consists of over 70 animals and has produced, to date, over 200 pups. Only one of the original 19 opossums has not died or been sacrificed - a male which is still reproductively active at 3.5 years of age.

All of the details given in this chapter pertain to *Monodelphis domestica*.

2.2: Housing.

The opossums were housed in plastic rat cages 57 x 39 x 19 cm (model RC1/F, North Kent Plastics). Wood shavings were used on the cage floor and the animals were given either ready-shredded or whole tissues to shred for themselves into nesting material. Females, after separation from their mates, were provided with a nest box 23 x 16 x 8 cm, with a 7 cm diameter "door" at one end. The cages were routinely cleaned every 2 to 3 days, taking care not to disturb females with litters. The temperature was maintained at 26^{o} to 27^{o}C and the room was subject to a 14 hour light/10 hour dark lighting regime.

2.3: Diet.

Food was mainly in the form of a powdered cat food (S.D.S. Powdered Carnivore Meat (Feline), code 825 510), which was mixed 1 : 1 by volume with

warm water, refrigerated in boxes until solid and then cut into cubes of approximately 2 cm^3. Twice weekly these cubes were sprinkled with a veterinary vitamin supplement, SA-37 (Intervet Labs. Ltd.), before being fed to the opossums. Also twice weekly the animals' meat diet was supplemented with various fruits, mainly bananas, apples and pears. The animals were already trained to take water from sipper-tube bottles. Fresh water was given every two days and clean bottles substituted every week.

2.4: Breeding.

Two breeding regimes were used successively, the first was abandoned in favour of the second, more efficient and less stressful regime:

i - The male was placed in a wire holding cage, 20 x 10 x 10 cm, within the female's cage for 2 to 4 hours on three consecutive days. On the fourth day, the male was released into the female's cage and left for 10 days. After this period the male was removed and the female was given a nest box for the next 14 days. If no litter was apparent by this time, the same male was re-introduced in the holding cage and the sequence was repeated until a litter was born.

ii - The female was placed directly into the male's cage, without a holding cage or restraint of any kind. The pair were left together for 14 days, then the female was removed to her own cage with a nest box and again checked for a litter over the next 14 days. If unsuccessful, this sequence was repeated using the same male until a litter was born.

All authors of *M. domestica* studies so far have used the convention of the day of birth being called "day 0" and this has been adhered to in the present study. Litters were separated from the female after 50 days and kept in one-sex litter groups for another 50 to 60 days, depending on individual and litter sizes. After this period the animals were caged separately until paired for the first time, at four to five months of age.

2.5: Chromosome Analysis.

Chromosome analyses were carried out on young aged from days 0 to 7 using a modification of a technique described for mice by Evans *et al.* (1972, 1987). The pup was killed by decapitation and the liver dissected in Ham's Nutrient Medium F12 (Gibco) at 37°C. The carcass was then fixed for either wax or resin processing. The liver was transferred to medium containing 0.4 mg/ml colchicine, chopped finely and aspirated by pipette. This was incubated for 2 to 4 hours at 37°C. After incubation the supernatant and small tissue pieces were centrifuged at 1000 r.p.m. for 10 minutes. The cell pellet was resuspended in 0.56% potassium chloride (KCl) for exactly 10 minutes and centrifuged at 1000 r.p.m. for 10 minutes (the timing is critical at this point). The pellet was then blotted dry and carefully fixed in three successive aliquots of freshly made up 3 : 1 methanol to glacial acetic acid. After fixation the pellet was resuspended in more fresh fixative, centrifuged again and finally resuspended in 60% glacial acetic acid and aspirated to disperse the cells. This aspirate was dropped onto ethanol-cleaned slides on a hotplate at 45° to 50°C from a height of 15 to 20 cm and left to dry. The slides were stained in fresh Giemsa stain (BDH), mixed 1 : 9 with phosphate-buffered saline (PBS) for 4 to 5 minutes, rinsed in PBS, air dried and then mounted in D.P.X. The slides were examined at 100x magnification under oil immersion and chromosome spreads were photographed. These photographs were enlarged, the chromosomes cut out and paired to obtain a karyotype.

2.6: Histology.

2.6.1: Follicular Staging.

The follicles observed in ovaries of all three species studied were staged according to a classification of mouse follicles by Pedersen & Peters (1968). This was based on oocyte size, the number of granulosa cells surrounding it and the

morphology of the follicle itself (Figure 3).

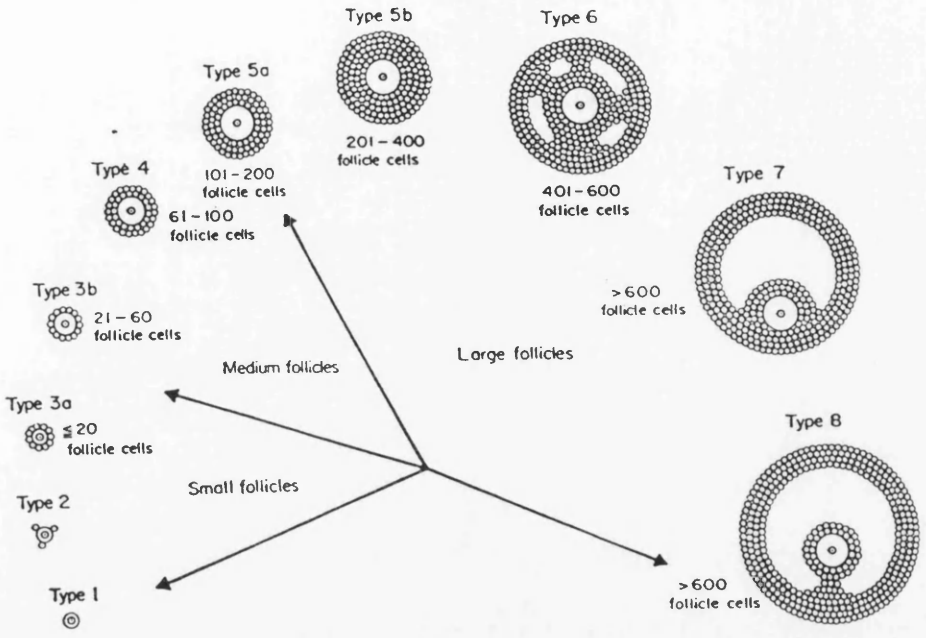


Figure 3:

Classification of follicles. (From Pedersen & Peters, 1968)

2.6.2: Paraffin Wax Histology.

Most of the wax sections were obtained from day 0 to 15 *M. domestica* specimens which were killed in chloroform, bisected and the posterior half processed. Embryos of approximately 8 days and 13 days of gestation were obtained when two females died and were treated similarly. Older specimens (day 20 to day 65) were chloroformed, then dissected to remove the gonads or gonads and mesonephroi, which were processed to paraffin wax. All animals were weighed and their head-lengths measured with callipers prior to processing.

The tissue required, either half pups or separate organs, was fixed either in alcoholic Bouin's fixative or 10% formalin for 24 hours then dehydrated through a graded ethanol series to xylene and then impregnated with 1 : 1

xylene/wax at 57°C. This was followed by two changes in either 57° or 60°C wax. These specimens were then blocked in fresh paraffin wax and stored in a refrigerator until cut. Sections were cut at 6 μm on a Beck "A 138" or Leitz "1512" rotary microtome and mounted on ethanol washed, air dried, albuminized glass slides. The slides were either stained with Mayer's haemalum and eosin (H&E); or alternate slides were stained with H&E and Mallory's triple stain (Appendix 1).

2.6.3: Resin Histology.

The specimens used for resin histology were *M. domestica* pups, aged mainly from day 0 to day 6. The animals were chloroformed, bisected and fixed in half-strength Karnovsky's solution (Karnovsky, 1965) for two hours at 4°C. The posterior halves were then dissected in cacodylate buffer to remove both mesonephroi with the gonadal ridges attached. After dissection the mesonephros/gonadal ridge complexes were fixed in 1% aqueous osmium tetroxide for two hours at 4°C, dehydrated in an ascending graded ethanol series. This was followed by impregnation which was achieved by treating the specimens with increasing ratios of propylene oxide to ethanol (25 : 75, 50 : 50, 75 : 25), then pure propylene oxide and finally a 1 : 1 solution of propylene oxide/araldite resin. The specimen was left in this solution on a rotator for 12 to 24 hours before being blocked in pure resin, which was placed in a 60°C oven for 48 hours to complete polymerisation.

From each animal, therefore, two resin blocks were made: one was used for light microscopy, cut at 1 μm on a Reichert "OM-U3" ultramicrotome and stained with toluidine blue (Appendix 1) and the second block was stored, to be cut later for electron microscopy, which is not included in this work.

All histological specimens and chromosome analyses were studied using a Wild M-20 microscope and photographs of the relevant sections were taken with

a Wild MEL-13 photo-extension unit on an M-20 microscope or an Olympus photomicroscope.

2.7: ....^{5}, 3β-Hydroxysteroid Dehydrogenase Staining.

2.7.1: Enzyme Histochemistry.

For 3β-HSD staining, ovaries were removed from CO_2 or chloroform asphyxiated animals, mounted on cork in O.C.T. compound (BDH) and then snap-frozen in iso-pentane (2, methyl butane, BDH) previously cooled in liquid nitrogen. These blocks were then wrapped in aluminium foil, labelled and stored at -70^{o}C for a maximum of four weeks. Longer storage resulted in a loss of enzyme activity. The ovaries were sectioned at 8 to 10 μm in a Bright "Starlet 2212" cryostat at a temperature of -25^{o} to -35^{o}C. The cut sections were stored at -20^{o}C for no more than three days before staining.

The 3β-HSD staining method was adapted from Hoyer & Andersen (1977), using dehydroisoandrosterone (DHA) as a substrate as literature suggested that this would give the strongest reaction in this technique, substituting tetranitro blue-tetrazolium (Tetra-NBT) for nitro blue-tetrazolium as the dye-producing salt and adding polyvinyl pyrrolidone (20% w/v) to improve the staining (Bjersing, 1967).

The incubation medium was:

DHA (0.3 mg/ml) in dimethyl formamide (DMF)	-	0.1 ml
Phosphate buffer (0.2 M, pH 7.2)	-	0.25 ml
Distilled water	-	0.65 ml
Tetra-NBT (1 mg/ml)	-	1.0 mg
β-nicotinamide adenine dinucleotide (NAD)(0.1 mg/ml)	-	0.1 mg
Polyvinyl pyrrolidone (PVP)(20% w/v)	-	0.2 mg

Positive controls were pregnant mouse ovaries and negative controls were incubated in the above medium, using either distilled water or DMF alone instead of the steroid dissolved in DMF. All chemicals used in the 3β-HSD demonstration were obtained from the Sigma Chemical Company Ltd.

2.7.1.1: Method.

i -	Extract lipids with cold acetone ($4^{o}C$)	-	2 mins
ii -	Wash in cold phosphate buffer ($4^{o}C$)	-	2 mins
iii -	Incubate in medium at $37^{o}C$ (control with microscope)	-	½ - 2 hrs
iv -	Rinse in distilled water		
v -	Fix in 10% formalin	-	15 - 30 mins
vi -	Rinse in distilled water		
vii -	Counterstain in 1% aqueous methyl green	-	10 secs
viii -	Rinse in distilled water		
ix -	Mount in an aqueous mounting medium (Hydramount, B.D.H. Ltd.)		

2.7.1.2: Results obtained.

3β-HSD positive cells - brown to purple/black cytoplasm.

All nuclei - green.

2.7.2: Immunocytochemistry.

The primary antibody used in the immunocytochemical study was a polyclonal rabbit anti-human placental 3β-HSD antibody, obtained from Dr. J.I. Mason (Depts. of Biochemistry and Obstetrics and Gynecology, Cecil H. and Ida Green Center for Reproductive Biology Sciences, University of Texas Southwestern Medical Center, Dallas, Texas, U.S.A.). Previously this antibody

had only been used on human tissues (Lorence *et al.*, 1990a, 1990b; Mack *et al.*, 1990; Sasano *et al.*, 1990a, 1990b).

The staining technique employed was that used by Sasano *et al.* (1990b), although the equivalent Rabbit ExtrAvidin Staining Kit (Sigma Chemical Co.; EXTRA-3) was used in place of the StrAviGen B-SA system employed in that study.

2.7.2.1: Method.

The diluent for all staining components, apart from the 3, 3'-diaminobenzidine (DAB), was 1% Bovine Serum Albumin (BSA, ICN Flow Ltd, 820451) in phosphate buffered saline (PBS, 0.01M, pH 7.4). The DAB was diluted in Tris-HCl buffer (pH 7.6). Specimens were fixed in 10% formalin, paraffin wax embedded and cut at 6μm.

i -	Xylene	-	2 x 5 mins
ii -	Ethanol series (100%, 95%, 90%, 70%, 30%)	-	3 mins each
iii -	Water	-	3 mins
iv -	Pre-incubation in 5% BSA	-	10 mins
v -	0.3% H_2O_2 in methanol	-	30 mins
vi -	PBS	-	3 x 5 mins
vii -	Drain and wipe around sections		
viii -	Normal Goat Serum (NGS) 1 : 5	-	30 mins
ix -	Drain off NGS but do not wash sections		
x -	Rabbit anti-human 3β-HSD 1 : 200	-	18 hours at 4^{o}C
xi -	PBS	-	3 x 5 mins
xii -	Drain and wipe around sections		
xiii -	Biotinylated goat anti-rabbit IgG 1 : 15	-	30 mins
xiv -	PBS	-	3 x 5 mins

xv -	Drain and wipe around sections		
xvi -	Peroxidase conjugated ExtrAvidin 1 : 15	-	20 mins
xvii -	PBS	-	3 x 5 mins
xviii -	DAB solution:	-	control under
	1 DAB tablet (Sigma Chemical Co.; D-5905)		microscope
	6 mls Tris-HCl buffer (pH 7.6)		(about 5 secs)
	12 μl H_2O_2		
xix -	Wash in water		
xx -	Counterstain in 1% methyl green	-	10 secs
	or in haematoxylin	-	5 mins
xxi -	Mount in D.P.X.		

Positive controls were pregnant mouse ovaries, where corpora lutea stained strongly and negative controls were treated with 1% BSA in PBS instead of the primary antibody.

2.7.2.2: Results Obtained.

3β-HSD positive cells - brown granules in cytoplasm.

All nuclei - green (methyl green) or purple/blue (haematoxylin).

CHAPTER 3.

Behavioural and Statistical Analyses.

3.1: Introduction.

Several studies have been undertaken on the behavioural aspects of *Monodelphis domestica*, mainly on scent marking (Fadem & Cole, 1985; Fadem & Schwartz, 1986), the behaviour of oestrus (Fadem, 1985; Trupin & Fadem, 1982; Fadem & Rayve, 1985; Fadem, 1987), mating behaviour (Trupin & Fadem, 1982) and on nest-building (Fadem *et al.*, 1986).

Several oestrous cycle lengths have been suggested for this animal: 28 days (Fadem *et al.*, 1982), 32 days (Fadem, 1985) and 14.4 and 32.3 days (Fadem & Rayve, 1985). However, it has been shown that in most *Monodelphis* females oestrus is induced by the presence of pheromonal and tactile cues from the male and occurs between 3 to 9 days after the introduction of a male (Fadem, 1985; Baggott *et al.*, 1987; Fadem, 1987; Phillips & Fadem, 1987). With a gestation length of 13 - 15 days, this means that all litters should be born between 16 to 24 days after pairing. Fadem (1985), however, states that all litters were born within 39 days of pairing. This would indicate that some animals take longer to come into oestrus than the 9 days stated.

The oestrous behaviour of *Monodelphis* has been described by Trupin & Fadem (1982), Fadem & Rayve (1985) and Fadem (1985, 1987) and consists of scent-marking, rump-dragging, tongue-clicking, mutual genital sniffing and licking and chasing around the cage. Trupin & Fadem (1982) went on to describe the mating behaviour which followed the above oestrous behaviour. The male would quickly mount the female, immobilise her in his grasp and the animals would collapse onto their right sides. These authors gave the coital duration as 4 to 6 minutes, usually ending with a post-coital lock for several minutes.

Reported litter sizes for laboratory colonies have been between 3 to 14 pups, with a mean ranging from 7 - 8.5 pups per litter (Fadem *et al.*, 1982; Fadem, 1985; Fadem & Rayve, 1985; Moore & Thurstan, 1990), and 8 - 14 pups per litter in wild animals (Fadem *et al.*, 1982). So far, no studies have mentioned the sex

ratios of *Monodelphis domestica*, either at birth or weaning.

Published data on neonatal weights and crown-rump lengths approximate 100 mg and 10 mm respectively (Fadem *et al.*,1982; Kraus & Fadem, 1987; Tyndale-Biscoe & Renfree, 1987; Tyndale-Biscoe & Janssens, 1988; Moore & Thurstan, 1988; Baker *et al.*, 1990). Only one study has given growth curves for *Monodelphis* (Cothran *et al.*, 1985) which were based on the weight of specimens. In the present study the head-length of pups, a parameter not previously considered, was measured.

Chromosome analyses, using a technique originally employed on mouse material (Evans *et al.*, 1972), have shown that the chromosome number for *Monodelphis domestica* is 2n = 18 (Hayman *et al.*, 1988), and have suggested that with few relatively large chromosomes this animal is ideal for genetic studies. Recently, VandeBerg (1990) published the karyotype showing the small size of the X chromosomes and the tiny male Y chromosome, making the task of chromosomal sexing relatively easy.

3.2: Breeding.

The breeding regime used initially for *M. domestica*, was applied from June 1989 to November 1989. During that period only 3 litters were born from 25 pairings (12% success rate), and 2 of those litters were lost within ten days of birth (67% loss). The major problem with this regime appeared to be the aggressiveness and territoriality of the female opossums. Several of the females used received injuries from the wire holding cage in which the males were presented. Males were attacked viciously when released unprotected into the females' cages, one being fatally injured. Due to these injuries, this regime was discontinued.

The second regime, used from December 1989 to present, appeared to be more successful. The territoriality and aggression of the female was curbed

severely when she was put into the male's cage. This meant that, as the female was out of her own territory, she was much more submissive to the male and, furthermore, much more tractable for handling.

Using this regime, up to August 1991, 44 litters have resulted from 114 pairings (39% success rate) and only four litters have been lost before the removal of several young from the teats (a 9% loss).

Breeding behaviour was watched in several pairs, in order to obtain timed embryos for sectioning. The animals were observed during the dark phase, with very subdued lighting from two 25 watt lamps, each covered by an Ilford 915 (light red) safelight filter. The behaviour was watched all night, taking only timed 3 minute breaks, so as not to miss any copulations.

On first exposure to each other, both males and females displayed much aggressive behaviour, but quietened after 3 to 4 hours. On the second and third nights' observation two of the males followed their females around, making quite loud clicking noises, presumably by tongue to palate contact. This clicking continued almost constantly and increased in frequency whenever the female showed any signs of aggression.

Much genital sniffing and licking was undertaken, mainly by the males, which would grasp hold of the hair on the female's rump, and not let go for periods of 5 to 10 minutes. On the fourth night of observation the males and females frequently took part in chasing behaviour, sometimes around the whole cage area but more often in a very tight, always anti-clockwise, circle. After 6 to 7 hours of this behaviour, often with the male still emitting the clicking sounds, copulation occurred: the male would mount the female very quickly, immobilising her by gripping her tightly just anterior to her hind legs with his forelegs, grasping the ankles of her hindlegs with his hind feet and biting into the fur of her neck. Invariably the pair collapsed onto their right sides and copulation lasted anywhere from 3 to 7 minutes. A lock was sometimes observed at the end of copulation. After mating both animals went to opposite ends of the cage and

groomed intensively for 4 to 5 minutes. The female was then, once again, very aggressive towards the male and would shriek and run off if the male approached her. If the male continued his advances, the female would attack him quite viciously.

No obvious diurnal rythms were observed for births (several females were disturbed whilst giving birth in the afternoon), but these mainly occurred during the dark phase. The neonates ascended unaided to the lower abdomen and fixed onto a teat. The pups were usually attached to the nipple for 14 days, although one litter separated as early as day 11 after birth. Once the young had detached, they either remained in the nest box while the mother came out to feed or, more often, were carried, clinging to their mother's fur with their feet, mouths and prehensile tails.

3.3: Litter Sizes and Sex Ratios.

The litters born so far in Glasgow have ranged in size from two to twelve, with an average of 7.5 (Figure 4 - distribution of litters in Glasgow). The litters for which we have information (n = 28) suggest a 1 : 1 ratio (88 males to 82 females).

3.4: Neonate Size and Growth.

The data collected on neonatal size and growth were limited, as only the pups removed for experimental work were measured. These data ranged from newly born specimens (day 0) to weaned adolescents (day 65), but were mainly concentrated from days 0 to 15.

M. domestica measured approximately 10 mm crown-rump length when born, but the softness and curvature of the posterior half of the animals made it almost impossible to take accurate measurements of length. The only parameter which was constantly measured, apart from weight, was head length. In the pups,

there were two obvious blood vessels which separated at the back of the head and then passed laterally around towards the anterior of the head. The separation of these vessels gave one reference point to measure from and the nostrils were used as the second point, so that all of the head length measurements were comparable. The age, sex, weight and head length data of the specimens collected is shown in Appendix 2.

When the weights were plotted against age (Figure 5a), the resultant graph appeared to be an exponential curve, so the $\log_{10}$ of the values was calculated, and then this was plotted against age. The graph of this analysis (Figure 5b) seemed to be a straight line, so the correlation coefficient was calculated, which turned out to be $r = +0.95$. Regression analysis gave the equation $\log_{10}$ weight = 0.04 x age + 2.18, $r^2 = 89.9\%$, $t = 35.21$, $p < 0.0001$.

The head lengths, when plotted against age, also appeared to create a straight line (Figure 6). When the correlation coefficient was calculated for these data it turned out as $r = +0.98$. Regression analysis gave a formula of head-length = 0.435 x age + 4.22, $r^2 = 96.7\%$, and $t = 63.53$ which is significant at $p < 0.001$.

Weight was then plotted against head length (Figure 7a). The correlation coefficient was calculated to be $r = +0.93$, although the r^2 value obtained from regression analysis was only an 86.2% fit. By transforming both axes to $\log_{10}$ (Figure 7b) the correlation coefficient rose to $r = +0.99$. Regression analysis on these transformed data gave a formula of $\log_{10}$ weight = 2.61 x $\log_{10}$ head-length + 0.741, $r^2 = 98.1\%$ and $t = 83.83$, which is significant at $p < 0.0001$.

3.5: Animal Sexing.

The *M. domestica* pups removed from the mother before day 8 were sexed in three ways. On their removal, and before they were sacrificed, the pups were examined under a dissecting microscope for signs of a scrotum just anterior

to the genital tubercle. These animals were putatively sexed as male (female *M. domestica* have no pouch to confuse the issue). In males the testis is differentiated at birth, so the animals used for light microscopy could also be sexed gonadally. Chromosome analyses were carried out to verify the visual sexing procedure and also to sex the animals taken but not used in this study. Sexing pups by staining for Barr bodies was ruled out as these structures do not stain in *Monodelphis* (Merry *et al.*, 1983). Table 1 compares the histological sexing of pups with both visual and chromosomal means. The chromosomes from the photographs taken (Figure 8) were cut up and karyotyped (Figure 9).

Figure 4:

Bar chart to show the disribution of *Monodelphis domestica* litter sizes obtained in Glasgow. The average litter size was 7.5 pups per litter.

Figure 5a:

Graph depicting the weight (mg) of *Monodelphis domestica* pups in relation to age (days). The results appear to form an exponential curve.

Figure 5b:

Graph depicting the $\log_{10}$ of weight of *M. domestica* pups in relation to age. The correlation coefficient was $r = +0.95$. Regression analysis gave the equation: Log_{10} weight $= 0.04 \times \text{Age} + 2.18$, $r^2 = 89.9\%$, $t = 35.21$ $p < 0.0001$.

Figure 5a.

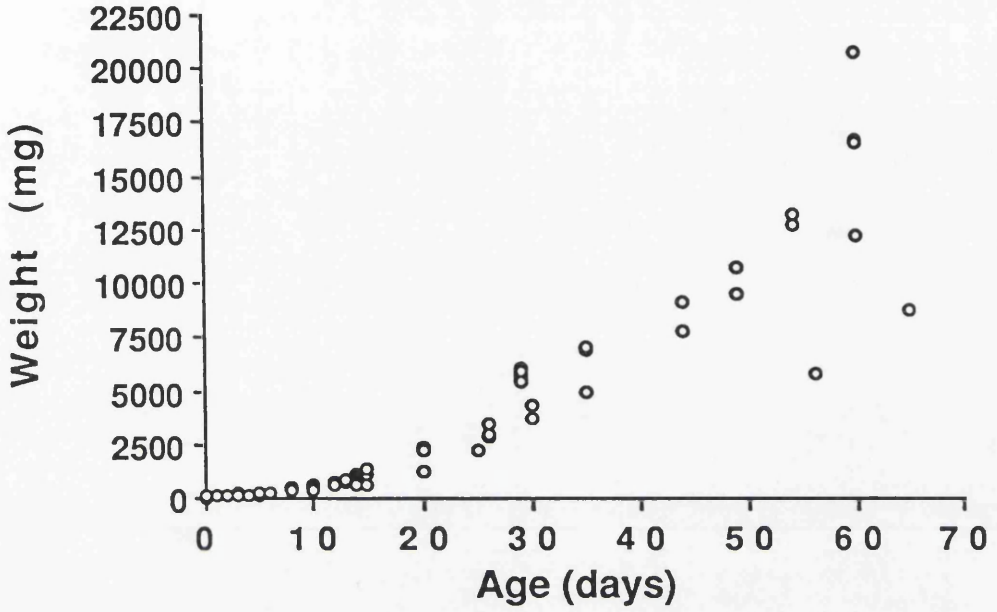


Figure 5b.

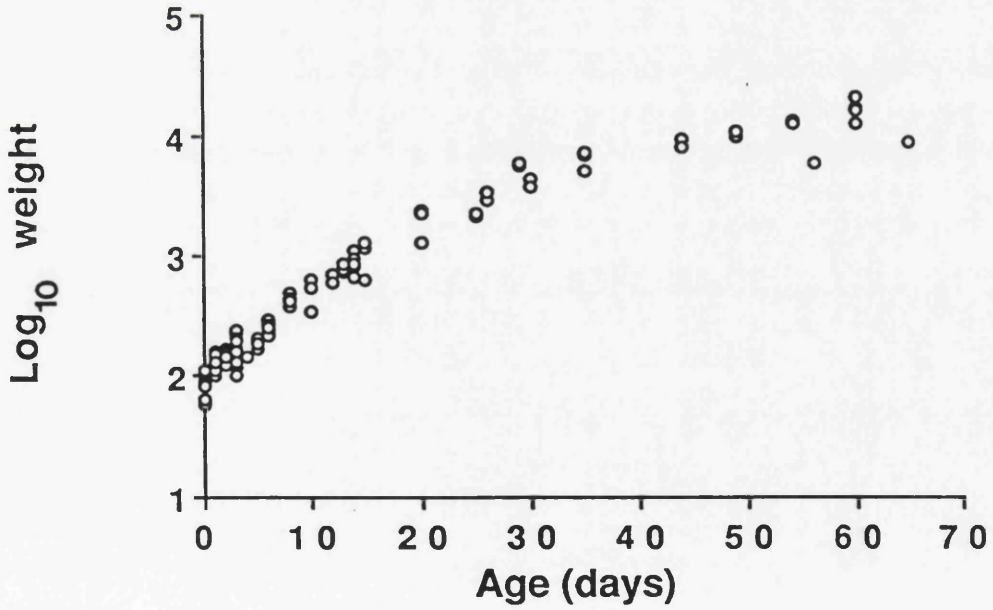


Figure 6:

Graph to show the relation of *M. domestica* pup head-length (mm) to age (days). The correlation coefficient was r = +0.98. Regression analysis gave an equation of: Head-length = 0.435 x Age + 4.22, r^2 = 96.7%, t = 63.53, $p < 0.0001$.

Figure 6.

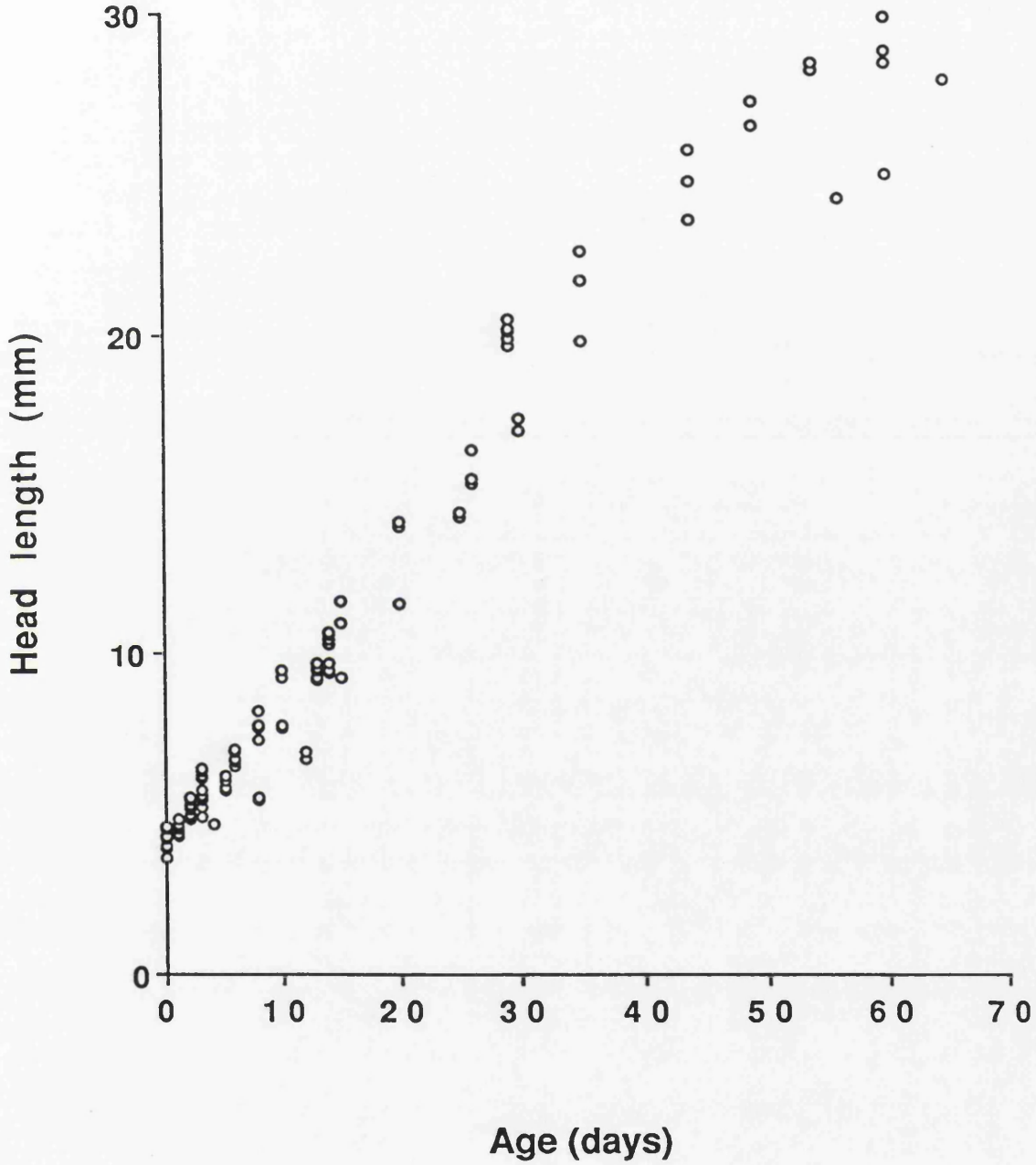


Figure 7a:

Graph depicting the relation of weight (mg) to head-length (mm). The correlation coefficient was r = +0.93. Regression analysis gave an equation of: Weight = 504 x Head-length - 3033, r^2 = 86.2%, t = 29.18, p < 0.0001.

Figure 7b:

Graph depicting the $\log_{10}$ of weight against $\log_{10}$ head-length. The correlation coefficient was r = +0.99. Regression analysis gave an equation of: Log_{10} Weight = 2.61 x Log_{10} Head-length + 0.322, r^2 = 98.1%, t = 83.83, p < 0.0001.

Figure 7a.

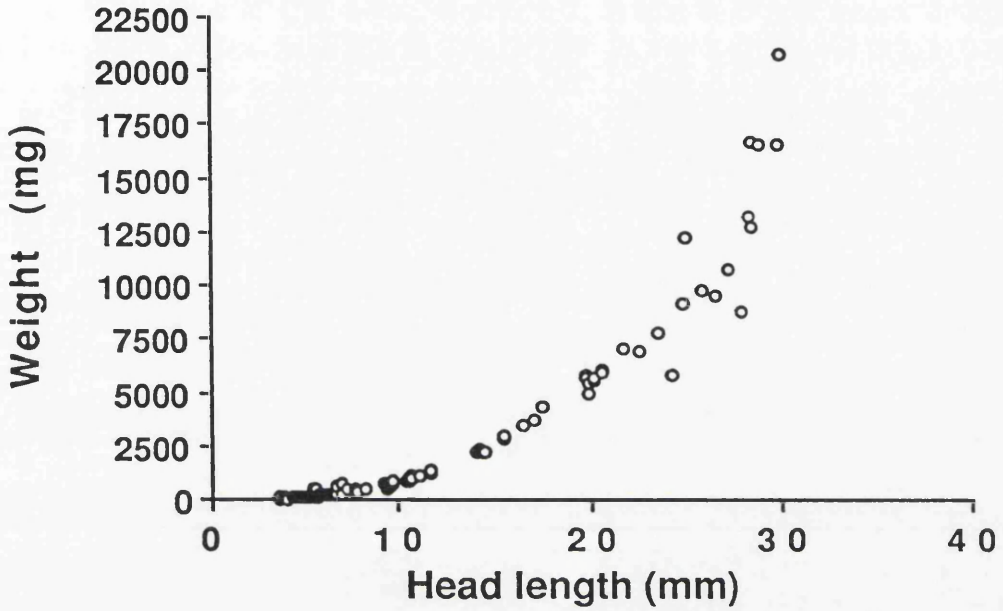


Figure 7b.

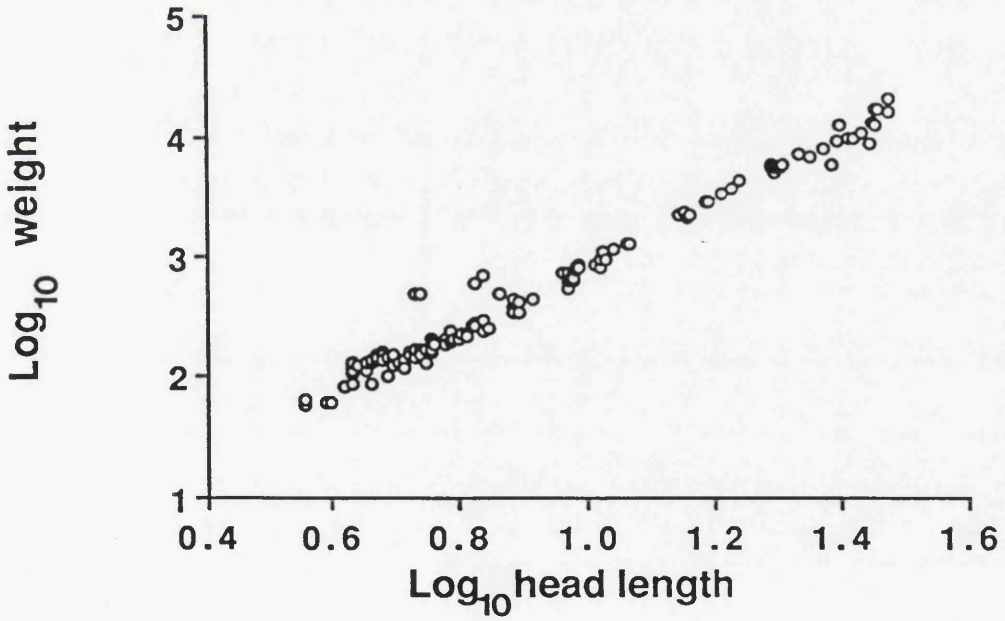


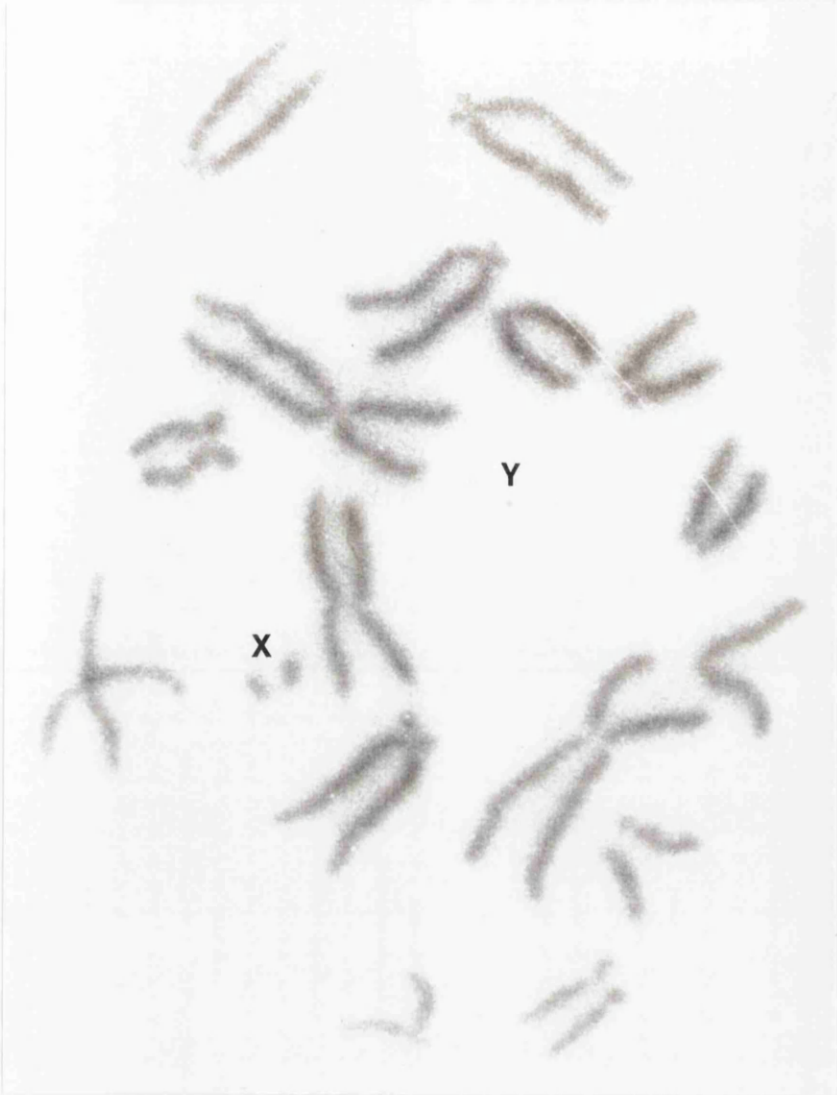
Table 2: Comparison of visual and chromosomal sexing methods against histological sexing of *M. domestica* pups.

Age	Visual				Histological			
	M	F	?	N	M	F	?	N
Day 0	5	1	1	7	2	3	2	7
Day 1	9	3	2	14	6	6	2	14
Day 2	8	6	4	18	9	5	4	18
Day 3	7	6	2	15	7	6	2	15
Day 4	0	1	0	1	1	0	0	1
Day 5	3	1	0	4	1	3	0	4
Day 6	3	1	0	4	2	2	0	4
Day 7	no			specimens			examined.	

Age	Histological				Chromosomal			
	M	F	?	N	M	F	?	N
Day 0	2	3	0	5	2	3	0	5
Day 1	2	3	2	7	2	5	0	7
Day 2	7	3	4	14	9	4	1	14
Day 3	1	3	2	6	2	4	0	6
Day 4	1	0	0	1	0	0	0	0
Day 5	1	3	0	4	1	3	0	4
Day 6	2	2	0	4	2	2	0	4
Day 7	no			specimens			examined.	

KEY: M = Male, F = Female, ? = Unknown, N = Number of pups sexed.

Figure 8.



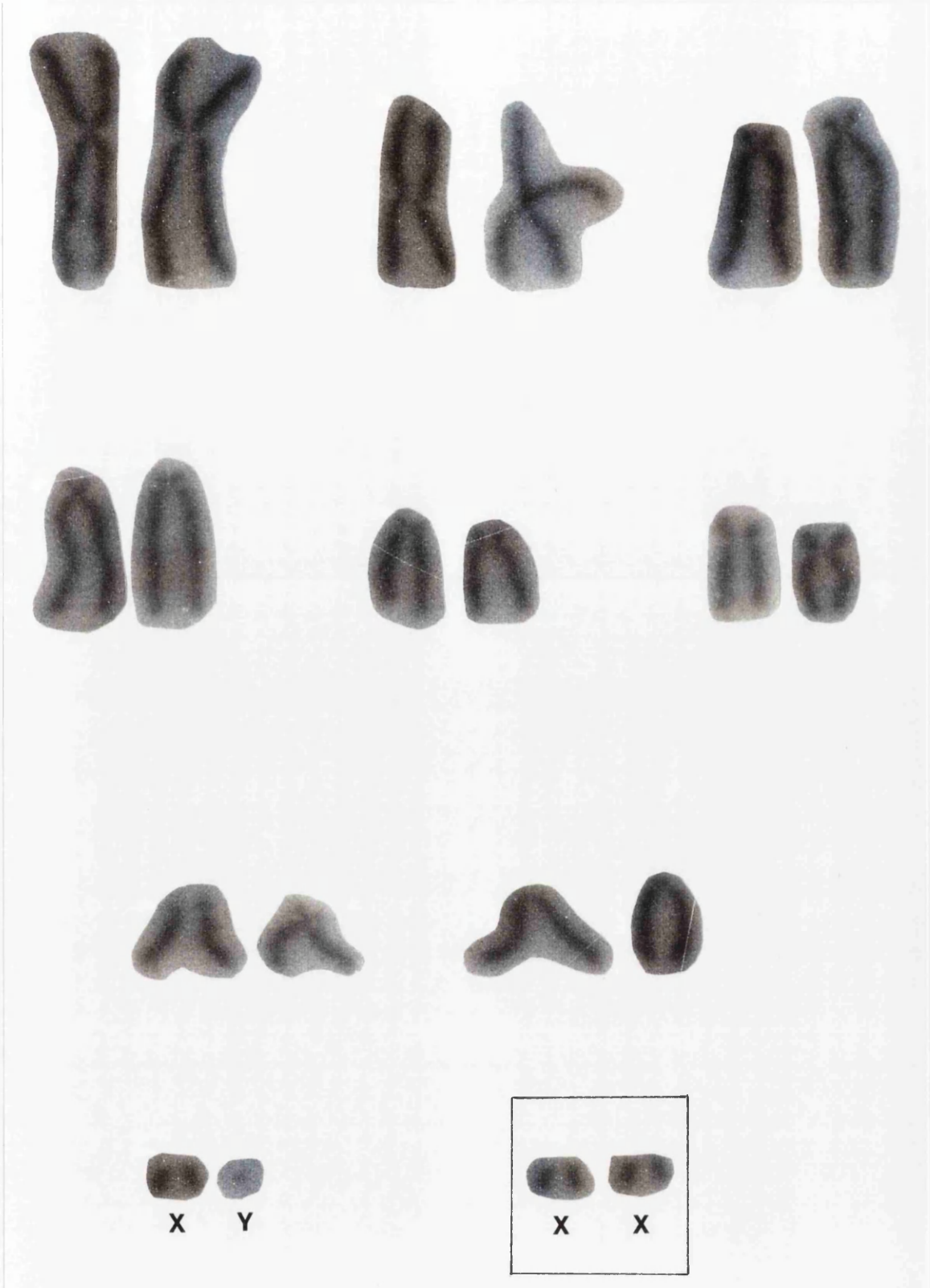
Enlarged photograph of a male chromosome spread.

Note the size difference of the marked X and Y chromosomes.

Figure 9:

Karyotype of a male *Monodelphis domestica*. Note the tiny size of the Y chromosome compared to the X chromosome. Inset is the XX sex chromosomes of a female .

Figure 9.



3.6: Discussion.

3.6.1: Breeding.

The first breeding regime used, where the male was initially introduced to the female in a holding cage, caused problems for both the animals and their handlers. The male appeared to be quite stressed after exposure to the female whilst in the holding cage, without being allowed access to her. The holding cage also meant that the male went for periods of time without access to food and water. After removal from the cage the male was very active, frantically scent-marking his own cage and drinking large amounts of water. The females were very aggressive, both when the males were introduced, with or without holding cages, and when they were being handled afterwards. When the males were presented without protection, the females attacked them without hesitation, often causing damage which could be fatal. It was decided to abandon this regime in November 1989, due to injuries to the animals.

The second mating regime was preferable to the first, showing an increased productivity of 27% and a reduced loss of litters within the first ten days of 58%. The females were in a strange territory from the outset and so were more timid towards their mates, as well as being much more tractable when they were removed. In using this regime, the females appeared much quieter on handling, even after having had their litters, than they were at any time during the first regime. These findings support those of Trupin & Fadem (1982) on the territoriality of *Monodelphis*, although this species appeared to be less territorially aggressive than its close relatives *Marmosa robinsoni* and *Didelphis virginiana*, neither of which can be housed in permanent male/female pairs (Jurgelski, 1974; Jurgelski & Porter, 1974; Godfrey, 1975; Rodger & Bedford, 1982; Trupin & Fadem, 1982).

A video recording system to observe the mating behaviour would have been preferable to a human presence, although the latter did not appear to affect

the animals' behaviour, whilst having the benefit of being able to identify which sounds were being produced by each animal. The opossums appeared to be unaffected by the lighting system used during observations.

The actual mating behaviour observed was similar to that described for *D. virginiana* (Jurgelski & Porter, 1974; Trupin & Fadem, 1982), and confirmed Trupin & Fadem's (1982) observations on *M. domestica*. Almost all of the behaviour patterns described by Trupin & Fadem (1982) were observed in the present study, including the tongue-clicking by the males, the mutual chasing around the cage and genital sniffing. In the lead-up to copulation the pairs observed always circled anti-clockwise, chasing faster and faster until the male caught and mounted the female. During coitus, the pair always collapsed onto their right sides, as in the case of *D. virginiana* (Jurgelski & Porter, 1974; Trupin & Fadem, 1982). The post-coital lock observed by Trupin & Fadem (1982) in several of their pairs, was seen in two of the six matings watched in the present study. As mentioned previously the aggression displayed by female *M. domestica* was greatly reduced whilst she was receptive to the male and although it increased again slightly after mating, apparently not as much as in the related species (Jurgelski & Porter, 1974; Trupin & Fadem, 1982).

It was observed that *M. domestica* gave birth at any time of the day, as many females were disturbed in the process at approximately 4.30 p.m. when they were being checked for litters. Although these findings did not directly contradict those of Fadem *et al.* (1982), that almost 70% of births occurred between 7 a.m. and 11 a.m., they suggested that the percentage stated may be rather higher than that of the Glasgow colony. This disturbance of the female giving birth did not affect the chances of the litter's survival, nor did it affect the reliability of the female for breeding at a later date.

The chronology of events after birth confirmed those given by Fadem *et al.* (1982), Tyndale-Biscoe & Renfree (1987) and VandeBerg (1990). The pouch young were usually attached to the teat until day 14, although three litters

detached earlier, at day 11; this, however, did not affect their development at all. As stated in the studies referred to, the pups, after detaching from the nipple, either stayed in the nest box, or were carried by the mother until they were fully mobile. The pouch young were separated from the female at day 50, by which time they were fully weaned, and were sexually mature by 4 to 5 months of age.

3.6.2: Litter Sizes and Sex Ratios.

The litter sizes obtained in Glasgow were similar to those given by Tyndale-Biscoe & Renfree (1987) of 7 pouch young per litter (average 7.5 in Glasgow), and ranging from 3 to 14 (range of 2 to 12 in Glasgow).

Sex ratios in several marsupials have been shown to be skewed in favour of male pouch young at birth (*Trichosurus vulpecula, Setonix brachyurus, Macropus robustus, Macropus canguru,* Caughley & Kean, 1964; *Trichosurus vulpecula*, Hope, 1972; *Antechinus swainsonii, Bettongia penicillata, Macropus giganteus, M. rufogriseus, M. rufus, Petrogale xanthopus*, Cockburn, 1990). Other studies have shown that the sex ratios of some metatherians can be altered to a significant degree by specific factors, in favour of males. These factors are dietary supplementation of the mother (*Didelphis marsupialis*, Austad & Sunquist, 1986) and increase in natural rainfall (*Macropus rufus, M. giganteus, M. fuliginosus*, Clutton-Brock & Iason, 1986). Parental investment and differential mortality in the pouch are factors which could affect the sex ratios at weaning.

Kraus & Fadem (1987) gave the sex ratios of these opossums at weaning as 1 : 1. This was confirmed in the present study; the total numbers of males and females obtained were very similar (88 males: 82 females).

In this study it was impossible to state whether or not differential mortality occurred during postnatal development as there were very few losses of pups within a litter from birth to weaning. To examine for this phenomenon, animals would need to be sexed at or near birth and then the remainder of the

litter sexed at weaning. As has been explained previously it is very difficult, if not impossible, to sex neonates of *Monodelphis* visually and even more so when they are attached to a struggling dam.

3.6.3: Neonate Size and Growth.

Several authors give an average birth weight of 100 mg for *Monodelphis* (Fadem *et al.*, 1982; VandeBerg, 1983; Cothran *et al.*, 1985; Kraus & Fadem, 1987; Tyndale-Biscoe & Renfree, 1987; Moore & Thurstan, 1990; Tyndale-Biscoe & Janssens, 1988; Baker *et al.*, 1990) which is close to that found in the present study of 83.9 ±13.0 mg. It is likely that the lower average weight obtained in this study was due to the fact that several of our neonatal pups were weighed before they had attached to the teat. This meant that they had not fed at all, thus being lighter than those which were weighed after attachment and probably a first meal. In all other studies so far, the "neonates" had to be detached from the dam and thus, presumably, had already been fed.

The weight of pups over the age range studied (day 0 to day 65) appeared to demonstrate an exponential growth curve which was tested by plotting the $\log_{10}$ of weight against age. The results of this analysis were significant, $r = +0.95$, $r^2 = 89.9\%$ and $t = 35.21$ ($p < 0.0001$), which confirmed that over this age range, the young are exponentially increasing in weight. A potential source of inaccuracy in the weights of pups which had to be removed from the teat (usually up to day 14 and in some cases up to day 20) resides in the fact that they often vomit and/or void excreta when detached, so lowering their actual weight (Appendix 2). Most of the weight data obtained in this study compared favourably with the standard weight growth curves suggested by Cothran *et al.* (1985), although the younger specimens (days 0 to 20) appeared to be lighter than their counterparts obtained by those authors. This presumably could be explained by the expulsion of gut contents during removal from the teat, where the dam was

not previously anaesthetized, and could imply that weight data obtainable from pups removed from the mother are neither particularly constant nor reliable.

The crown-rump length, a common parameter used in measuring vertebrate embryos and neonates, was disregarded in this study as a viable measurement in *Monodelphis* due to the curvature and the softness of the posterior half of neonates. This meant that crown-rump length was not very accurate and varied greatly in specimens of the same age. The head length of pups, however, appeared to give a more constant measurement for young of the same age.

Head length was more reliable in that two obvious features, the nostrils and blood vessels at the rear of the head, marked constant reference points for measurements until the pups were furred, when the blood vessels were hidden from view. By this stage in development the juveniles were large enough to measure head-length with confidence. When statistically analysed these data turned out to be highly significant ($r = +0.98$, $r^2 = 96.7\%$, $t = 63.53$, $p < 0.0001$). This demonstrates that *M. domestica* pup head length is directly proportional to age and suggests that a juvenile of indeterminate age could be staged more reliably by extrapolation from the head length graph than from either the $\log_{10}$ of weight or weight graphs.

3.6.4: Sexing of Young.

Three methods of sexing *Monodelphis* pups, up to day 7, were used in the present study: firstly by the visual criterion of looking for a scrotal bulge in male specimens, secondly by looking for differentiated testes in histological sections and thirdly, by karyotyping, looking for an XX pair or single X and Y chromosomes.

Previous studies on *M. domestica* (Fadem *et al.*, 1982; Moore & Thurstan, 1988) stated that males displayed a scrotal bulge at birth and could, therefore, be

visually sexed accurately before histological examination. These findings could not be confirmed by the present study as the scrotal bulge was not evident until around day 3, in accordance with the results obtained by Baker *et al.* (1990). The major problem with visual assessment was that pups removed from the mother tended to remain curled up. Therefore, to examine them for scrotal rudiments, the specimen had to be uncurled. When the hind limbs and tail were at the required angle to observe the relevant area, the skin on the abdomen, including the scrotal skin, was so stretched that the latter was rendered indiscernible. The present study confirms the observations of Baker *et al.* (1990) that the testis is already differentiated at birth; male neonates could be sexed histologically, by the presence of testis cords and an almost complete *tunica albuginea*.

Hayman *et al.* (1988) gave the chromosome number for *M. domestica* as 2n = 18, using a technique described for mice by Evans *et al.* (1972), although no karyotype was given. A slighltly revised method to that used by Evans *et al.* (1972, 1987) was employed in the present study which confirmed the chromosome number and the karyotype published by VandeBerg (1990).

The pups sexed by visual and chromosomal criteria, in the present study, were verified where possible by histological examination. As it turned out, the sexing of pups visually was unreliable. At several ages, the specimens sexed thus were the exact opposite of what was found histologically.

Chromosomal analyses proved to be the more accurate method of sexing pups. In all but one specimen, the pups were correctly sexed as confirmed by histological examination. The one opossum which could not be sexed in this way was due to a lack of examinable chromosome spreads.

CHAPTER 4.

Histological Study of Gonadal Development.

4.1: Introduction.

The gonads, in all mammals, initially form as thickenings of the mesothelium on the dorso-lateral ridge of the mesonephros. The mesonephros is the intermediate fetal kidney which is functional in some eutherians (pig, sheep, cattle, cat, rabbit: Grinsted, 1981, 1982; Grinsted & Aagesen,1984) and all marsupials (Tyndale-Biscoe & Renfree, 1987), although in several eutherians it is never more than a rudimentary structure (mouse, rat, man: Grinsted & Aagesen, 1984). The mesonephros is very important in the gonadal differentiation in many species and has been shown to influence the development of these organs in several ways: it donates cells to the gonad of both sexes, secretes substances which affect the onset of meiosis, influences the gonadal sex differentiation of mice *in vitro* (Nikitin & Byskov, 1981) and also affects the steroid production of fetal and neonatal rabbits (Grinsted & Aagesen, 1984).

There are three constituents of the indifferent gonad: the investing mesothelium, the gonadal blastema and the primordial germ gells (PGCs) (Peters & McNatty, 1980; Byskov, 1986). The blastema (the derivation of which is still controversial but could be the mesonephros, the mesothelium or both (Byskov, 1986)) develops behind this mesodermal thickening, so making it protrude into the body cavity as the gonadal anlage (Byskov, 1986). PGCs may be present in the mesothelium before the blastema arises (mouse: Byskov, 1986), or may not appear in the gonadal rudiment until around birth (bandicoots: Ullmann, 1989).

The PGCs originally appear extragonadally, normally in the yolk-sac endoderm and migrate through the dorsal mesentery to the gonads and can be followed using their characteristically high alkaline phosphatase enzyme activity. Several methods of transportation have been suggested including active movement, morphogenic movements of the tissues they are embedded in and chemotaxis (reviewed in Byskov, 1986). Many PGCs get "lost" during their migration and can end up almost anywhere in the embryo. The vast majority of

these "lost" PGCs disappear soon after sexual differentiation and those that remain may cause problems, in the form of teratomas and other tumours which may be fatal (Byskov, 1986). The PGCs within the presumptive gonad proliferate mitotically, lose their enzyme activity and are given the suffix -gonia (oogonia, spermatogonia). The blastema is also very active mitotically and so quickly increases the size of the gonad. It is at this point gonadal sex differentiation is initiated.

Invariably, in mammals, the testis is the first of the gonads to differentiate. Four morphological events take place: testicular cords are formed, enclosing the germ cells along with Sertoli cells; Leydig cells differentiate outwith the cords; and a *tunica albuginea* develops (Byskov, 1986). The testis also becomes more rounded in shape. The latter is thought to occur to reduce the feminizing mesonephric influence on the gonad (Nikitin & Byskov, 1981; Grinsted & Aagesen, 1984)). As a result of the aforementioned facts, a developing gonad is recognized as an ovary when none of these testicular characteristics are present, implying that: "if it is not a testis, it must be an ovary" (Byskov, 1986).

Ovarian differentiation usually occurs several days after that of the testis (2 to 4 days in the mouse: Rugh, 1968; Dairi, 1988). The events leading to the unambiguous recognition of an ovary are the clustering of the germ cells (now called oogonia) around the cortex of the gonad and the initiation of the meiotic prophase. The onset of meiosis, relative to sexual differentiation however, differs in some species and is discussed more fully in section 1.10.

The gonadal development of most eutherians available for study has been described. For common laboratory species, such as the mouse, accounts have been given of sex differentiation both *in vivo* (Rugh, 1968) and *in vitro* (MacKay & Smith, 1985, 1986). The gonadal differentiation of several marsupial species, though they are rarely laboratory bred, has been described in some detail. The species studied so far have been: *Bettongia gaimardi, Dasyurus viverrinus,*

Didelphis virginiana, Isoodon macrourus and *Perameles nasuta, Macropus eugenii, Monodelphis domestica* and *Potorous tridactylus* (for references see section 1.6).

McCrady (1938) published a complete account of the embryology of the Virginia opossum (*Didelphis virginiana*) and found that at 13 days *post coitum* (stage 34, one half-day before birth) the gonads were still undifferentiated. By birth, however, the testis was recognisable, having acquired testicular cords and a *tunica albuginea*, although the ovary remained indifferent.

The work described in this chapter refers to *Monodelphis domestica* and deals mainly with the ovary. The testis is already differentiated at birth and has previously been described at that stage by Baker *et al.* (1990). In this study, too few embryonic specimens were available to give a description of that event. The gonadal sex differentiation of the ovary is described for the first time and this is also the first histological study to be undertaken on the normal postnatal gonadal development of *M. domestica*.

4.2: Prenatal Gonadal Development.

The embryos obtained at approximately 8 days *post coitum* (n = 7) contained very few, poorly developed structures. No mesonephroi or gonadal ridges were evident and the somites were just beginning to develop. The embryos of approximately 13 days *post coitum*, however, displayed obvious gonadal ridges. These appeared as small protuberances of mesothelial and, possibly, some mesonephric cells overlying several of the mesonephric glomeruli (Figure 10). At its maximum dimension, estimated from serial sections, the gonadal ridge was approximately one-third the length of the mesonephros.

At birth, 13.5 days *post coitum* or day 0 (n = 11), the testis was already obvious, whereas the ovary showed no signs of differentiation. A great size difference was also apparent (Figure 11); the testis was consistantly larger than the presumptive ovary.

4.3: Testicular Development.

The testicular mesothelium in day 0 specimens (n = 3; Figure 12) appeared to be very flattened except at the distal tip where it was more cuboidal in shape and had several PGCs embedded in it. An almost continuous *tunica albuginea*, 1 to 4 cells thick, was present. The testicular cords were well developed, each with a single boundary layer of flattened, sustentacular cells. PGCs were found within the cords, although they were by no means obvious amongst the similar looking pre-Sertoli cells. Leydig cells could not be distinguished amongst the other somatic cells.

By day 2 (n = 11; Figure 13) the mesothelial layer of the testis was entirely flattened and almost completely devoid of PGCs. The *tunica albuginea* had become continuous and had widened to between 2 and 7 cells in thickness.

The putative *rete testis* was possible to trace in day 3 specimens (n = 6). By day 4 (n = 1; Figure 14a), the *rete* was obvious and could be followed from a tubular structure in the degenerating mesonephros to connect with the testicular cords in the gonad (Figure 14b). The cords were all patent, had enlarged to occupy most of the gonad and had aquired several surrounding layers of sustentacular cells. The germ cells within the cords lay mainly internal to the Sertoli cells. Outwith the cords, the Leydig cells or their precursors could be identified: they contained a very granular nucleus which lacked nucleoli.

From day 4 to day 60, the oldest male pups studied (total n = 35, Appendix 2; Figure 15), several differences were found: the testis had greatly increased in size, the testicular cords almost completely filled the gonad and the Leydig cells had become more obvious between the cords. The epididymes were apparent by day 49 (n = 1). The majority of spermatogonia in day 60 specimens (n = 3) lay on the basement membrane of the cords, intermingled with the Sertoli cells (the adult condition). The epididymes in these pups were obviously separated into *caput* and *cauda* (Figure 16).

4.4: Ovarian Development.

4.4.1: General Development and Folliculogenesis.

The only developments of the ovarian rudiment in the first five days of neonatal life (total n = 27, Appendix 2) were : the ovarian rudiment had increased in size, although it was was still much smaller than the testis of the same age, the germ cells had mainly become distinguishable as oogonia and a discontinuous *tunica albuginea ovarii* of one to two cells thick had appeared. Apart from the latter structure the ovary, at day 5, still appeared undifferentiated (Figure 17). In contrast to the male condition, the mesothelium surrounding the gonadal rudiment was cuboidal, apparently loosely connected, and contained many primordial germ cells. The hilus, connecting the ovary to the mesonephros, was broader than in the testis at the same stage.

Conspicuous ovarian differentiation started in day 6 pups (n = 3), in which the oogonia had begun to congregate in the periphery of the ovary so forming a rudimentary cortex.

From day 6 until day 14, the only observed development of the ovary was an increase in size and the number of germ cells accumulating in the periphery of the gonad. The cortex was relatively expansive in comparison with the medulla, though these regions were not well delineated (Figure 18). Meiosis commenced on day 14 and connective tissue septae had begun to delimit germ cell "nests" (Figure 19).

Between days 14 and 26 small cords had developed in the medulla ("medullary cords") and several of these cords appeared to infiltrate the aforementioned nests (Figure 20). At day 29 several of the more central oogonia were surrounded by cells from the medullary cords, so forming the first type 2 (primordial) and even type 3a (early primary) follicles (Figure 21).

Folliculogenesis proceeded centrifugally, the more central follicles reaching type 3b (late primary) by day 35, type 4 (early secondary) by day 44, type

5a (mid-secondary) by day 49 and type 5b (late secondary) by days 54 to 56. By day 60 the majority of the outermost oogonia had become at least type 2 follicles (Figure 22).

In the adult opossum ovary (n = 9) the mesothelium was almost completely flattened, with an intact basement membrane, and enclosed several PGCs. At its distal aspect, the ovarian mesothelium in some specimens, was cuboidal in shape. A *tunica albuginea ovarii* of 2 to 4 cells thickness was observed, but appeared to be discontinuous. Very few type 2 (primordial) follicles were apparent, but all other stages up to types 6/7/8 (tertiary, antral follicles) were observed (Figure 23). Corpora lutea were present in 7 of the 9 specimens. These structures measured up to 550 μm in diameter and consisted of two cell types: the granulosa lutein cells, and the theca lutein cells (Figure 24). The medulla of the ovary (Figure 25) was ill-defined and contained medullary cords, several large blood vessels and stromal cells. Interstitial cells could also be distinguished between follicles: these cells were similar in appearance to the granulosa lutein cells, and occurred in small aggregations.

4.4.2: Development of the *Rete Ovarii.*

The *rete ovarii* was first observed in day 5 pups, before ovarian differentiation had begun, as a condensation of columnar cells in the hilar region of the gonad (Figure 26). By day 10, the *rete* had become patent and its diameter had decreased and by day 13 it had been invested with a flattened cellular wall of one to two cells in width and had begun to infiltrate the ovary (Figure 27).

The infiltration of the *rete* into the gonad appeared to be at its most expansive between days 26 and 29 where it reached around two-thirds of the ovarian length. In the day 26 pups, the connecting *rete* contained cellular debris in its lumen. Several of the day 29 specimens had a *rete* which was observed almost to spiral through the hilus into the gonad. Although by day 29 medullary cords

were present in the gonad, connections between these and the blind-ending *rete ovarii* could not be distinguished (Figure 28).

At day 35, several days after folliculogenesis had begun, the *rete* was observed to abut onto the medullary cords although the cellular wall of the former always remained intact (Figure 29). This state was maintained up to day 65 (the oldest female pups examined).

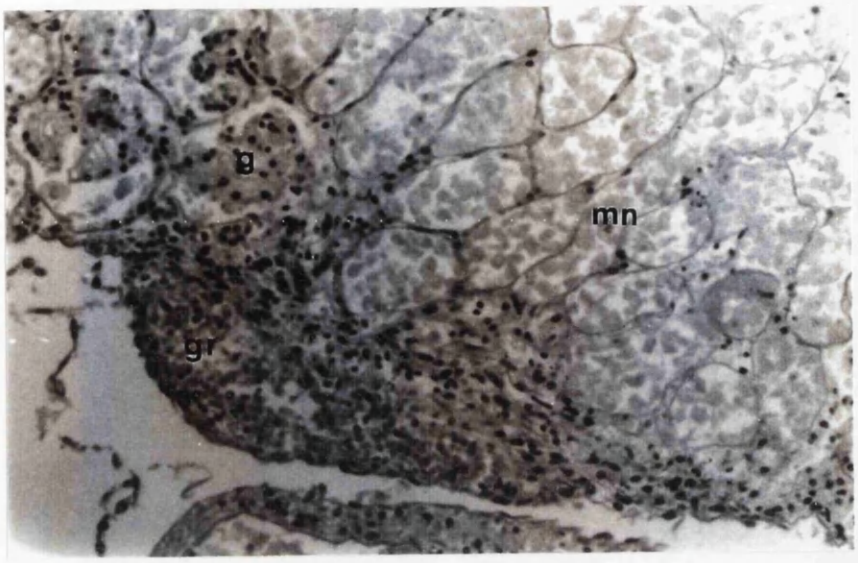
The *rete ovarii* could not always be distinguished in the adult gonads. In the 6 specimens which did demonstrate the structure, however, it appeared to be less conspicuous than in any of the younger specimens. The *rete* in the adult was observed to consist of a single, patent tube in the hilar region of the gonad, with no obvious connections to either the medullary cords or any of the follicles present.

During the entire development of the *rete ovarii*, no associations were observed between the *rete* and either mesonephric tubules or glomeruli.

ABBREVIATIONS USED IN FIGURES.

2	-	type 2 follicle	m	-	medulla
3a	-	type 3a follicle	mc	-	medullary cords
3b	-	type 3b follicle	mn	-	mesonephros
4	-	type 4 follicle	mt	-	metanephros
5a	-	type 5a follicle	o	-	ovary
5b	-	type 5b follicle	og	-	oogonia
6	-	type 6 follicle	ro	-	*rete ovarii*
7	-	type 7 follicle	rt	-	*rete testis*
af	-	atretic follicle	s	-	Sertoli cell
c	-	cortex	sg	-	spermatogonia
cts	-	connective tissue septae	st	-	stroma
g	-	glomerulus	t	-	testis
gc	-	germ cell(s)	ta	-	*tunica albuginea*
gcn	-	germ cell nests	tao	-	" " *ovarii*
gl	-	granulosa lutein	tc	-	testicular cord
gr	-	gonadal ridge	ti	-	theca interna

Figure 10.



Embryonic gonadal ridge of 13 days gestation (x 250). Note the lack of any internal organization.

Figure 11a:

Neonatal testis (x 300) showing testicular cords and a *tunica albuginea*. Compare the size with that of the ovary in figure 11b.

Figure 11b:

Undifferentiated neonatal ovary (x 300).

Figure 11a.

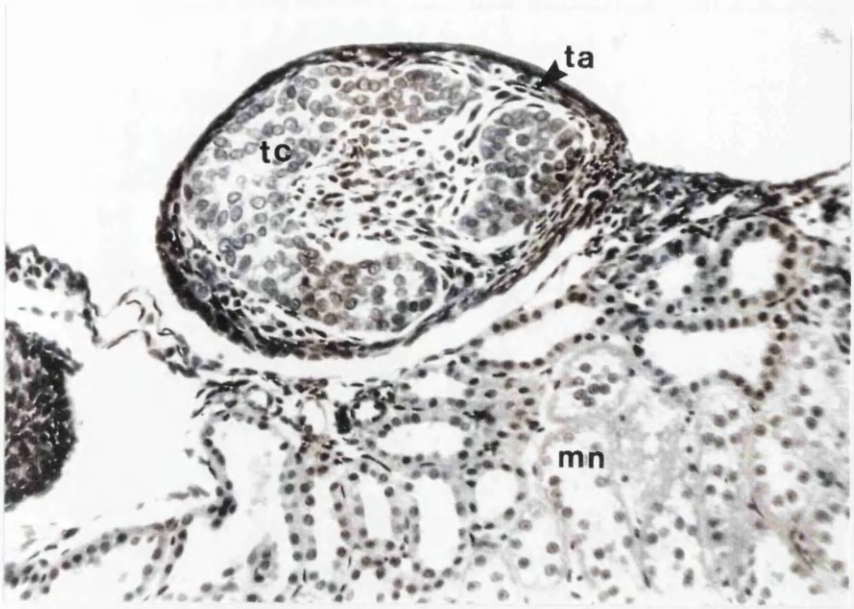


Figure 11b.

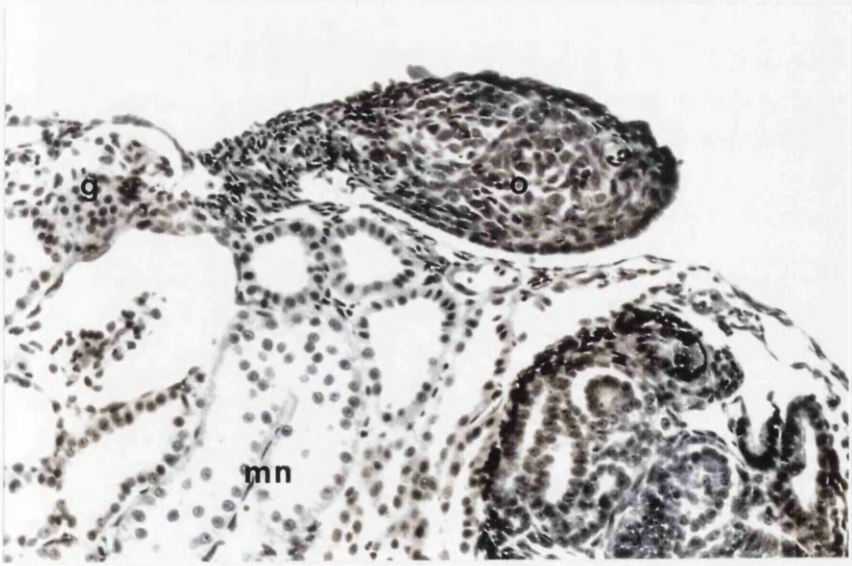


Figure 12:

Neonatal testis (x 500). A *tunica albuginea* and testicular cords are present. The germ cells and Sertoli cells within the cords are difficult to distinguish from each other. A PGC is seen to be trapped in the mesothelial layer (arrow).

Figure 13:

Day 2 testis (x 300), showing a continuous *tunica albuginea* and clear demarkation of the cords by sustentacular cells (arrows).

Figure 12.

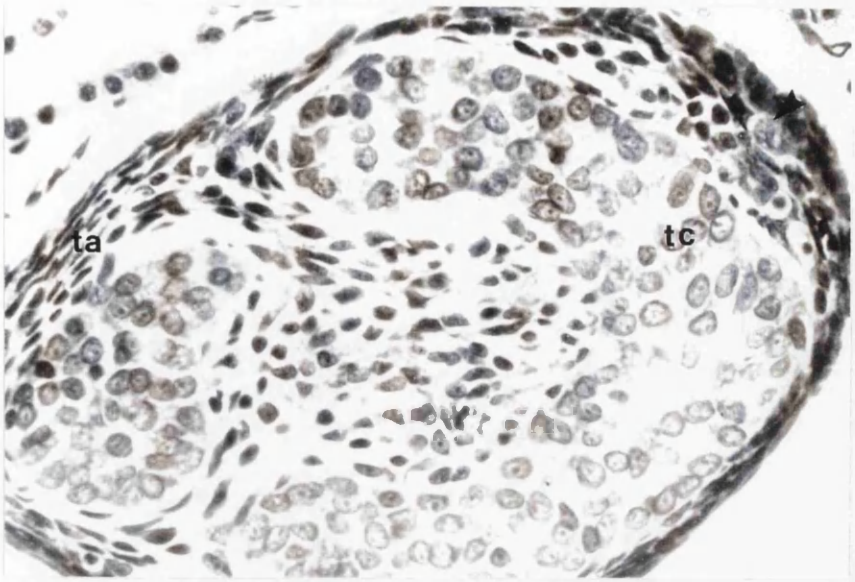


Figure 13.

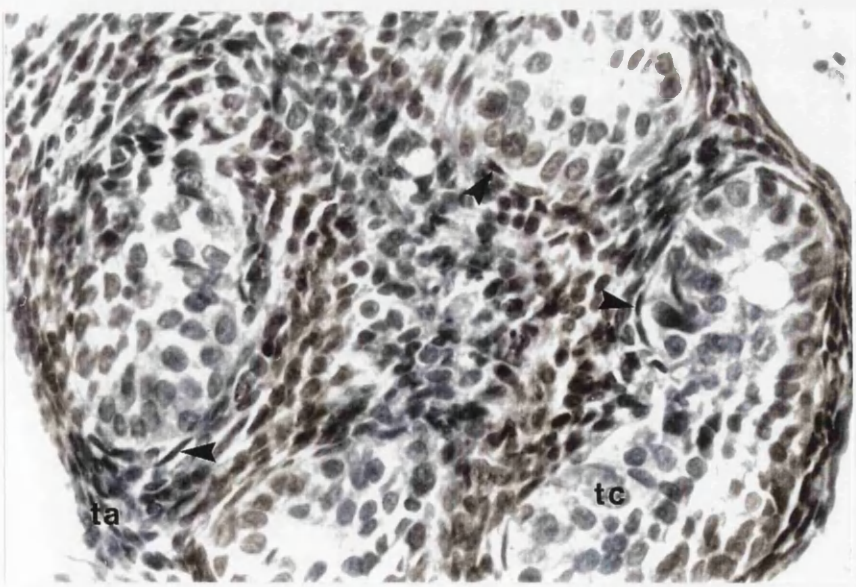


Figure 14a:

Day 4 testis (x 250) showing the patency of the testicular cords.

Figure 14b:

Day 4 testis (x 300) showing the *rete testis* contact the testicular cords, presumptive Leydig cells and the distinction between Sertoli cells and germ cells in the cords.

Figure 14a.

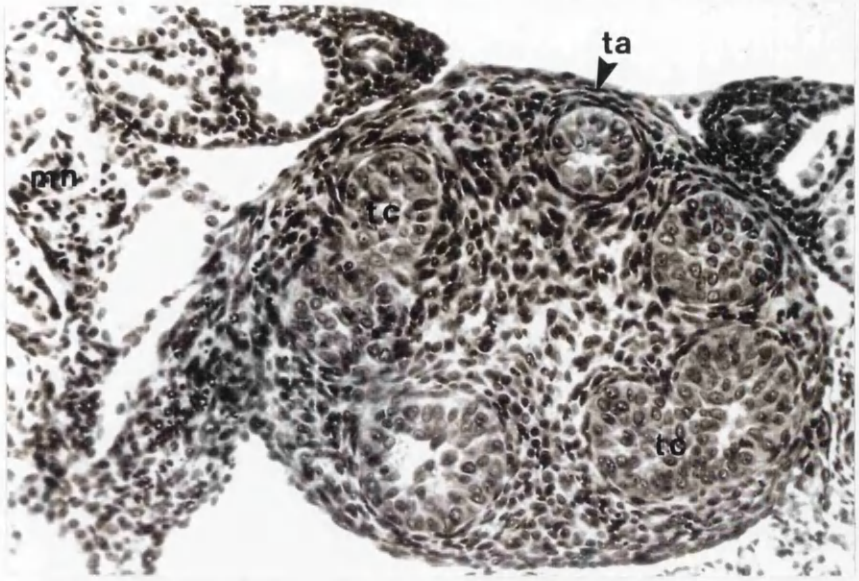


Figure 14b.

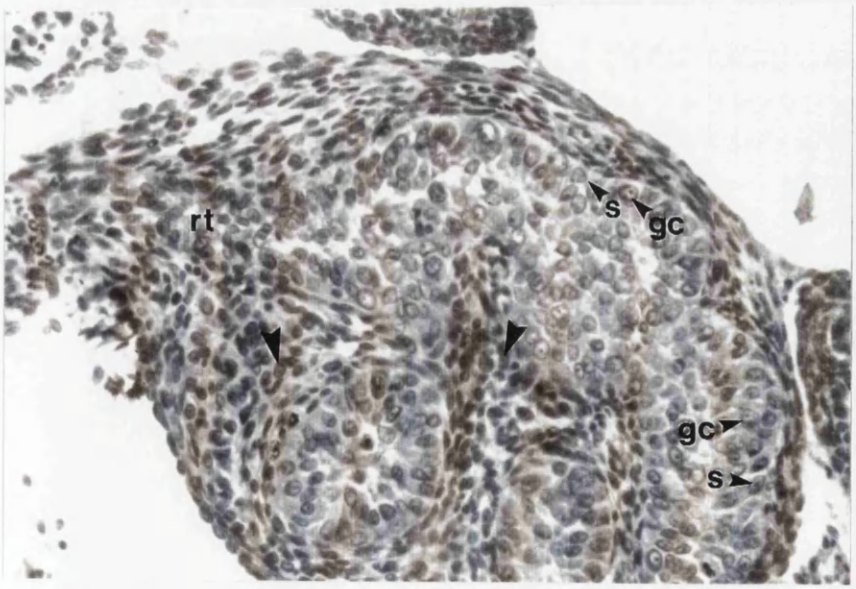


Figure 15:

Day 60 testis (x 300) showing the extent of cord formation. Spermatogonia lay on the basement membrane of the cords, intermingled with Sertoli cells. Leydig cells can be seen between the cords.

Figure 16:

Section from a day 60 male (x 125) to show the *caput* (cp) and *cauda* (cd) *epididymes*.

Figure 15.

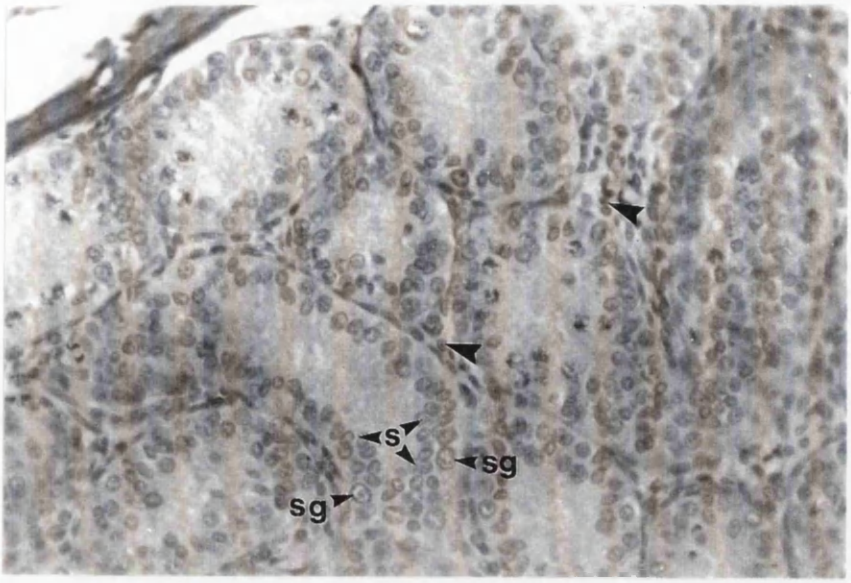


Figure 16.

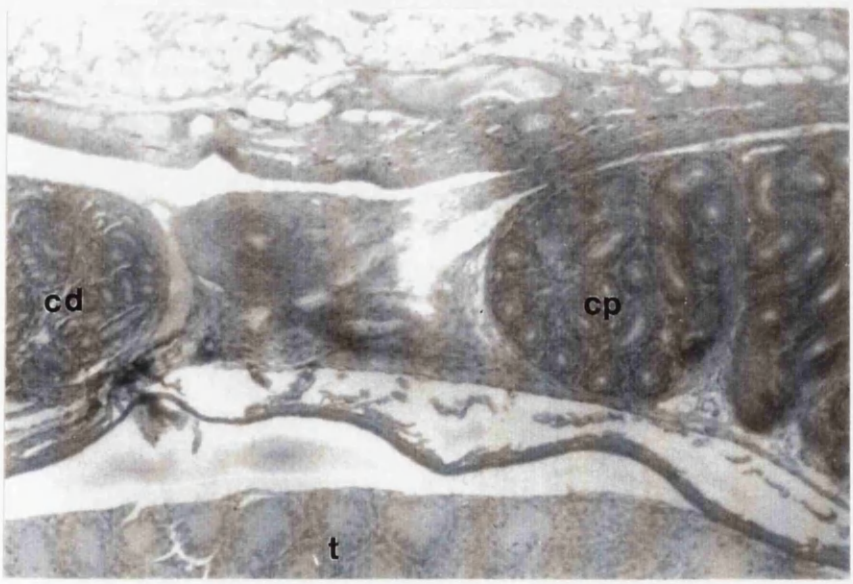


Figure 17a:

Day 5 ovary (x 125) showing a lack of organization.

Figure 17b:

Detail of figure 18a (x 250) showing a discontinuous *tunica albuginea ovarii* and recognizable oogonia. A PGC (arrow) can be seen embedded in the ovarian mesothelium.

Figure 17a.

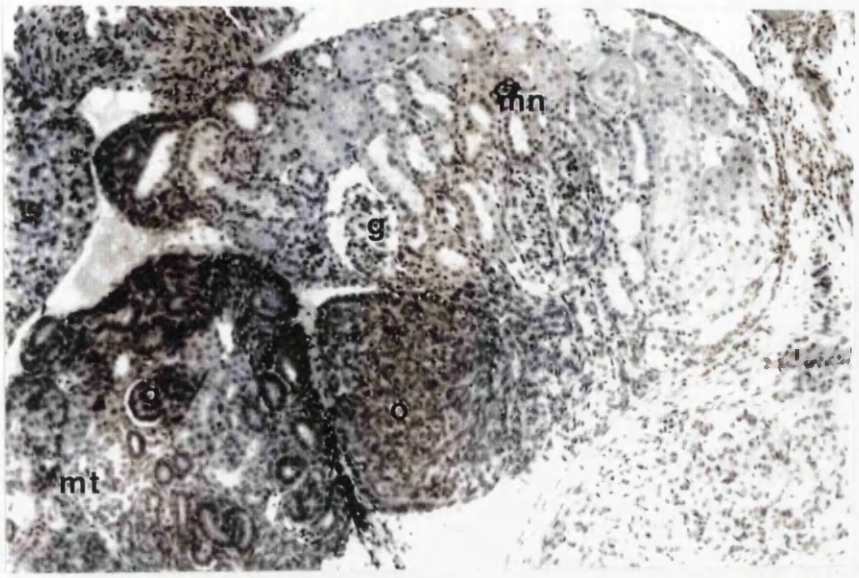


Figure 17b.

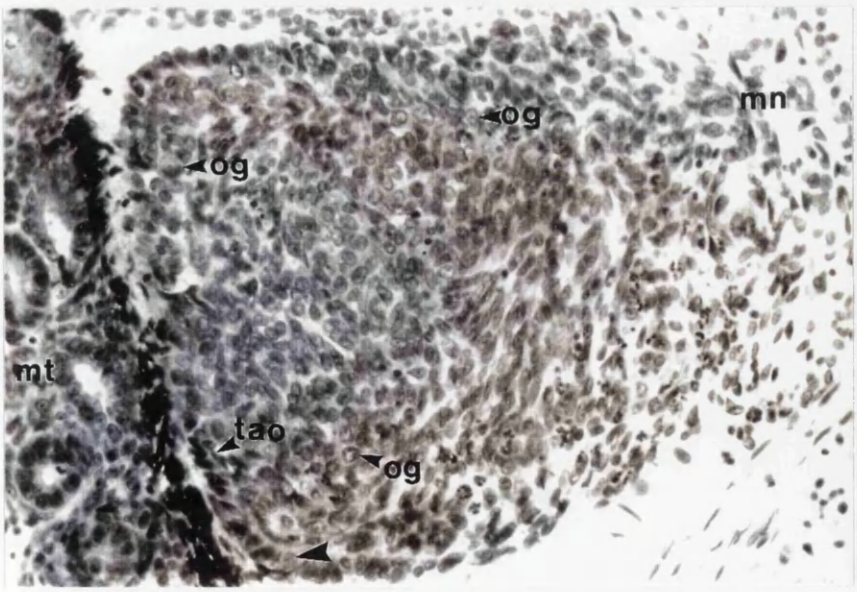


Figure 18:

Day 13 ovary (x 250) showing organization into an indistinct cortex and medulla. Several PGCs are still recognizable in the cortex.

Figure 19:

Day 14 ovary (x 300) showing the division of the oogonia into germ cell nests by connective tissue septae. Several meiotic germ cells are apparent (arrows).

Figure 18.

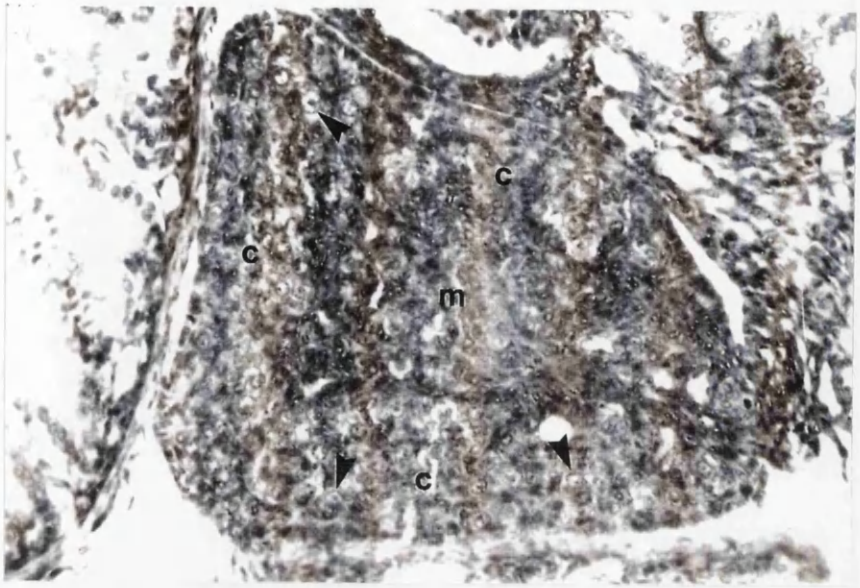


Figure 19.

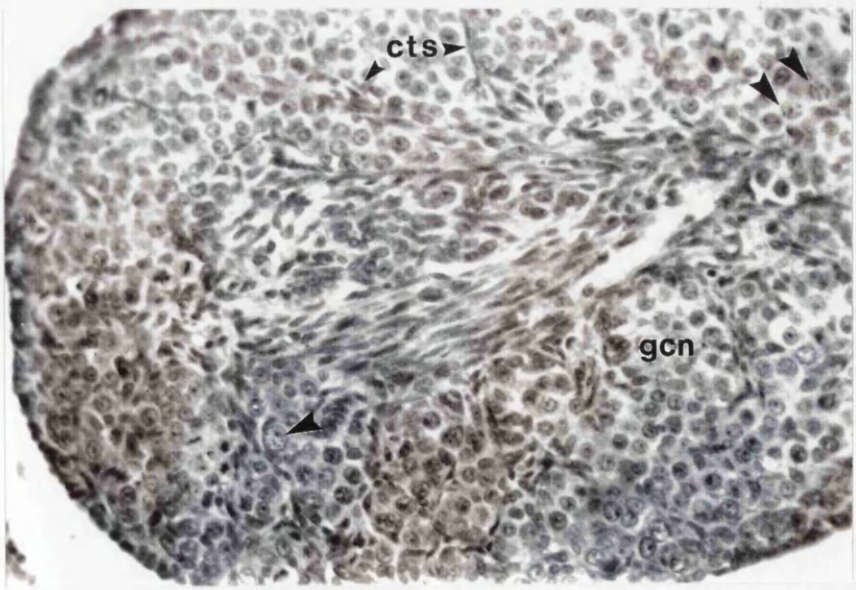


Figure 20:

Medulla and inner cortex of a day 26 ovary (x 300) to show medullary cords infiltrating the germ cell nests.

Figure 21:

Medulla and inner cortex of a day 29 ovary (x 300) to show the first formation of type 2 and type 3a follicles.

Figure 20.

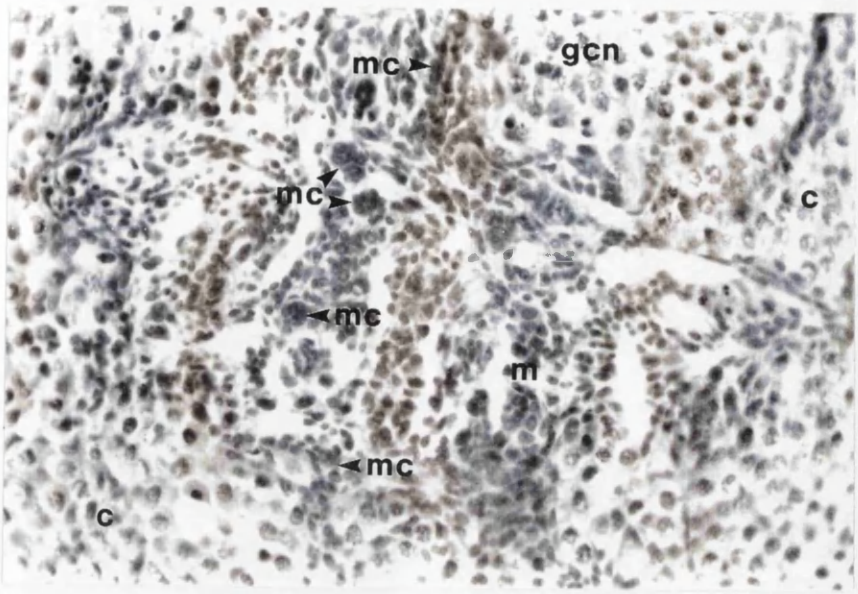


Figure 21.

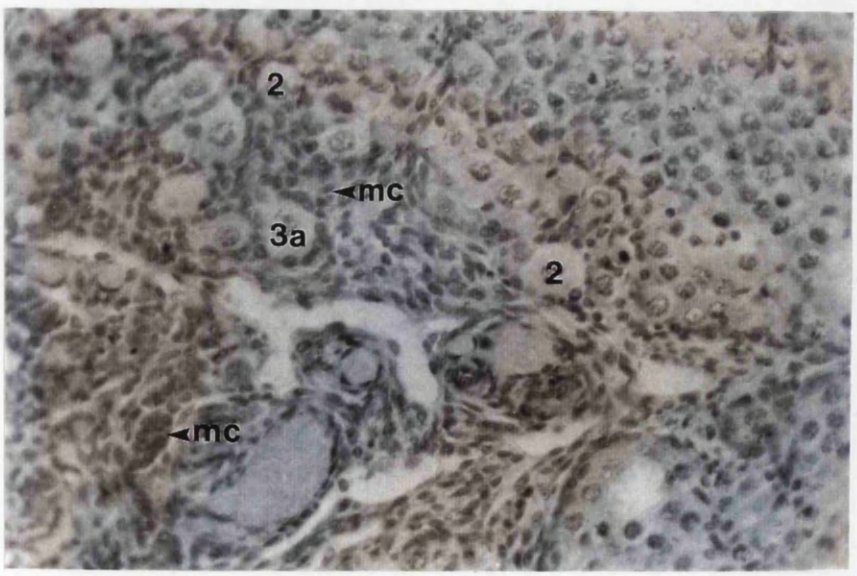


Figure 22:

Day 60 ovary (x 300) showing a range of follicle types.

Figure 23:

Adult ovary (x 125) showing a range of follicle types.

Figure 22.

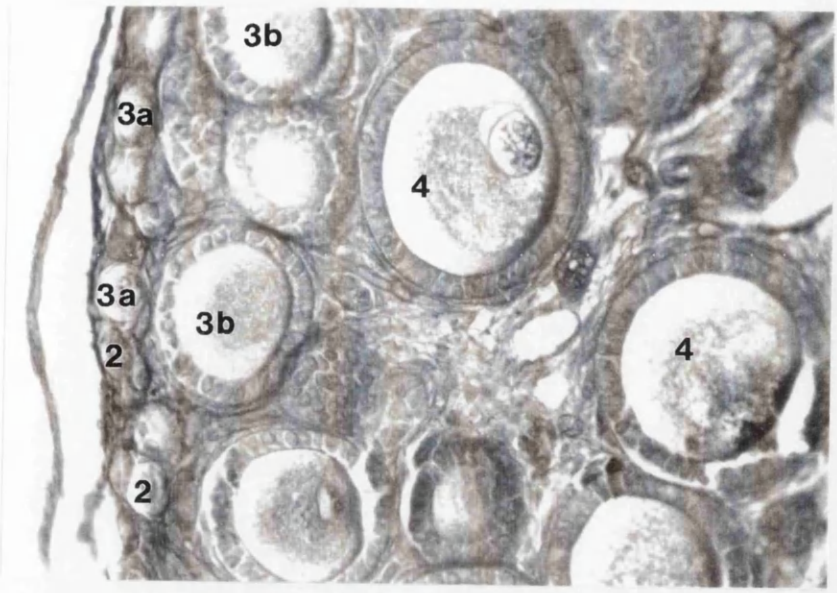


Figure 23.

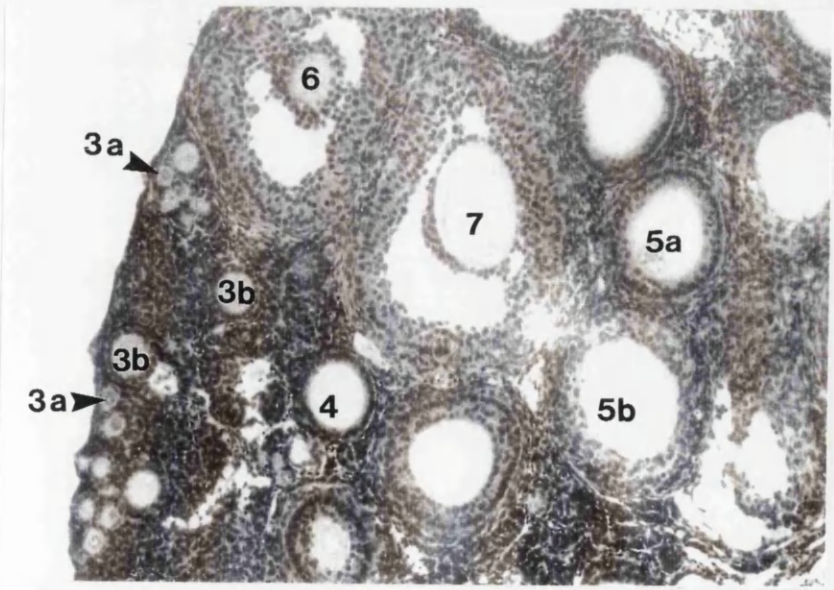


Figure 24:

Corpus luteum in an adult ovary (x 125). Distinct granulosa lutein and theca lutein cells are visible.

Figure 25:

Medullary region of an adult ovary (x 125) showing the stroma and interstitial cells.

Figure 24.

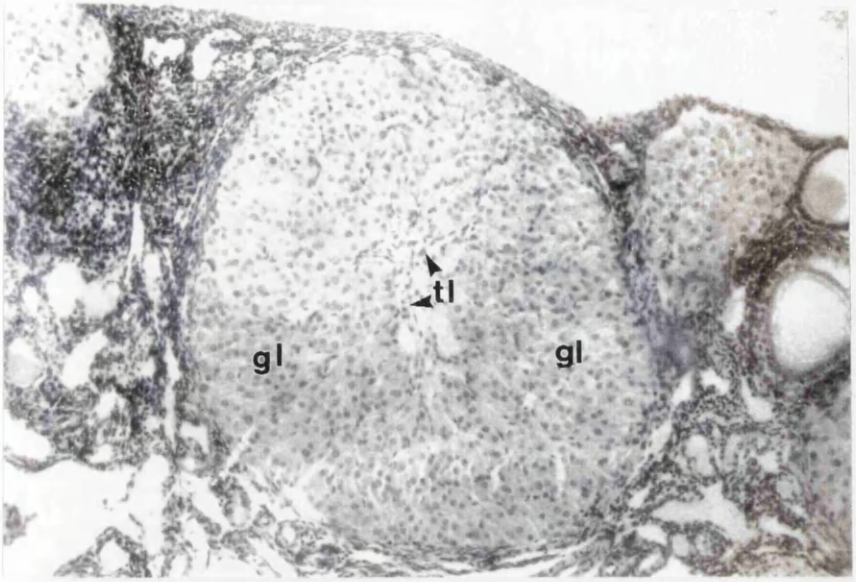


Figure 25.

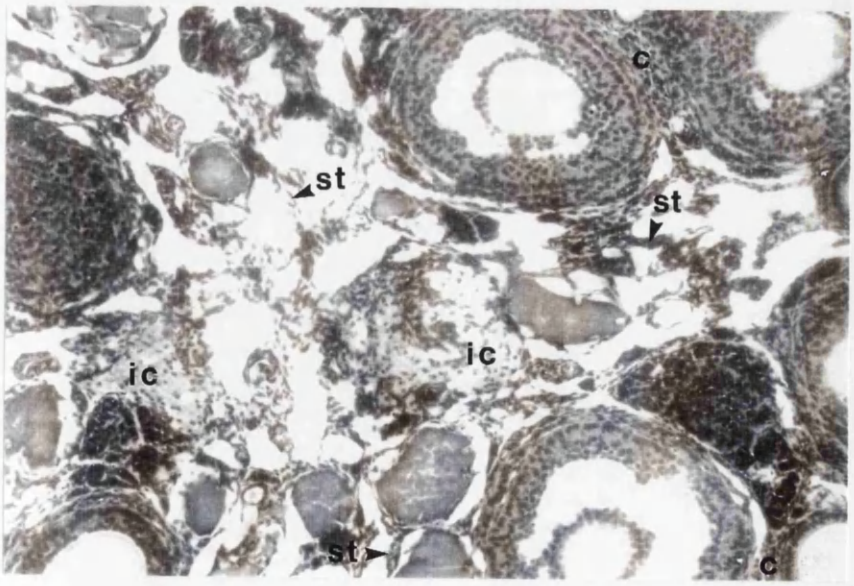


Figure 26:

Anterior gonadal ridge of a day 5 female (x 300). The *rete ovarii* can clearly be seen as a condensation of columnar cells.

Figure 27:

Day 13 ovary (x 300) with the patent *rete ovarii* prominent. The *rete* has been invested with a surrounding layer of flattened cells (arrows).

Figure 26.

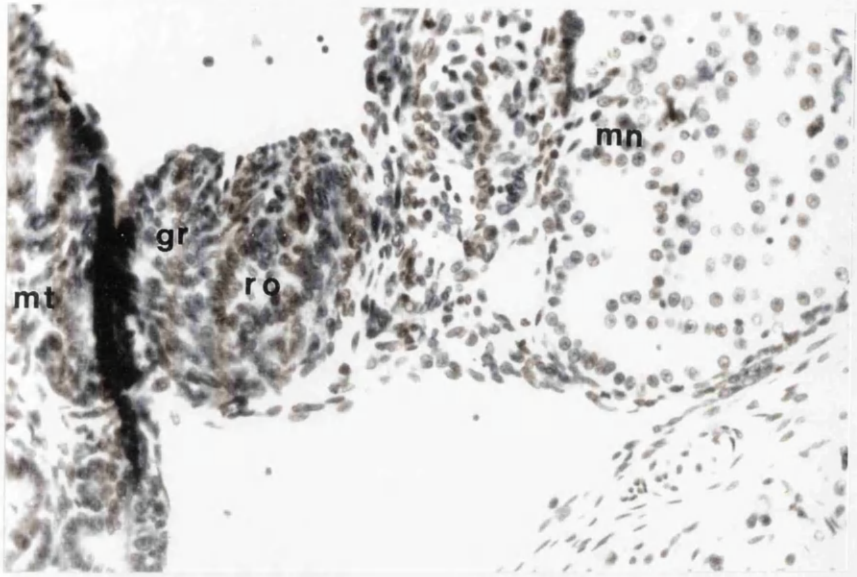


Figure 27.

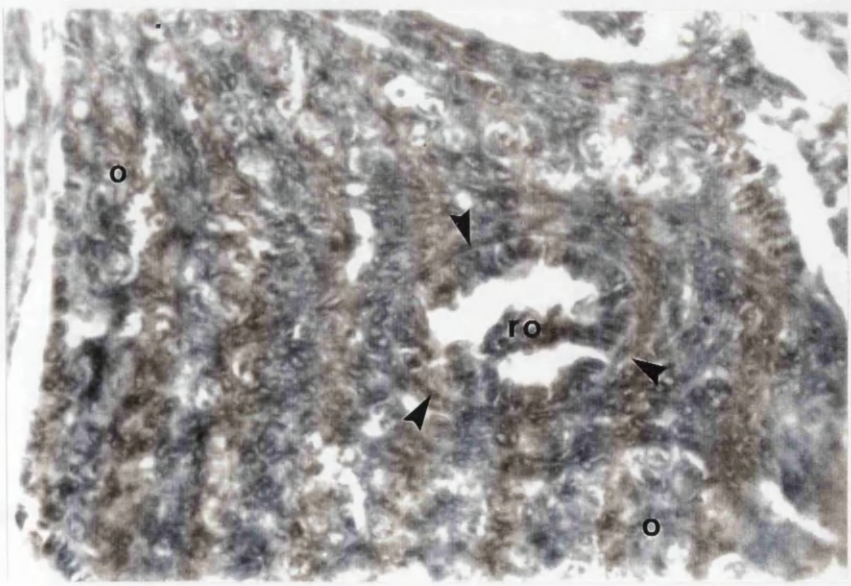


Figure 28:

Medulla of a day 29 ovary (x 300) showing the distal part of the *rete ovarii*. Medullary cords are also present but do not make contact with the *rete*.

Figure 29:

Medulla of a day 35 ovary (x 300) showing the *rete ovarii* with its complete cellular wall (arrows). Medullary cords are present but do not contact the *rete* in this section.

Figure 28.

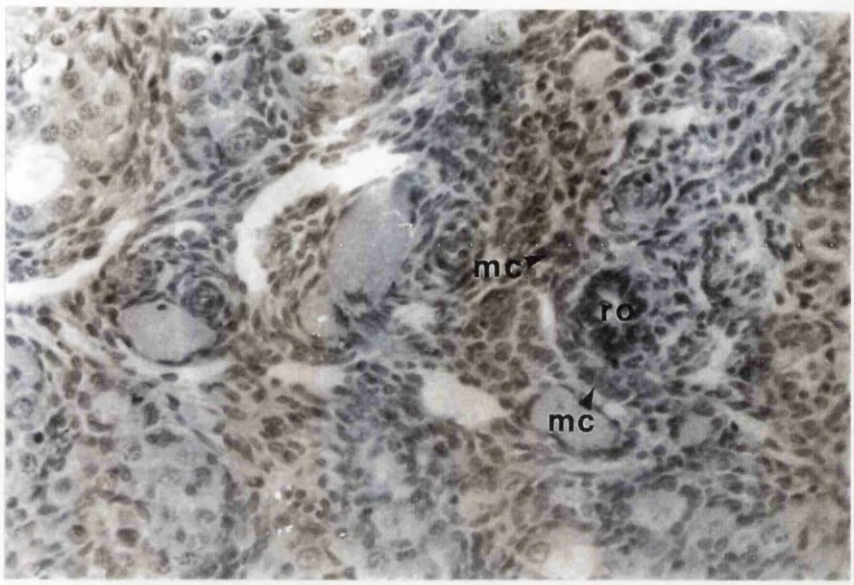
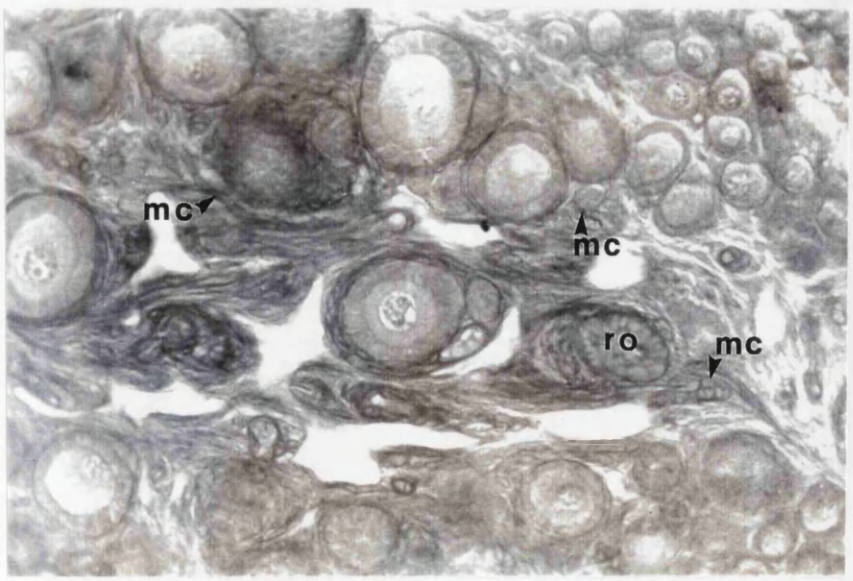


Figure 29.



4.6: Discussion.

4.6.1: Prenatal Gonadal Development.

The embryological specimens obtained, at days 8 and 13 of gestation, appeared to be very similar histologically to specimens of the same age described by McCrady (1938) in *Didelphis virginiana*. Early on day 13 of gestation (stage 34: McCrady, 1938), when these specimens were collected, there were no obvious signs of gonadal differentiation. On the day of birth however (day 13.5 of gestation or day 0 of pouch life), the testis was differentiating and showed signs of testis cord formation and a discontinuous *tunica albuginea*. These findings confirmed those of Baker *et al* (1990), as well as supporting their statement on the similarities of *M. domestica* and *D. virginiana*. The gonadal development of *Monodelphis* and *Didelphis* at birth resembles that of a 12 day mouse fetus (Rugh, 1968; Dairi, 1988). Moore & Thurstan (1988, 1990), however, stated that *Monodelphis* testes did not differentiate until day 3, which was repudiated by both this study and that of Baker *et al.* (1990).

The facts that ovarian differentiation occurs several days after birth and the pups are readily available for experimentation imply that *Monodelphis domestica* is an especially good model for ovarian studies of all kinds.

4.6.2: Testicular Development.

The postnatal development appeared to follow closely that described for most eutherians (Clegg & Clegg, 1975; Pelliniemi, 1975; Pehlemann & Lombard, 1978; Pelliniemi *et al.*, 1979; Peters & McNatty, 1980; Johnson & Everitt, 1983; MacKay & Smith, 1985, 1986; Byskov, 1986) as well as marsupial species previously studied (McCrady, 1938; Renfree *et al.*, 1987; Tyndale-Biscoe & Renfree, 1987; Shaw *et al.*, 1988; Short *et al.*, 1988; Tyndale-Biscoe & Janssens, 1988).

Unfortunately, the "arbitrary event" of birth occurs too late in *Monodelphis* to study testicular differentiation postnatally. Embryonic studies have to be undertaken to describe this occurrence in this species (Baker *et al.*, 1990). Therefore, it is suggested that, although *Monodelphis* displays many of the desirable marsupial characteristics for ease of study, better marsupial models could be used in the examination of male gonadal differentiation. These species would be those in which the gonads become differentiated after birth (*Dasyurus viverrinus*: Ullmann, 1984; *Isoodon macrourus, Perameles nasuta*: Ullmann, 1981b; *Macropus eugenii*: Alcorn, 1975; Renfree *et al.*, 1987; Dairi, 1988).

4.6.3: Ovarian Development.

The development of the ovary in *Monodelphis* was comparable, in various respects, to several other species, both eutherian and metatherian. Firstly, in common with all other mammals so far studied (Peters & McNatty, 1980; Byskov, 1986; Renfree *et al.*, 1987), ovarian development lagged behind that of the testis by several days.

The granulosa cells in the present study, in contradistinction to the majority of eutherians studied, were observed to be derivatives of the medullary cord cells which differentiated from the ovarian blastema, as in the bandicoots (Ullmann, 1989), potoroo and bettong (*Bettongia gaimardi*: Dairi, 1988).

Monodelphis domestica, similarly to the potoroo, bettong and the bandicoots, appeared to have ovaries of the "immediate meiosis" type, that is, the germ cells were not partitioned into cords before meiosis began. The bandicoots' oogonia, however, did not enter meiosis until 2 to 3 weeks after ovarian differentiation (Ullmann, 1989). In this respect, *Monodelphis domestica* resembles the bandicoots rather than the macropods: meiosis did not begin until 8 days after the ovary had differentiated. The steroidogenesis of *Monodelphis* ovaries, in relation to the onset of meiosis, will be discussed in the following

chapter.

Histologically, it could not be determined in *Monodelphis domestica* whether or not the mesonephros plays a part in gonadal development as it does in many eutherians *via* the *rete* system (Wartenberg, 1981; Byskov *et al.*, 1985; Byskov, 1986). Marsupial mesonephroi at birth, contrary to the case of eutherians, are functional excretory organs (Tyndale-Biscoe & Renfree, 1987) and thus, it is not surprising that they do not contribute to the gonadal development (Alcorn, 1975; Ullmann, 1984, 1989). In embryonic eutherians, it has also been shown that the mesonephros does not influence gonadal differentiation until its excretory function has ceased (Grinsted, 1981; Grinsted & Aagesen, 1984).

The *rete ovarii* in *Monodelphis* appeared to develop similarly to that in the bandicoots (*Isoodon macrourus & Perameles nasuta*: Ullmann, 1989), the potoroo (*Potorous tridactylus*: Dairi, 1988) and the eutherian Galago (*Galago crassicaudatis crassicaudatis*: Yoshinaga *et al.*, 1988). It first appeared in the hilar region of the gonad as a condensation of columnar cells. This structure then infiltrated the gonad proper and became patent. In day 35 specimens of *Monodelphis*, the *rete* abutted onto several of the already present medullary cords, but remained intact at all times. In the mesonephros of *Monodelphis*, as in the bandicoots and Galago, the *rete* only makes secondary connections to the degenerating glomeruli and mesonephric tubules. This contrasts with the condition in the mouse, cat, mink and ferret where the *rete* is formed by cells of the degenerating mesonephros, infiltrates the gonad and then breaks down to forms the granulosa cells where it contacts oogonia (Byskov, 1975, 1978, 1986).

CHAPTER 5.

Study of Δ^5, 3β-Hydroxysteroid Dehydrogenase.

5.1: Introduction.

The major steroids produced in the gonads, progestagens, androgens and oestrogens, are all indirect derivatives of cholesterol which gives them their basic four-ring (cyclopentanoperhydrophenanthrene) structure (Peters & McNatty, 1980). Cholesterol is found in lipid droplets within the steroid-producing cells and is converted to the different steroids by numerous enzymes (Baird, 1977, Peters & McNatty, 1980, Brodie, 1983, Johnson & Everitt, 1983).

Δ^5,3β-hydroxysteroid dehydrogenase (3β-HSD) is one of the essential enzymes in the biosynthesis of steroid hormones (Wattenberg, 1958; Levy *et al.*, 1959; Ferguson, 1965; Goldman *et al.*, 1965, 1972; Presl *et al.*, 1965; Pupkin *et al.*, 1966; Schlegel *et al.*, 1967; Goldman & Kohn, 1970; Mori & Matsumoto, 1970; Hoyer & Andersen, 1977; Peters & McNatty, 1980; Hoyer & Byskov, 1981; Brodie, 1983; Johnson & Everitt, 1983; Brook & Clarke, 1989; Sasano *et al.*, 1990a, 1990b) which acts by oxidising the hydroxyl group at the 3β- position, so producing a ketone group (Wattenberg, 1958; Ferguson, 1965; Bjersing, 1967; Goldman *et al.*, 1972; Peters & McNatty, 1980; Troyer, 1980; Brodie, 1983; Johnson & Everitt, 1983; Brook & Clarke, 1989). This means that pregnenolone, dehydroisoandrosterone (DHA) and androstenediol are respectively converted to progesterone, androstenedione and testosterone.

Wattenberg (1958) first demonstrated 3β-HSD as evidence of steroid synthesis in ovaries of the mouse, rat, rabbit and human. In 1959 Levy *et al.*, working on the rat, made major modifications to the technique. Since then 3β-HSD has been studied by many authors on various eutherian mammals using the exact methods of Wattenberg (1958) and Levy *et al.* (1959) or minor modifications of either (mouse: Ferguson, 1965; Hart *et al.*, 1966; Müller, 1975; Hoyer & Byskov, 1981; wood mouse: Brook & Clarke, 1989; rat: Presl *et al.*, 1965; Pupkin *et al.*, 1966; Schlegel *et al.*, 1967; Goldman & Kohn, 1970; rabbit: Mori & Matsumoto, 1970; Goldman *et al.*, 1972; George *et al.*, 1979; Grinsted *et*

al., 1982; pig: Bjersing, 1967; human: Goldberg *et al.*, 1963; Goldman *et al.*, 1965; Baillie *et al.*, 1966; Seegar-Jones *et al.*, 1968; Nagai, 1985; Sasano *et al.*, 1990). Only one major methodological study has been undertaken, by Hoyer & Andersen (1977), on the rat.

The results which emerged from these analyses show that in the *post partum* eutherian mammal 3β-HSD is found in the corpora lutea, stroma, interstitial cells and theca interna of the follicle (Levy *et al.*, 1959; Ferguson, 1965; Pupkin *et al.*, 1966; Müller, 1975; Brook & Clarke, 1989). Goldman *et al.* (1965), Hoyer & Andersen (1977) and Sasano *et al.* (1990a, 1990b) all reported definite positive enzyme activity in the granulosa cells of type 6/7/8 (tertiary, antral) follicles. The granulosa staining in other studies was reported as either negative (Wattenberg, 1958; Presl *et al.*, 1965; Seegar-Jones *et al.*, 1968; Goldman & Kohn, 1970) or weakly positive in atretic or pre-ovulatory follicles (Levy *et al.*, 1959; Goldberg *et al.*, 1963; Ferguson, 1965; Pupkin *et al.*, 1966; Bjersing, 1967; Schlegel *et al.*, 1967; Müller, 1975; Hoyer & Andersen, 1977; Brook & Clarke, 1989).

Most authors state that fetal ovaries do not contain this enzyme activity, although Goldman *et al.* (1965), Hart *et al.* (1966), Goldman *et al.* (1972) and George *et al.* (1979) working on human, mouse and rabbit respectively, all reported positive staining in the interstitial cells and/or stroma of mid- to late-gestational specimens.

The study of 3β-HSD activity, which is often present in cells before they begin secreting the hormones, can therefore be used to identify the future steroidogenic cells in the gonad, as well as those which have started steroid production (Guraya, 1968a, 1968b; Hoyer & Andersen, 1977; Brook & Clarke, 1989). Nagai (1985) combined studies of ultrastructure and 3β-HSD activity in the granulosa cell of the human ovary and found intermediate stages of ultrastructural development from the secondary follicle to the post-ovulatory follicle (pre-corpus luteum). Nagai (1985) showed that the 3β-HSD activity was present on the cristae and inner membrane of the mitochondria and on the

smooth endoplasmic reticulum. Therefore, it is suggested, in agreement with Pupkin *et al.* (1966) and Schlegel *et al.* (1967), that there is a steroidogenic capability in the granulosa of the pre-ovulatory follicle.

Sasano & Sasano (1989) and Sasano *et al.* (1990a, 1990b) used a polyclonal antibody against human 3β-HSD in the adult human ovary and found that type 2, 3, 4 and 5 (primordial, primary and secondary) follicles showed no enzyme activity. Type 6 (early tertiary, or antral) follicles showed theca interna staining which progressed with follicular development to the granulosa cells. This confirmed the findings of Pupkin *et al.* (1966), Schlegel *et al.* (1967) and Nagai (1985).

The significance of the latter findings (Pupkin *et al.*, 1966; Schlegel *et al.*, 1967; Nagai, 1985; Sasano & Sasano, 1989; Sasano *et al.*, 1990a, 1990b) is clarified when it is taken into account that the corpus luteum, one of the major steroid synthesising tissues of the ovary, is derived mainly from the granulosa cells of the ovulated follicle with the addition of some thecal or stromal elements (Mossman & Duke, 1973; Clegg & Clegg, 1975; Harrison & Weir, 1977; Peters & McNatty, 1980; Baker, 1982; Johnson & Everitt, 1983). This transformation, differentiation or re-differentiation has been noted by many authors and has shown, unequivocally, the origins of some of the ovarian steroidogenic cells.

Marsupials, due to their relatively undeveloped state at birth and availability for experimentation as pouch young (Burns, 1941; Alcorn, 1975; Catling & Vinson, 1976; Alcorn & Robinson, 1983; George *et al.*, 1985; Renfree *et al.*, 1987; Tyndale-Biscoe & Renfree, 1987; Dairi, 1988; Hutson *et al.*, 1988; Moore & Thurstan, 1988, 1990; Shaw *et al.*, 1988; Short *et al.*, 1988; Tyndale-Biscoe & Janssens,1988), would make good models for research into the time and place of origin of an enzyme such as 3β-HSD.

5.2: Histochemical Staining for 3β-HSD.

The histochemical staining for 3β-HSD activity was performed on pregnant mouse ovaries (n = 4) and non-pregnant *M. domestica* ovaries (n = 4), all from adult females. Sections of mouse ovary were used as a positive control in all cases, and one slide from each species was treated without steroid as a negative control.

5.2.1: Positive Controls.

All of the positive control sections (mouse) showed very strong positive reactions after an incubation period of 45 minutes. Positive staining was discerned in the corpora lutea (Figure 30), some theca interna cells around type 5b to type 8 (late secondary and tertiary) follicles and in some interstitial cells.

5.2.2: Negative Controls.

Negative control sections for mouse were incubated for 45 minutes as a strong Positive control reaction was apparent by that time. The negative control sections for *Monodelphis* ovaries were incubated for 1 hour 30 minutes as this was the longest time quoted in the literature for a possible reaction.

i - Mouse:- The mouse negative control section (45 minutes incubation) was negative, with a little light background staining in some areas, which was masked when the slide was counterstained.

ii - *M. domestica*:- The opossum negative control section (1 hr. 30 mins. incubation), was also negative, but due to the longer incubation period, background staining was much heavier than for the mouse, but still not as

strong as a positive stain. Most of this background was masked by the counterstain (Figure 31).

5.2.3: Experimental Sections

i - Mouse:- Corpora lutea stained very strongly and it was possible to distinguish successive generations of corpora lutea due to their staining intensity. Some follicles, mainly types 5b to 8 (large secondary or tertiary), had positive staining at their outer edges which was probably the theca interna or the first signs of follicular atresia. Light positive staining was noted in some interstitial cells (Figure 32).

ii - *M. domestica*:- The ovaries stained in the opossum specimens were from non-pregnant females, so no new corpora lutea of pregnancy were present. However, several old corpora lutea showed mid-positive staining. Follicular staining was restricted to a few type 6 (early antral) follicles which were at various stages of atresia. Outwith the old corpora lutea most positive staining was found in individual, or groups of, interstitial cells which showed stronger positive staining than several of the old corpora lutea (Figure 33).

5.3: Immunocytochemical Staining for 3β-HSD.

The immunocytochemical staining for 3β-HSD was performed on adult female mouse ovaries as a positive control and on a developmental series of *Monodelphis domestica* ovaries. This series was comprised of pups taken at various ages up to day 35, at day 80, a stage between weaning and puberty, and adult opossums. As for histochemical staining, adult mouse ovaries and mouse and opossum adrenal glands were used as positive controls, as these tissues were

expected to be strongly positive for 3β-HSD. Negative controls were treated with 1% BSA-PBS instead of the primary antibody.

5.3.1: Positive Controls.

i - Adult mouse ovary (Figure 34):- All of the positive control sections of mouse ovary showed strong positive reactions with the strongest being in the corpora lutea and the weakest in the majority of both type 5a and atretic follicles. Healthy follicles of types 2 to 4 were completely devoid of positive staining. Types 5b to 8 all showed a little theca interna staining but very heavy granulosa staining. This positive reaction of the granulosa cells decreased in amount, not staining intensity, towards the oocyte of the follicle. The interstitial tissue in the mouse ovary, which mainly resembled luteinized granulosa cells, was extensive and stained quite strongly throughout the gonad.

ii - Adrenal glands (Figure 35):- In the positive control sections of both these species the whole cortex stained strongly and extensively.

5.3.2: Negative Controls.

In all of the negative controls, both mouse and opossum ovary and adrenals, no positive staining was observed at all (Figure 36).

5.3.3: Experimental Sections.

i - Day 0 (Figure 37):- These specimens, which were the intact posterior halves of pups, demonstrated positive staining in the developing adrenals as well as in several of the ovarian blastema cells. Several patches of mesonephric

cells also stained positively.

ii - Days 10 and 15 (Figure 38):- Although there were several positive cells in the mesonephroi of these pups, no staining whatsoever was found in the gonads.

iii - Day 20 (Figure 39):- Many strongly positive cells were observed in both the mesonephroi and oviducts of these specimens although the ovary was, on the whole, negative. A very few cells in the ovarian medulla demonstrated positive staining - these did not appear to be in the *rete ovarii*, but could have been interstitial cells or very early medullary cords. The external *rete ovarii* could be distinguished in the mesonephros and hilus of the gonad and could be seen to be negative for 3β-HSD.

iv - Days 25 and 30 (Figure 40):- As in day 20 specimens, positive cells were observed in the mesonephros, oviduct and a few in the ovarian medulla. The latter were probably part of the medullary cords which could be seen alongside the negatively staining internal *rete ovarii*. What appeared to be the connecting *rete*, in the hilar region of the gonad, also stained positively.

v - Day 35 (Figure 41):- In these, and later, specimens only the ovaries of the animals were processed and stained. At day 35, positive cells were observed in the medulla (probably in the medullary cords) and several were noted within the nests of oogonia.

vi - Day 80 (Figure 42):- This animal was pre-pubertal: accordingly, no corpora lutea were present. Follicles were present up to type 5b and several of these later stages demonstrated positive staining in their granulosa cells.

The staining of these follicles was similar to that observed in the mouse positive controls, although the enzyme in this species appeared to be more evenly distributed throughout the granulosa layers.

Several of the type 5a/b follicles had luteinized and only granulosa lutein cells were obvious in the resulting structure. All of these luteinized follicles stained strongly for 3β-HSD.

Also observed at this age were several individual and small groups of interstitial cells which resembled luteinized granulosa cells of atretic follicles. These cells stained quite heavily for the enzyme.

The peripheral tissue in these sections, mainly oviducts and their fimbriae, also demonstrated the enzyme in isolated or small groups of cells.

vii - Adult ovaries (Figure 43):- The granulosa lutein cells of the adult corpora lutea stain strongly, similar to that found in the mouse, although no positively staining theca lutein cells were observed.

Positive staining was only noted in follicles above type 5a, although the amount of staining in each of these was not constant. Several follicles had only one or two positive cells whereas others had few unstained granulosa cells. There was little theca interna staining, but most positive follicles had a few stained thecal cells.

No interstitial tissue similar to that in the mouse was apparent, but a few individual and small scattered groups of weakly positively stained cells were observed. These interstitial cells, as in the mouse, resembled granulosa lutein cells, probably derived from dissociated corpora lutea.

Figure 30: Histochemically stained positive control sections of mouse ovary.

a: (x 300) Corpus luteum demonstrating heavy positive staining.

b: (x 300) Corpus luteum and interstitial cells stain positively, type 4 follicles remain unstained.

Figure 30a.

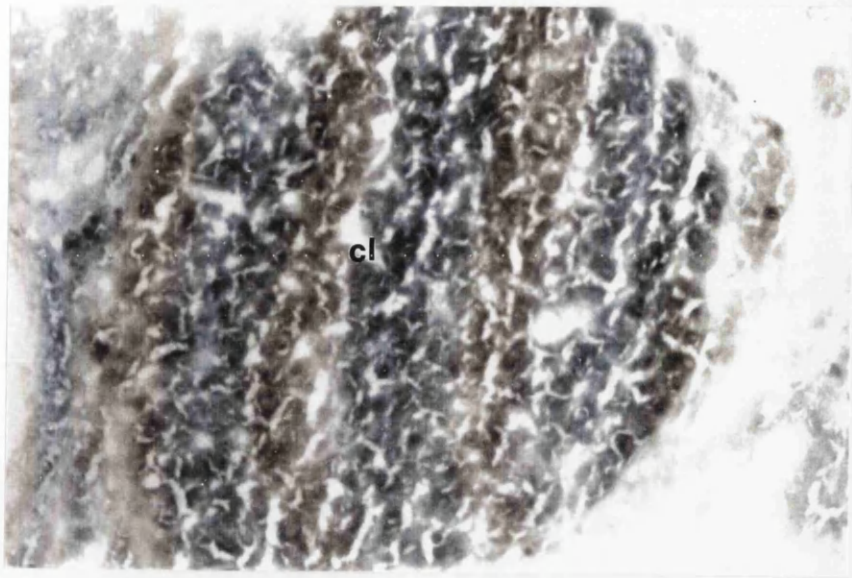


Figure 30b.

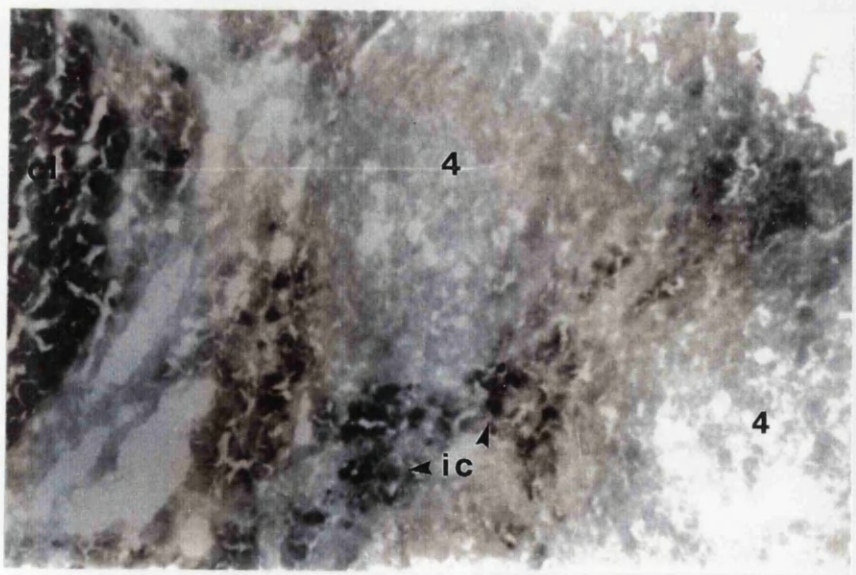


Figure 31: Negative control section for histochemistry.

Adult *Monodelphis domestica* ovary (x 250). No staining apparent.

Figure 31.

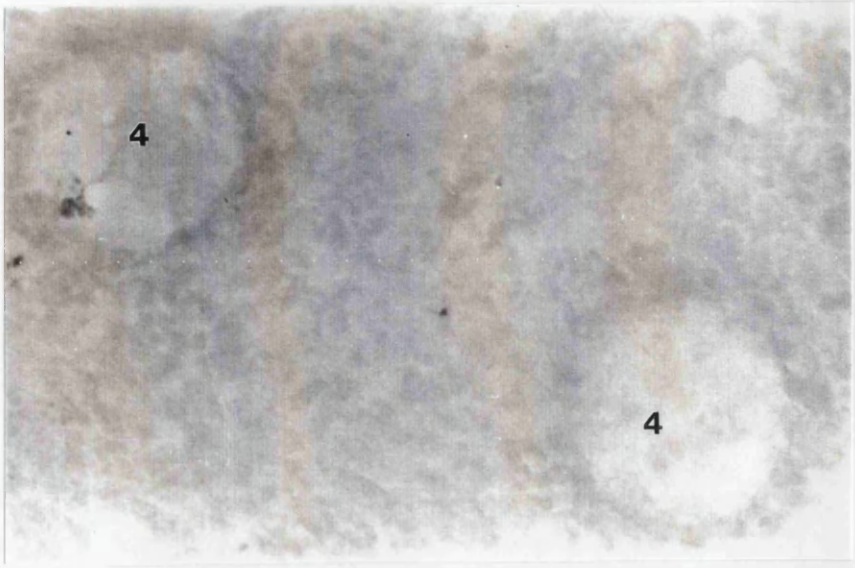


Figure 32: Experimental sections of adult mouse ovary (x 300).

a: A heavily stained corpus luteum.

b: Corpus luteum and interstitial cells stained strongly. The type 4 follicle present did not stain positive for 3β-HSD.

Figure 32a.

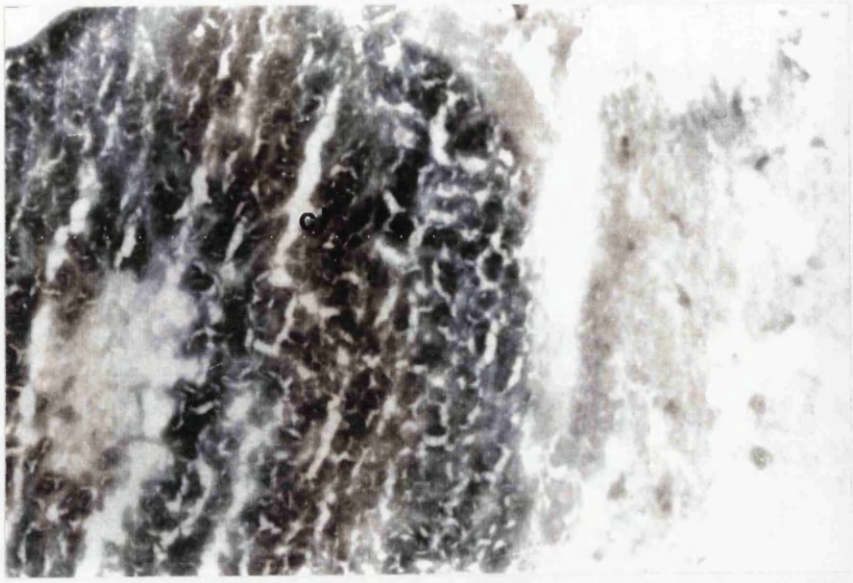


Figure 32b.

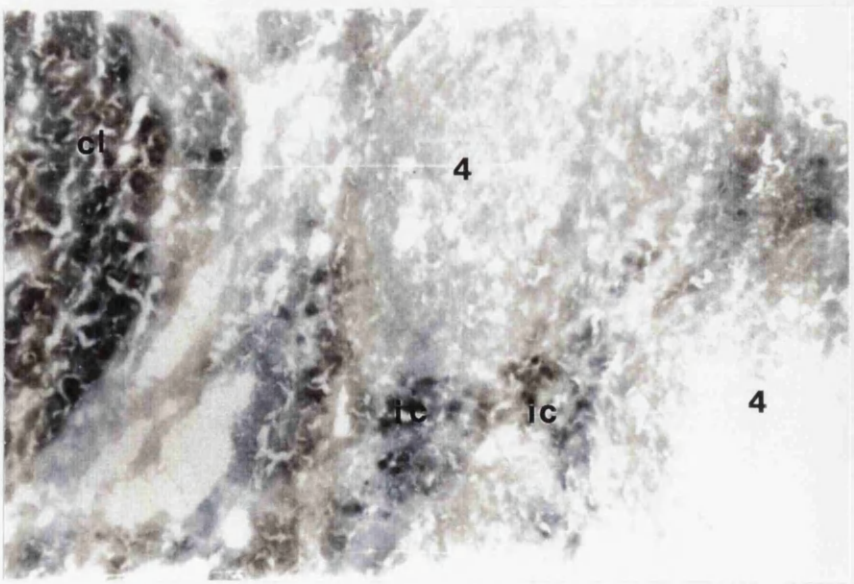


Figure 33: Experimental sections of adult *Monodelphis* ovary (x 300).

a: Corpora lutea and interstitial cells stain positively.

b: The theca interna cells of a type 5b follicle stained intensively.

Figure 33a.

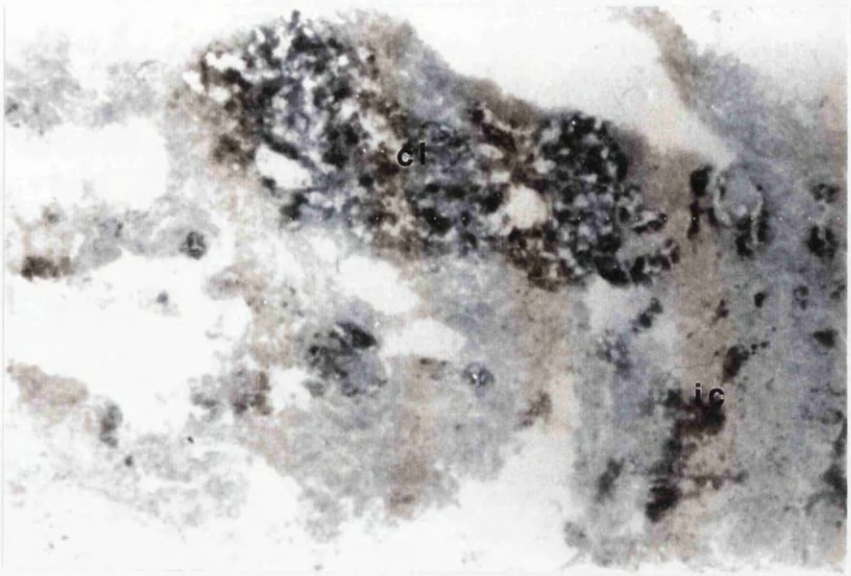


Figure 33b.

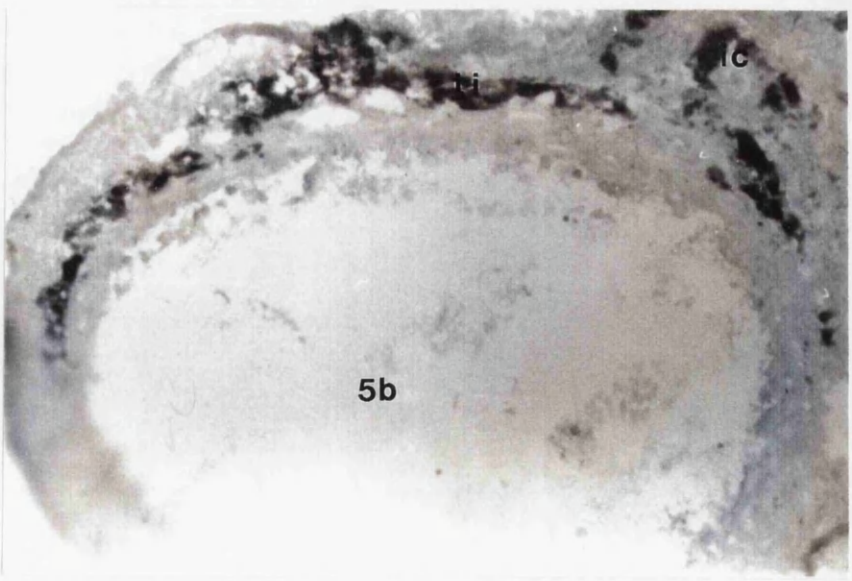


Figure 34: Immunocytochemistry positive control sections of a mouse ovary.

a: (x 425) Positive staining is present in corpora lutea, interstitial cells and type 6 follicles.

b: (x 1062.5) Type 6 follicle showing little theca interna staining and the differential staining of the granulosa towards the oocyte.

Figure 34a.

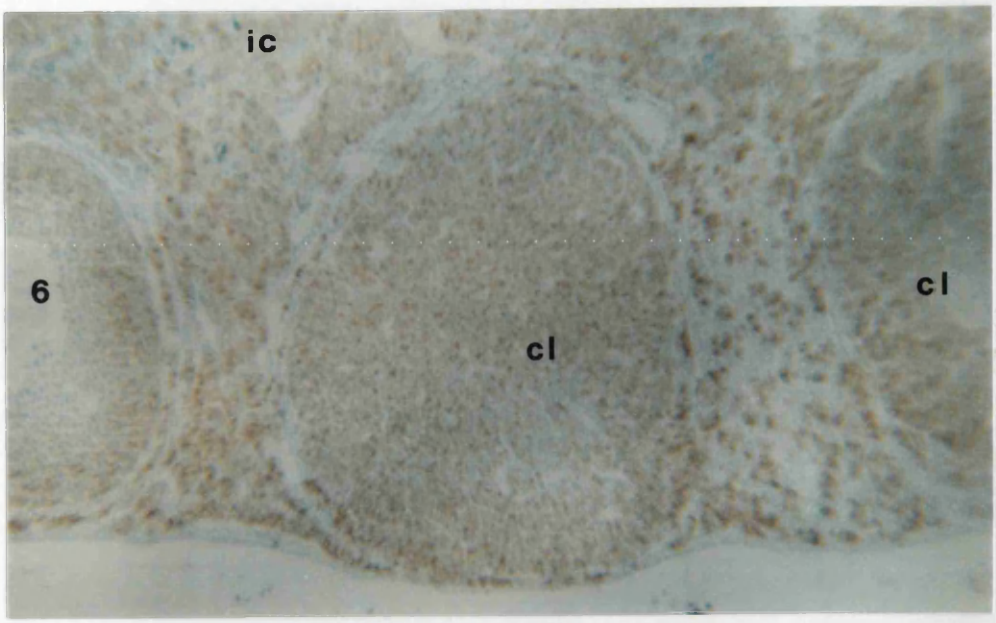


Figure 34b.

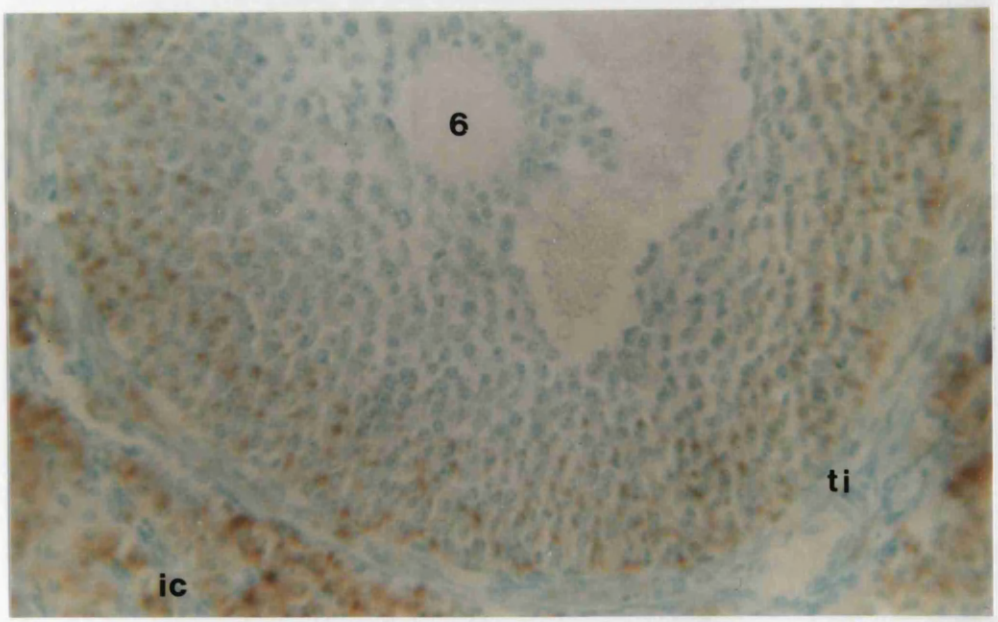


Figure 35: Adrenal positive control sections.

a: Mouse (x 425). The cortex stains heavily but the medulla remains negative.

b: *Monodelphis domestica* (x 425). The cortex stains heavily but the medulla remains negative.

Figure 35a.

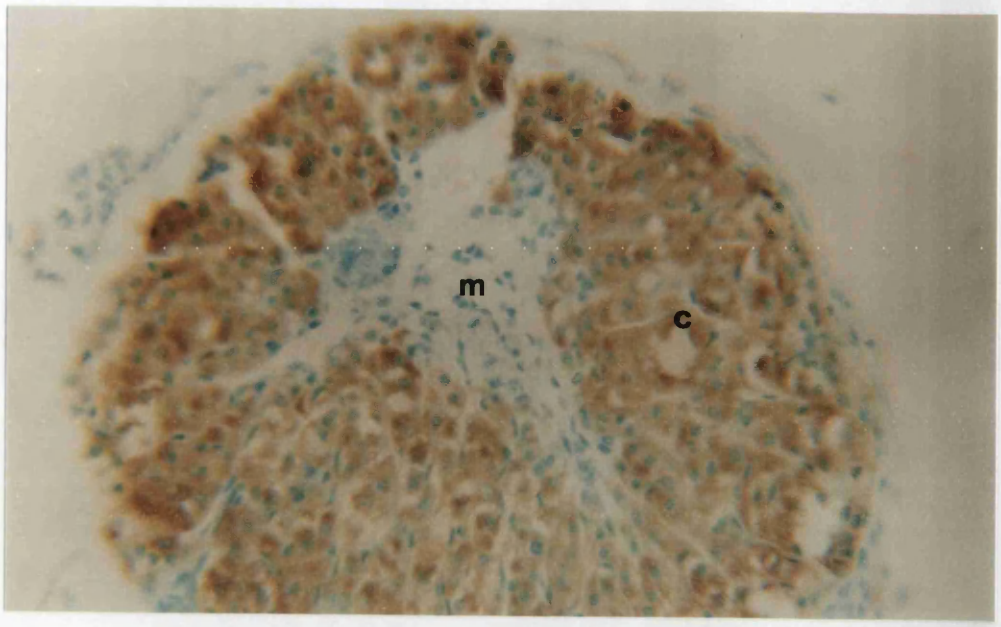


Figure 35b.

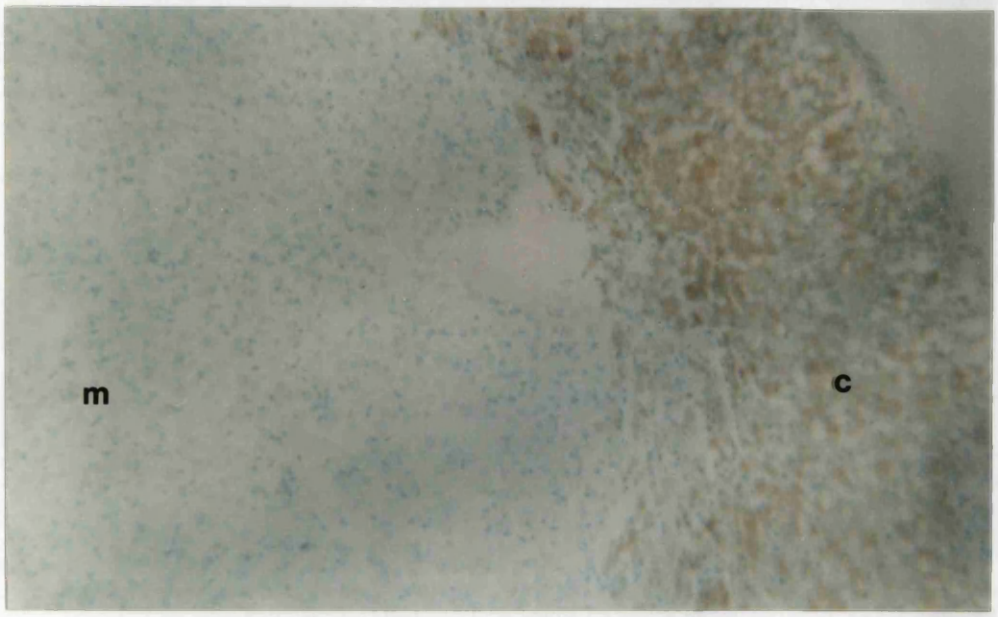


Figure 36: Adrenal negative control sections.

a: Mouse (x 425). No positive staining observed.

b: *Monodelphis domestica* (x 425). No positive staining observed.

Figure 36a.

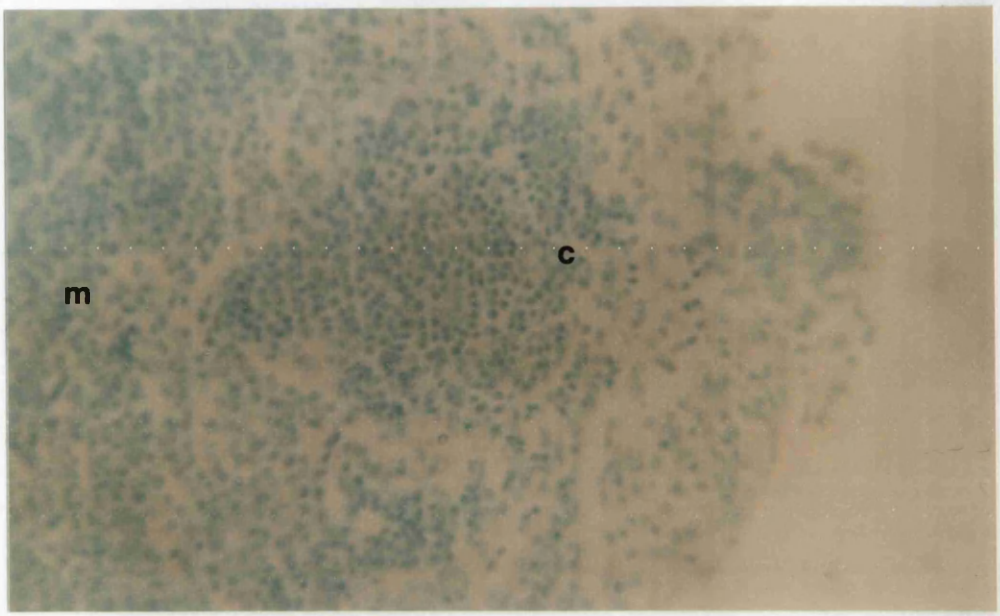


Figure 36b.

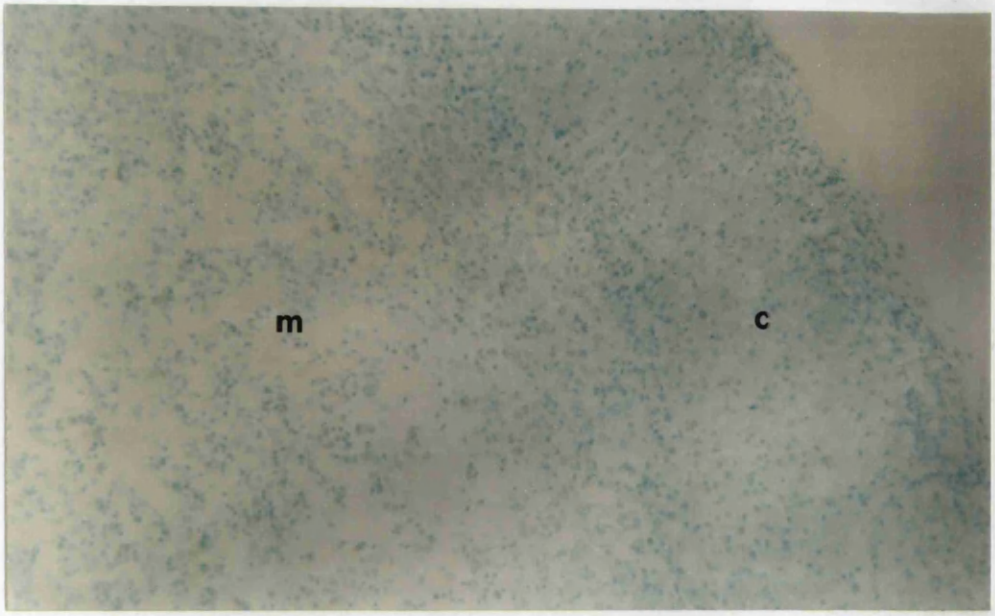


Figure 37:

Day 0 *M. domestica* ovary (x 425). The developing adrenal glands stain strongly. Few cells in the undifferentiated ovary stain positively (arrows).

Figure 38:

Day 15 *M. domestica* ovary (x 425). At this age the ovary is completely devoid of 3β-HSD staining.

Figure 37.

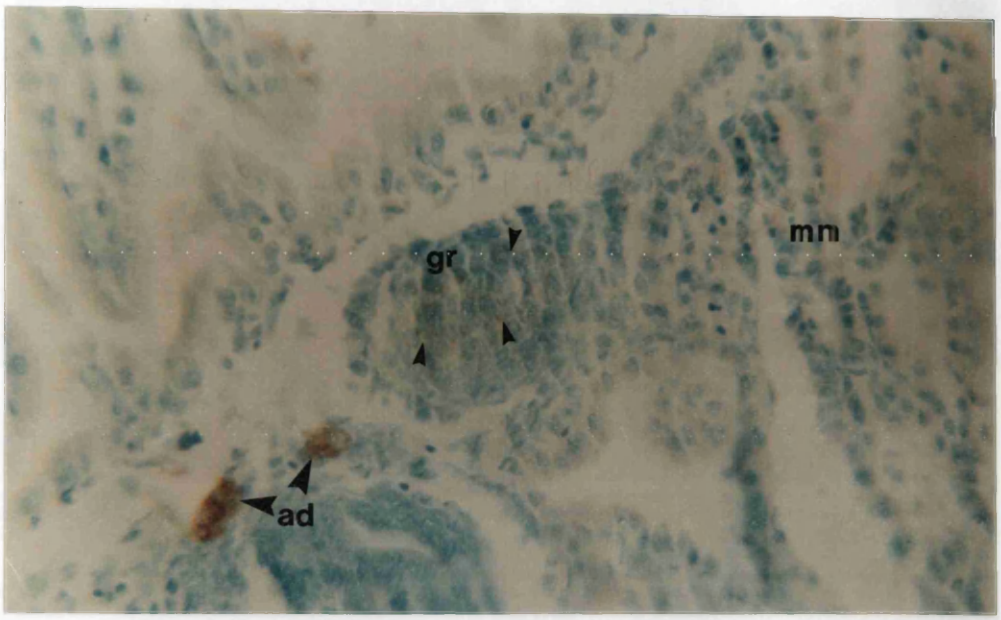


Figure 38.

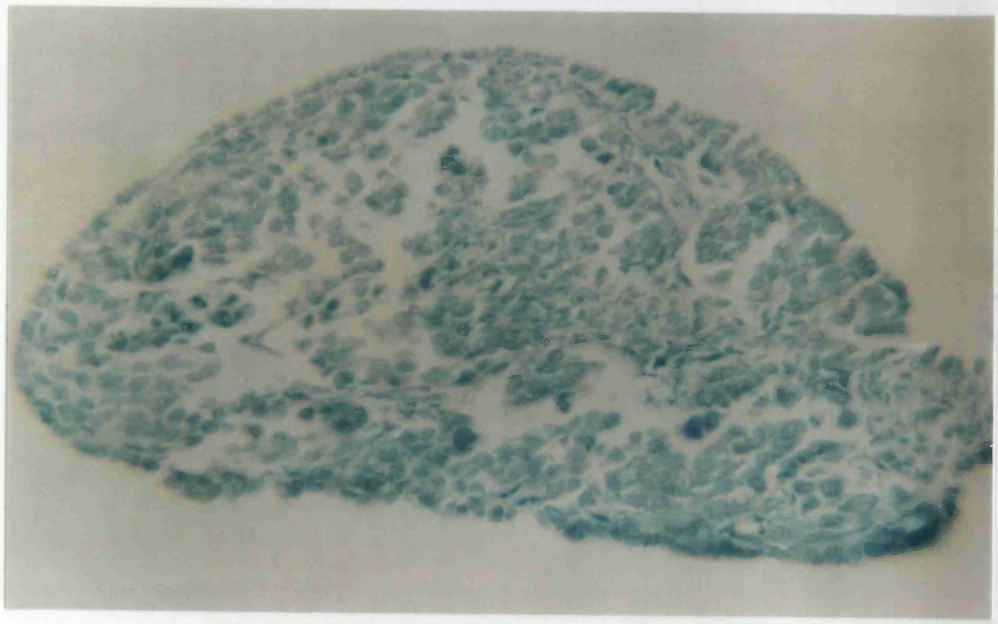


Figure 39:

Day 20 *M. domestica* ovary (x 425). The *rete ovarii* can be distinguished in the medulla of this ovary, but stains negatively. A few positively staining cells are present in the ovarian medulla (arrows).

Figure 40:

Day 25 *M. domestica* ovary (x 425). Several cells in the ovarian medulla stain positively (arrows).

Figure 39.

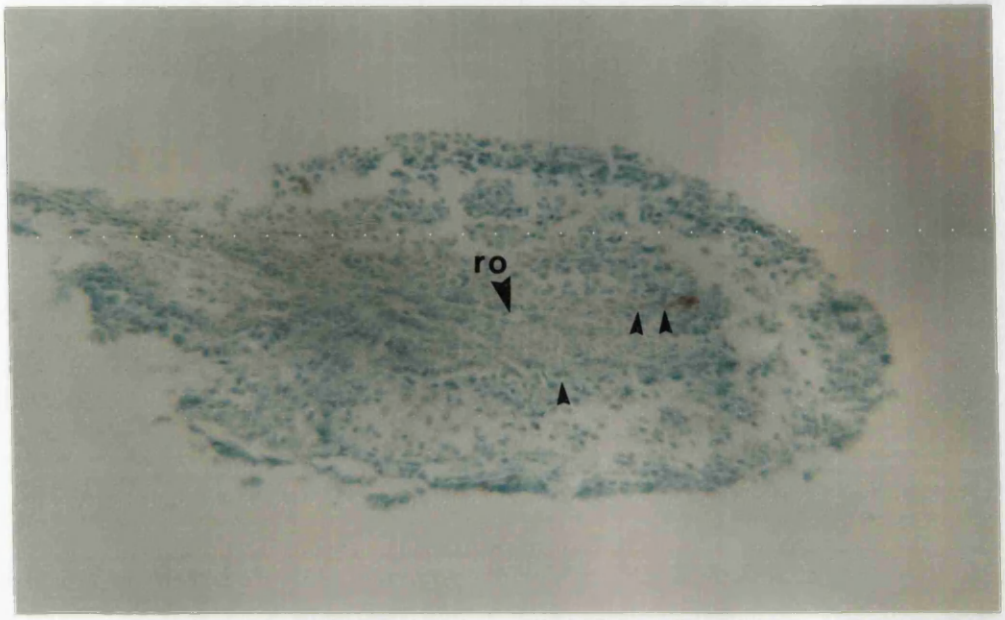


Figure 40.

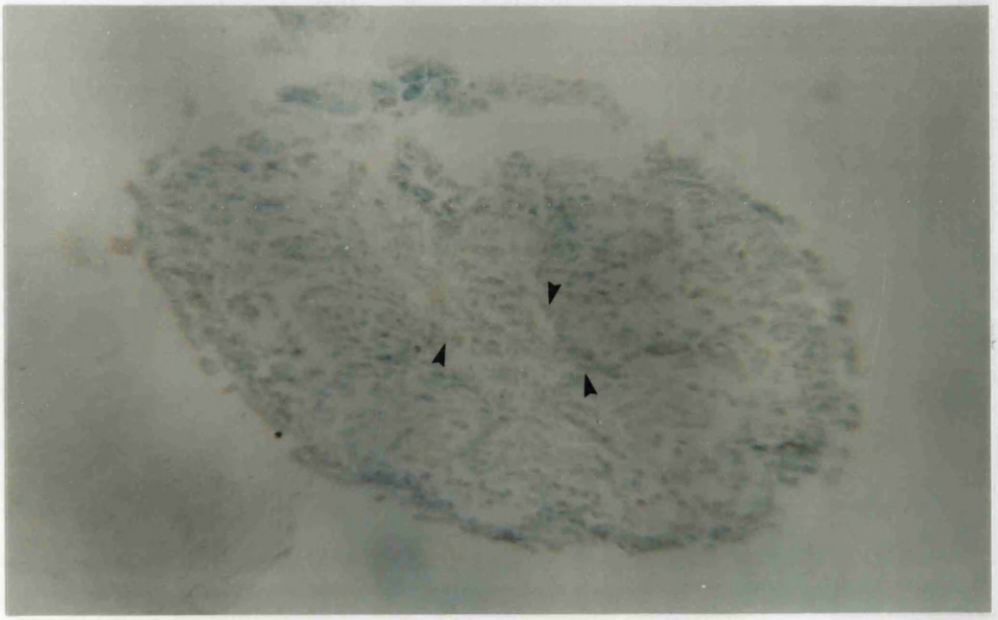


Figure 41:

Day 35 *M. domestica* ovary (x 425). Positively stained cells (arrows) are found both in the medulla (probably the medullary cords) and amongst the oogonia (possibly where medullary cord cells have invaded the germ cell nests).

Figure 42:

Day 80 *M. domestica* ovary (x 1062.5). The granulosa cells of type 5b follicles stains positively, although their theca interna does not stain. The granulosa cells of all earlier follicles is negatively staining.

Figure 41.

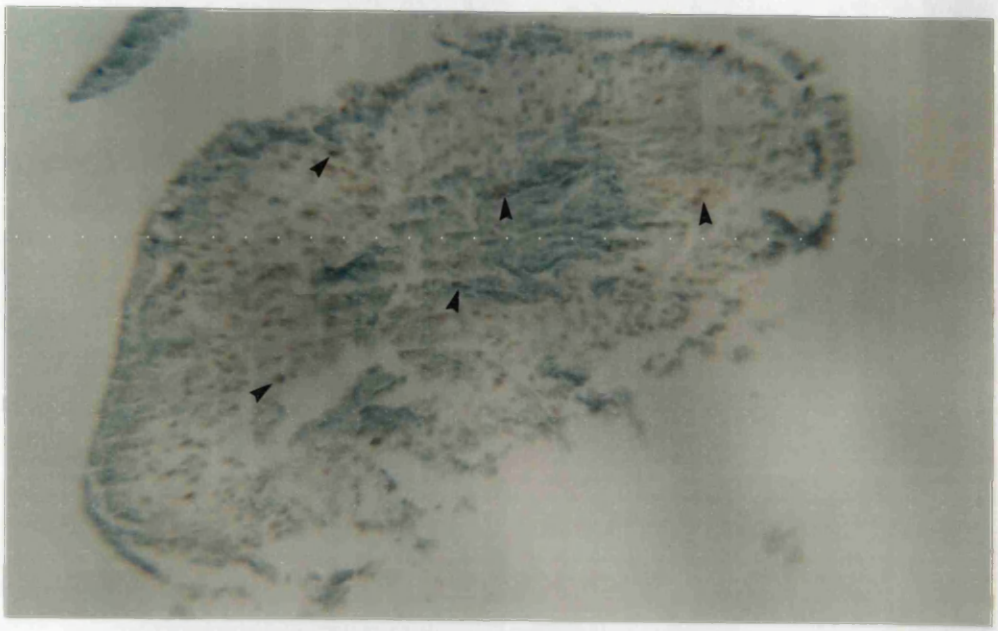


Figure 42.

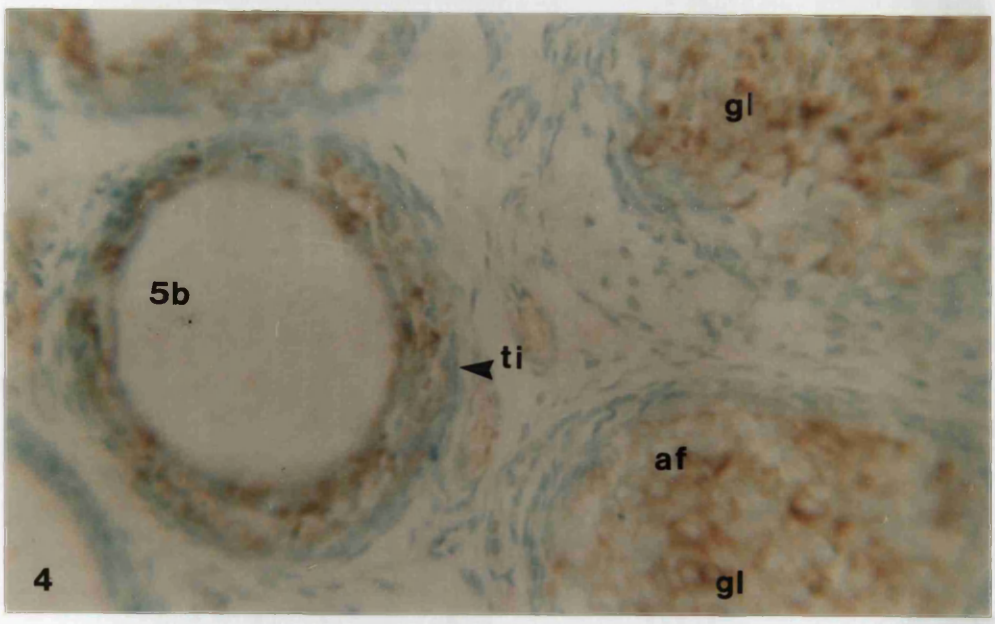


Figure 43 Adult *Monodelphis domestica* ovary.

a: This corpus luteum (x 1062.5) shows that the granulosa lutein cells stain positively, though the theca lutein cells are negative for the enzyme.

b: This type 5b follicle (x 1700) shows positive granulosa cell staining, though not around the whole follicle, and negative theca interna staining.

Figure 43a.

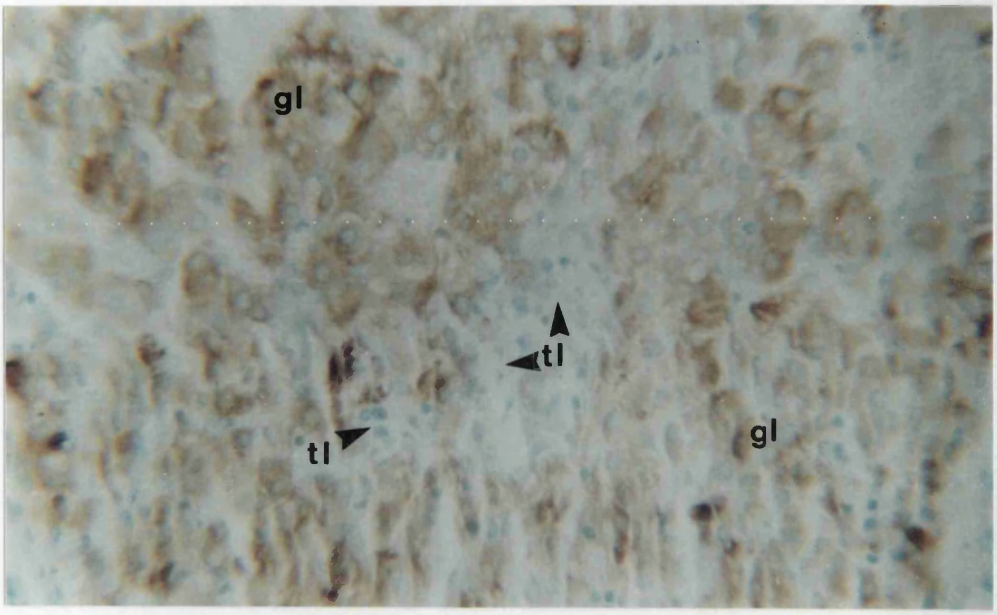
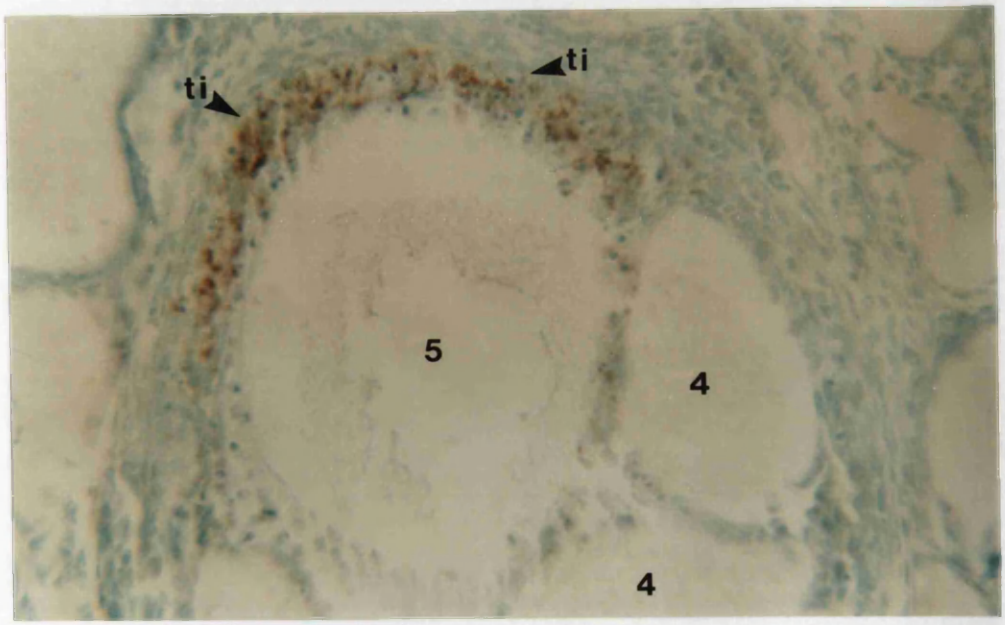


Figure 43b.



5.4: Discussion.

5.4.1: Comparison of Techniques.

Several problems had to be faced in the course of histochemical studies. The gonads to be examined had to be snap-frozen as soon as possible after the animals death, storage had to be at -70^{o}C, and the blocks could not be stored for more than two weeks without losing some enzyme activity. Cryomicrotomy could also affect the procedure, as relatively thick sections of the gonad had to be cut, and many sections were lost by curling, chattering, premature thawing and transfer onto the slides. Once stained histochemically, the reaction products faded rapidly (as little as two days after staining) so that analysis and photography had to be carried out immediately. Staining alternate sections with H & E would have helped with cell identification which was difficult with this technique.

The majority of these problems could be overcome by using immunocytochemistry as did Sasano & Sasano (1989) and Sasano *et al.* (1990a, 1990b). With the latter technique, formalin fixed, paraffin wax processed, thinner sections could be stored indefinitely without losing enzyme activity. Another apparent benefit with immunocytochemistry was that the localization of the staining was very specific and could even be used at the electron microscope level. The same problems with cell identification occurred in immunocytochemical staining, but were less pronounced: H & E stained slides would help in this matter.

It could be argued that the two techniques utilized in this study were not directly comparable. Histochemistry involves adding a substrate to a tissue section and then staining that section for a reaction product. This means that if a particular product is produced outwith the cell and transported into it, that individual cell will falsely stain positive (i.e. the product is present, but the cell did not produce it). Alternatively, immunocytochemistry tags the actual enzyme which is responsible for the reaction taking place.

Immunocytochemistry is the more preferred of the two techniques in that it is the easier to perform, is more specific and the resulting stained sections can be stored indefinitely. One possible problem with this technique is that the primary antibody used, raised against an antigen in one animal, may not cross-react with that of a different species.

5.4.2: Enzyme Histochemistry.

The majority of histochemical 3β-HSD studies on eutherian mammals researched for this work agreed on the distribution of the enzyme in the adult ovary (Levy *et al.*, 1959; Ferguson, 1965; Pupkin *et al.*, 1966; Müller, 1975; Brook & Clarke, 1989). The results obtained from adult mouse ovaries in this study confirmed the earlier observations, in both presence and intensity of staining in distinct parts of the ovary. In decreasing order of intensity these were: the interstitial cells, corpora lutea and theca interna cells. Several follicles had positively staining granulosa cells, confirming the results of Goldman *et al.* (1965) and Hoyer & Andersen (1977).

The histochemical results obtained for *M. domestica* were very similar to those described above in eutherians. The corpora lutea, interstitial cells and theca interna cells all stained strongly positive for the enzyme, and there was also activity recorded in the granulosa of several follicles.

This study shows, therefore, that both *Monodelphis domestica* and its close relative *Didelphis virginiana* (Guraya, 1968b) compare favourably with the eutherians so far studied in respect to their histochemically demonstrable 3β-HSD distribution.

These findings are in contrast, however, to those of Dairi (1988) and personal observations on the histochemistry of the ovary of the macropod marsupial, *Potorous tridactylus*. Dairi (1988), working on pouch young up to day 86, reported negative results on all specimens. Unexpectedly, adult potoroo

ovaries (personal observations) were also completely devoid of 3β-HSD activity. This is unusual, as it is thought that enzymes such as 3β-HSD are universal in mammals. Further studies need to be carried out on the potoroo to ascertain the reasons for these unusual results.

5.4.3: Immunocytochemistry.

Until now the localization of 3β-HSD by immunocytochemistry has only been carried out on the human ovary (Sasano & Sasano, 1989; Sasano *et al.*, 1990a, 1990b) and bovine adrenal cortical cells (Ishimura *et al.*, 1988). The latter authors' results were confirmed in this study, both in the mouse and in *Monodelphis*: the cortical cells in all three species stained intensely and no adrenal medullary staining was evident.

The results obtained by Sasano *et al.* (1990) on the human ovary, however, differed from those in this study of mouse and *Monodelphis* gonads. In the latter animals thecal demonstration of the enzyme was minimal and the granulosa layers of the follicles that were positively stained were much stronger than those in the theca. This also appeared to be the case in the corpus luteum where luteinized theca cells either did not stain or stained much weaker than the granulosa lutein cells.

There are two possibilities for these differences. Firstly, the antibody used was against a human placental enzyme antigen, therefore, the cross reaction with mouse and opossum material may not have been complete. Secondly, as is probably the case, the distribution of the enzyme is slightly different in each species which would also account for the differences in the opossum and mouse results.

The steroidogenic staining of developing *Monodelphis* ovaries, being negative until day 15 (the day after resumption of meiosis) and thereafter increasing, supports the suggestion in section 4.6.3 that the ovaries of this species

are of the type displaying "immediate meiosis".

The facts that in the developmental series of *Monodelphis* the *rete ovarii* rarely stained positive and never within the gonad, combined with the positive staining of medullary cords at the time of follicle formation (days 25 to 35), suggests that the granulosa cells are derivatives of the medullary cords, not the *rete ovarii*, a source favoured by several authors (Byskov & Lintern-Moore, 1973; Byskov, 1975; Byskov & Saxén, 1976; Byskov *et al.*, 1977; Byskov, 1978; Stein & Anderson, 1979; Fajer, 1981; Byskov, 1986).

General Conclusions.

Since individual chapters include discussions on the work undertaken, the purpose of this section is to draw together the general conclusions from each part of this work and to make recommendations for further study.

The aims of this examination of the Grey short-tailed opossum, *Monodelphis domestica*, were several and varied: to compare two previously uncompared mating regimes; to verify other authors' accounts of postnatal development; to describe the normal postnatal development of the gonads and investigate the origins of the ovarian constituents; and finally, to attempt to recognise, through histochemical and immunocytochemical techniques, the temporal and spatial origins of steroidogenesis in the ovary of this species.

Of the two mating regimes used sequentially, the second was preferable. By introducing the female to the male in his own cage, her territoriality was subdued, and she was more amenable to handling. The productivity of matings increased by 27% when this regime was implemented and neonatal losses were reduced by 58%. The stress, discomfort and fatalities incurred with the initial regime, by introducing male to female, within a holding cage, preclude any comparative study of the two regimes.

The breeding behaviour, litter sizes averaging 7.5, and a sex ratio at weaning of unity all confirmed earlier works on *Monodelphis* (Fadem, 1985, 1987; Fadem *et al.*, 1982; Fadem & Rayve, 1985; Fadem & Tesoriero, 1986; VandeBerg, 1983, 1990), as did the chronology of neonatal events (VandeBerg, 1990).

The neonatal birth weight of 100 mg stated by several authors (Fadem *et al.*, 1982; VandeBerg, 1983; Cothran *et al.*, 1985; Moore & Thurstan, 1988, 1990; Baker *et al.*, 1990) fell within the range attained in this study (83.9 ± 13.0 mg), although it was found that pups obtained before attachment to the nipple had a much lower weight than that average (around 62 mg). Therefore, it is suggested that further research into actual birth weight, rather that post-detachment "birth weight" would yield new and interesting results.

Cothran *et al.* (1985) published "normal" growth curves for *Monodelphis*. The animals in the present study adhered reasonably well to these, although there was wide variation in the weight of pups of any one age. Head-length, a parameter not normally measured in mammals to produce growth curves, yielded much smaller variations than did weight and so was more reliable than the latter for ageing unknown specimens. Further study in this area could produce growth curves by head-length, which would be much more accurate for ageing than would the normal weight growth curves.

The sexing of pups, by the presence or absence of a scrotum at birth, as described by Moore & Thurstan (1988, 1990) was found, in this study, to be impossible - a scrotal rudiment was not seen in any of the male neonates examined. To assess correctly the sex of neonates, either histological or chromosomal analyses were required.

Testicular sex differentiation occurs *in utero* in *Monodelphis*, as described by Baker *et al.* (1990), similar to its close relative *Didelphis virginiana* (McCrady, 1938). Morphologically, at birth, the gonads resemble those of a 12 day mouse foetus. Large size differences in the neonatal gonads were observed with the testis being as much as twice the size of an ovary at the same age.

The corpora lutea and interstitial cells of the ovary are derived similarly to those of most mammals: the corpora lutea were comprised of luteinized granulosa and theca interna cells (Mossman & Duke, 1973; Johnson & Everitt, 1983), although in young animals, follicles which had not attained a theca interna layer could also luteinize, forming similar structures (Byskov *et al.*, 1985); the interstitial cells were derived mainly from degenerated corpora lutea.

The granulosa cells and theca interna, however followed the marsupial course of development, in that they developed, indirectly, from the gonadal blastema, *via* the medullary cords (Ullmann, 1984; Dairi, 1988), rather than from the mesonephric derivative, the *rete ovarii* as in the majority of eutherians (Byskov, 1975, 1978, 1986; Stein & Anderson, 1979; Fajer, 1981).

The *rete ovarii* in *Monodelphis* also followed the marsupial rather than eutherian course: in most eutherians, the *rete* develops from the mesonephros (Byskov, 1986), whereas in marsupials (and the prosimian Galago) the *rete* develops from a condensation of cells in the hilar region and may not contact mesonephric structures until later on in development, if at all (Ullmann, 1981b; Dairi, 1988; Yoshinaga *et al.*, 1988). Electron microscopy would facilitate the further study of such structures.

The results of the histochemical studies undertaken closely resembled those of Guraya (1968b) for *Didelphis virginiana*, Hoyer & Andersen (1977) for the rat and Goldman *et al.* (1965) for the human. The potoroo (Dairi,1988; personal observation), however, did not demonstrate the action of 3β-HSD histochemically. As enzymes tend to be universal, this begs further study on the potoroo ovary.

The immunocytochemical study on the ovaries of *Monodelphis* yielded different results to that of Sasano *et al.* (1990a, 1990b) on the human ovary. There are two possibilities for these differences: the antibody used was to a human placental antigen, so that cross-reactivity between the species may have been compromised or, which is more likely, the actual distribution of the enzyme differs from species to species. The latter idea could be tested by raising an antibody to an opossum antigen and testing that on opossum, mouse and human tissue.

The results obtained in both the steroidogenic and histological parts of this work, when considered together, lead to the conclusion that *Monodelphis domestica* has ovaries of the "immediate meiosis" type described by Byskov (1979).

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APPENDIX 1.

Common Staining Procedures.

A1.1: Haematoxylin and Eosin (H & E).

A1.1.1: Solutions Required.

i - Mayer's haematoxylin: dissolve 1 g of haematoxylin in 1 litre of distilled water, add 0.2 g sodium iodate and 50 g potassium aluminium sulphate and filter. Add a few crystals of thymol.

ii - Alcoholic eosin: dissolve 5 g of eosin (yellowish) in 1 litre of absolute alcohol. Do not filter.

iii - Scott's tap water substitute (STWS): dissolve 3.5 g of sodium bicarbonate and 20 g of magnesium sulphate in 1 litre of distilled water. Add a few crystals of thymol. Do not filter.

A1.1.2: Procedure.

i -	Xylene	- 5 to 10 mins
ii -	Descending alcohols (100%, 95%, 90%, 70%, 30%)	- 3 mins each
iii -	Water	- 3 mins
iv -	Mayer's haematoxylin	- 6 mins
v -	Rinse in water	
vi -	STWS until blue/purple in colour	
vii -	Ascending alcohols (30%, 70%, 90%)	- 3 mins each
viii -	Alcoholic eosin	- 2 mins
ix -	95% alcohol	- 45 secs
x -	100% alcohol	- 15 secs
xi -	Xylene	- > 5 mins
xii -	Mount in DPX	

A1.1.3: Results Obtained.

Nuclei - purple/blue.

Cytoplasm - pink.

A1.2: Mallory's Triple Stain.

A1.2.1: Solutions Required.

i - Saturated mercuric chloride in 5% glacial acetic acid.

ii - 1% aqueous acid fuchsin.

iii - 1% phosphomolybdic acid.

iv - Mallory's stain: dissolve 2.5 g aniline blue (w.s.), 10 g orange G and 10 g oxalic acid in 500 mls of distilled water. Do not filter.

A.1.2.2: Procedure.

i -	Xylene	- 5 to 10 mins
ii -	Descending alcohols (100%, 95%, 90%, 70%, 30%)	- 3 mins each
iii -	Water	- 3 mins
iv -	Sat $HgCl_2$ /acetic acid	- 10 mins
v -	Rinse in distilled water	
vi -	1% acid fuchsin	- 1 min
vii -	Differentiate in distilled water	- 10 secs
vii -	Fresh 1% phosphomolybdic acid	- 1 min
viii -	Distilled water	- 10 secs
ix -	Mallory's stain	- 75 secs
x -	Distilled water	- 10 secs
xi -	90% alcohol	- 10 secs
xii -	100% alcohol	- 2 x 10 secs

xiii - Xylene - > 5 mins

xiv - Mount in DPX.

A1.2.3: Results Obtained.

Muscle - pink.

Connective tissue - blue.

Red blood cells - yellow.

A1.3: Toluidine Blue.

A1.3.1: Solution Required.

Add 1 g toluidine blue and 1 g sodium tetraborate to 100 mls of distilled water. Mix then filter. This remains viable for several months at room temperature.

A1.3.2: Method.

i - Cover 1μm resin sections with stain

ii - Place on 60^{o} to 70^{o}C hotplate for 5 to 15 seconds

iii - Wash off excess stain with hot water (this saves cracking the slide)

iv - Air dry on hotplate

v - Mount in D.P.X.

A1.3.3: Results Obtained.

All tissues are various shades of blue/black.

APPENDIX 2.

Actual Data from *Monodelphis domestica.*

A2.1: Note on Animal Coding.

The coding of pups for this study took the form of a three number code with the addition of a letter at the end if there were more than one pup taken from a single litter at one time. For example, Md 51.1.5C - Md 51 is the code of the female from which the pup was taken. The second number (1) shows from which of the female's litters the pup was taken and the third number (5) gives the age (day 0 being the day of birth). The letter on the end of the code shows which animal this is when several were taken from a single litter. Weights marked with an asterisk denota pups taken before attachment to the nipple.

A2.2: Table.

CODE	SEX	WEIGHT (mg)	HEAD-LENGTH (mm)
Md 12.1.0A	M	100	--
Md 12.1.0B	F	93	--
Md 11.4.0A	F	110	4.3
Md 11.4.0B	M	112	4.5
Md 11.4.0C	F	90	4.3
Md 11.4.0D	F	87	4.6
Md 11.4.0E	M	84	4.2
Md 63.1.0A	?	62*	3.9
Md 63.1.0B	?	61*	4.0
Md 63.1.0C	?	60*	3.6
Md 63.1.0D	?	64*	3.6
Md 12.1.1A	M	110	--
Md 12.1.1B	M	102	--
Md 2.4.1A	M	125	4.4

CODE	SEX	WEIGHT (mg)	HEAD-LENGTH (mm)
Md 2.4.1B	F	121	4.3
Md 2.4.1C	M	123	4.4
Md 2.4.1D	M	136	4.7
Md 2.4.1E	?	114	4.3
Md 2.4.1F	?	135	4.5
Md 20.2.1A	F	135	4.3
Md 20.2.1B	F	130	4.6
Md 20.2.1C	M	160	4.8
Md 20.2.1D	F	141	4.6
Md 20.2.1E	F	135	4.5
Md 20.2.1F	M	152	4.7
Md 20.2.1G	F	132	4.5
Md 21.1.2A	F	143	5.0
Md 21.1.2B	M	145	4.9
Md 21.1.2C	M	153	4.8
Md 21.1.2D	F	154	5.0
Md 24.1.2A	M	153	5.5
Md 24.1.2B	M	165	5.5
Md 24.1.2C	F	166	5.5
Md 24.1.2D	F	164	5.5
Md 24.1.2E	M	160	5.4
Md 24.1.2F	M	162	5.3
Md 24.1.2G	F	138	4.8
Md 24.1.2H	M	153	5.3
Md 24.1.2J	M	135	5.1
Md 37.2.2A	M	127	5.2
Md 37.2.2B	F	131	5.2

CODE	SEX	WEIGHT (mg)	HEAD-LENGTH (mm)
Md 37.2.2C	M	137	5.2
Md 37.2.2D	M	126	5.0
Md 37.2.2E	F	144	5.4
Md 12.2.3A	F	174	5.4
Md 12.2.3B	?	155	5.5
Md 12.2.3C	M	166	5.7
Md 12.2.3D	M	160	5.7
Md 12.2.3E	M	172	5.6
Md 36.1.3A	?	235	6.4
Md 36.1.3B	F	210	6.3
Md 36.1.3C	M	238	6.1
Md 36.1.3D	F	212	6.2
Md 36.1.3E	F	196	6.1
Md 12.3.3A	F	117	5.2
Md 12.3.3B	F	133	5.6
Md 12.3.3C	M	160	5.7
Md 12.3.3D	M	104	4.9
Md 13.1.4	M	146	4.7
Md 3.2.5A	F	208	6.0
Md 3.2.5B	F	204	5.7
Md 3.2.5C	M	170	5.7
Md 3.2.5D	F	201	5.8
Md 51.1.5A	F	193	6.0
Md 51.1.5B	M	210	6.2
Md 51.1.5C	M	190	5.8
Md 20.1.6A	M	276	6.7
Md 20.1.6B	M	270	6.6

CODE	SEX	WEIGHT (mg)	HEAD-LENGTH (mm)
Md 20.1.6C	F	290	6.9
Md 20.1.6D	F	275	6.7
Md 44.1.6A	M	242	6.9
Md 44.1.6B	M	226	6.5
Md 44.1.6C	M	224	6.5
Md 44.1.6D	F	250	7.0
Md 11.1.8A	M	500	5.5
Md 11.1.8B	M	494	5.4
Md 23.2.8A	F	483	7.3
Md 23.2.8B	M	434	8.2
Md 23.2.8C	M	383	7.7
Md 23.2.8D	M	435	7.7
Md 23.2.8E	M	426	7.8
Md 11.1.10A	F	630	9.5
Md 11.1.10B	F	547	9.3
Md 53.1.10A	F	344	7.7
Md 53.1.10B	M	340	7.7
Md 53.1.10C	M	347	7.8
Md 13.1.12A	F	692	6.9
Md 13.1.12B	M	609	6.7
Md 21.2.13A	F	743	9.3
Md 21.2.13B	F	742	9.2
Md 21.2.13C	M	823	9.6
Md 21.2.13D	M	748	9.5
Md 21.2.13E	F	768	9.6
Md 21.2.13F	M	850	9.7
Md 21.2.13G	F	853	9.7

CODE	SEX	WEIGHT (mg)	HEAD-LENGTH (mm)
Md 13.1.14A	F	832	10.5
Md 13.1.14B	M	953	10.7
Md 11.3.14A	M	1100	10.6
Md 11.3.14B	F	932	10.5
Md 11.3.14C	F	824	9.7
Md 11.3.14D	F	881	10.3
Md 24.3.14A	M	680	9.5
Md 24.3.14B	F	659	9.5
Md 24.3.14C	F	665	9.4
Md 52.1.15	F	631	9.3
Md 62.1.15A	F	1170	11.0
Md 62.1.15B	F	1314	11.7
Md 10.1.20	M	1295	11.6
Md 62.1.20A	F	2410	14.2
Md 62.1.20B	F	2275	14.0
Md 63.1.25A	F	2165	14.3
Md 63.1.25B	F	2235	14.5
Md 11.2.26A	F	2880	15.4
Md 11.2.26B	F	2957	15.5
Md 11.2.26C	M	3400	16.4
Md 22.1.29A	F	5781	19.7
Md 22.1.29B	F	5687	19.7
Md 22.1.29C	F	5999	20.5
Md 22.1.29D	M	5602	20.1
Md 22.1.29E	M	5671	20.2
Md 22.1.29F	M	5453	19.9
Md 22.1.29G	M	5975	20.5

CODE	SEX	WEIGHT (mg)	HEAD-LENGTH (mm)
Md 63.1.30A	F	4315	17.4
Md 63.1.30B	F	3760	17.0
Md 11.1.35	F	6980	22.6
Md 11.1.44A	M	9188	24.8
Md 11.1.44B	F	7828	23.6
Md 12.1.49A	M	9524	26.4
Md 12.1.49B	F	10800	27.3
Md 13.1.54A	M	13175	28.3
Md 13.1.54B	F	12718	28.5
Md 36.1.60A	F	20743	30.1
Md 36.1.60B	F	16645	28.5
Md 36.1.60C	M	12270	25.0
Md 36.1.60D	M	16625	28.9
Md 36.1.60E	M	16563	29.9
Md 13.1.65	F	8733	28.0