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Sexual Differentiation of the Reproductive System in the Marsupial *Monodelphis domestica*

BY

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DECLARATION

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

Qingchun Xie

July 1997

DEDICATION

This work is dedicated to my Father and my Mother.

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ABSTRACT

This study, on testis development and descent, reproductive tract development and the sex steroid hormones in the grey short-tailed opossum, *Monodelphis domestica*, investigates sex differentiation in this important biomedical model.

Testis development in *Monodelphis* has been investigated by light and electron microscopy. On the day of birth, gonads in both sexes are elongated in shape, extending along the medial aspect of the large mesonephroi. Half the karyotyped males are found to have histologically differentiated testes at birth. By day 1 testicular cords are clearly distinguished in all XY gonads and the *tunica albuginea* is fully developed. At this stage the large and pale primordial germ cells can be easily differentiated from dark pre-Sertoli cells. From day 3 onwards, the testis becomes more rounded than the ovary. Leydig cells can be first distinguished at day 3 showing expected ultrastructural features of steroidogenically active cells. These cells form clusters surrounded by envelope cells until week 12 (pre-pubertal stage). By 4 months (pubertal stage), seminiferous tubules are now patent and various spermatogenic stages, including spermatozoa, are seen for the first time. At this time, Leydig cells are of adult type, greatly outnumbering other interstitial tissue cells and are closely-packed around blood vessels.

Investigation of reproductive tract development and testicular descent in *Monodelphis* has demonstrated that 1) young are born with a fully functional mesonephros and at a sexual indifferent stage of urogenital development tract (e.g. patent Wolffian ducts are seen in both sexes); 2) Müllerian ducts were identified on day 1, grow in a caudal direction and reach the urogenital sinus on day 6; 3) in the male, regression of the Müllerian duct (presumably due to the production of Müllerian Inhibiting Substance at this time) occurs between days 12-16, the onset of production period; 4) female Wolffian ducts start to regress at day 15, reflecting the fact that testosterone secretion must occur in the male at this time; 5) the testis begins its transabdominal descent at day 13, starts inguinal descent by 16 and reaches its final position at the base of the scrotum by day 28

The findings of this study also demonstrate functional characterisation of Leydig cells in *Monodelphis* by immunocytochemical identification of the enzyme 3β-hydroxysteroid dehydrogenase and by measurement of testosterone levels using radioimmunoassay. Three β-hydroxysteroid dehydrogenase immunostaining is first detected in a few Leydig cells on day 16, increases by day 24; reaches a peak at 4 months and is present even in senescent (3 years) animals. Plasma testosterone is first measurable at pre-puberty (3.5 months). Prior to that, plasma testosterone concentrations are uniformly below the level of detection in both sexes from day 5 to 2.5 months. By four months (puberty) plasma testosterone levels in males have risen significantly, continuing to increase at six months and peaking in the adult (1-2 years). Ovarian testosterone concentrations were consistently lower than those in the testis as were those of adrenals of both sexes. Thus the testis would appear to be the major source of androgen production throughout life in this species.

Puberty is reached in *Monodelphis* at 4 months as shown by the expression of 3β- hydroxysteroid dehydrogenase, a rise in circulating testosterone levels and the first appearance of sperm at this time.

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Chapter 1: General Introduction

1.1. Sexual Differentiation in Eutherians

1.1.1. History

In 1947, Alfred Jost established a theory for the fundamental mechanism of phenotypic sexual differentiation in mammals. Jost proposed that sexual differentiation in eutherians is a sequential process beginning with the establishment of chromosomal sex at fertilisation, followed by the development of gonadal sex, and culminating in the formation of the sexual phenotype (Fig. 1.1). Each step in this process is dependent on the preceding one and, under normal circumstances, phenotypic sex conforms to chromosomal and gonadal sex.

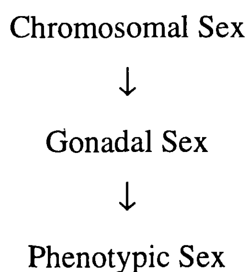


Fig. 1.1. Jost's model for sexual differentiation (1947; 1953).

According to Jost's formulation, chromosomal (or genetic) sex, established at the time of conception, directs the development of either ovaries or testes. If testes develop, their subsequent hormonal secretions elicit the development of male primary and secondary sex characteristics, therefore collectively forming the male phenotype. If an ovary develops (or if no gonad is present), development of phenotypic sex is female in character.

1.1.2. Chromosomal sex

In eutherians, the genetic nature of the spermatozoon penetrating and activating the oocyte is the primary determinant of the sex. The sex chromosome constitution of the zygote is accepted as having been imposed at fertilisation by the penetrating spermatozoon. The chromosomal sex-determining system in eutherians is XX : XY system, where females are chromosomally XX and males are XY.

The chromosomal basis for sex determination was first established between 1910 and 1916 by Morgan and his American colleagues. According to their work, sex determination is controlled by the number of X chromosomes; the Y chromosome plays no role in the process except for a contribution to male fertility. However, when karyotyping techniques for mammalian chromosomes were developed in the late 1950s and early 1960s, it became apparent that the presence of the Y chromosome is male determining and there is a vital segment on the Y chromosome that acts as the primary signal for male development in both mouse and human (for a succinct historical review see Hunter, 1995).

No matter how many X chromosomes are present in the embryo, the presence of a single Y chromosome (as in XY, XXY, XXYY, XXXY, etc.) dictates formation of a testis and, therefore, phenotypic male development (Davis, 1981). In the absence of a Y chromosome and its primary signal (a presumptive gene or sequence of genes), development will proceed in a female direction (Jost *et al.*, 1973). The primary sex-determining signal on the Y chromosome is a testis-determining signal referred to as TDF in human and Tdy in mouse (Davis, 1981).

One early proposal was the involvement of a protein - H-Y antigen, a term used for a male-specific cell-surface transplantation antigen originally thought to be controlled by the Y chromosome (Eichwald & Silmser, 1955; Billingham & Silvers, 1960). Wachtel *et al.* (1975) used serological assays to detect antigen as the elusive factor, and proposed that the antigen had a testis-determining effect (Wachtel *et al.* 1975; Ohno, 1976). The male-specific cell-surface antigen would act as a diffusible molecule to mediate transformation of the sexually indifferent gonad into a testis, that is to switch cells of the indifferent gonad into the testicular pathway. Lack of H-Y antigen in the female would result in the differentiation of ovaries.

In 1990, Sinclair *et al.* and Gubbay *et al.* found comparable genes located in the sex-determining region of man (SRY) and mouse (Sry) respectively, which are the best candidates for TDF. The presence of SRY or Sry on the Y chromosome acts as a transcriptional switch affecting the expression of subsidiary autosomal genes to control the formation of the testis, while the absence of the gene in the female (or the deletion of the gene in a male) leads to ovarian development.

1.1.3. Differentiation of the Gonads

Differentiation of the gonads is the first phenotypic evidence of sexual dimorphism and involves the migration, organisation and differentiation of both germinal and somatic cells (Fig. 1.2) (numerous reviews: Jost *et al.*, 1973; Wartenberg, 1989a & b; Jost & Magre, 1993; Frederick & Wilson, 1994; Hunter, 1995). The presumptive gonads, or genital ridges, first appear as a stratification of the coelomic epithelium on the medial aspect of the paired mesonephroi during early embryonic development (Gardner *et al.*, 1985). At this time the sex of the developing gonads cannot be determined morphologically and the indifferent gonads become populated with the primordial germ cells (PGCs).

The indifferent gonad is composed of three distinct cell types: germ cells, supporting cells of the coelomic epithelium of the gonadal ridge, and stromal (interstitial) cells derived from the mesenchyme of the gonadal ridge (for review see Frederick & Wilson, 1994). If the embryo is genetically male (XY), the PGCs preferentially colonise the medullary region of the presumptive gonads whereas, if genetically female (XX), the PGCs become concentrated in the cortical region (for review see Hunter, 1995).

The earliest PGCs are characterised by their ability to synthesise glycogen and alkaline phosphatase which can be demonstrated by appropriate histochemical techniques (McKay *et al.*, 1953). Primordial germ cells - progenitors of the sex cells - were first identified by Witschi in the wall of the yolk sac (Witschi, 1948). More recently, in the human embryo, Fujimoto *et al.* (1977) demonstrated PGCs in the dorsal endoderm of the yolk sac membrane at 4 weeks post-conception, in the hindgut epithelium and mesentery at 5 weeks and then in the genital ridge at 6 weeks. In the mouse embryo, PGCs can be identified at an earlier stage (7-7.5 days post coitum) as a cluster in the mesoderm posterior to the primitive streak (Ginsburg *et al.*, 1990).

Primordial germ cells are distinguishable from other (somatic) cells of the developing embryo because of their significantly greater size, their large spherical nuclei and their distinctive cytoplasm which contains a substantial amount of glycogen, numerous lipid droplets, ribosomes and mitochondria (Fujimoto *et al.*, 1977; McLaren 1983). These cells increase in number by mitotic division during

migration (e.g. 13-day mouse fetal gonads may contain > 10,000 germ cells), but most conspicuously once they reach the presumptive gonads (Tam & Snow, 1981).

In eutherians, formation of the gonad may involve contributions from four distinct lineages: the coelomic epithelium, mesenchymal cells, the mesonephros and the germ cells themselves. However, there is no consensus as to whether the somatic cells of the gonad are derived from the coelomic epithelium by ingrowth (Yoshinaga *et al.*, 1988) or from the mesonephros by outgrowth (Mackay *et al.*, 1989; Smith & Mackay, 1990; Buehr *et al.*, 1993), or from both coelomic epithelium and mesonephros (Pelliniemi, 1975; Wartenberg 1981; 1982).

Prior to differentiation of the testis, there is an incomplete separation between the mesothelium and the underlying tissue, because the basal lamina of the coelomic epithelium is not yet fully formed (for review see Byskov & Hϕyer, 1994). The earliest histological indication of gonadal differentiation is the development of primordial Sertoli cells and their organisation as presumptive seminiferous cords in genetic males (Jost, 1972b; Jost & Magre, 1984), supported by a basal lamina. In addition, the genital ridges are larger in XY than in XX embryos even before Sertoli cells can be recognised histologically (Mittwoch, 1970; 1985). The significance of the more rapid growth of gonads in the male is that testicular products such as androgens and Müllerian Inhibiting Substance (MIS) are necessary to prevent phenotypic feminization as the 'default' pattern (Jost *et al.*, 1973).

Differentiation of the Testis

Differentiation begins at the centre of the genital ridge in the human and at the cranial end of the genital ridge in the mouse. As testis formation proceeds, germ cells migrate into the medulla and the sex cords become a prominent early feature (at about 12.5 days *post coitum* (dpc) in the mouse (Ginsburg *et al.,* 1990), 13.5 dpc in rat and 6-7 weeks gestational age in the human (Jost, 1970a). Germ cell mitosis continues in the developing testis, but the initiation of meiosis in the male is delayed until the time of puberty (Jost & Magre, 1993). The XY germ cells in the testis enter a state of mitotic arrest as (pro) spermatogonia at the same time as female germ cells enter meiosis (at 13 - 15 dpc in the mouse), and they resume mitotic proliferation in the immediate postnatal period (McLaren, 1983; Jost & Magre, 1993).

The primordial Sertoli cells are specialised somatic cells with clear abundant cytoplasm and surround the germ cells achieving intimate contact by long cytoplasmic processes. Differentiation and alignment of Sertoli cells are the first steps in testicular cord formation in genetic males, simultaneous with a rapid proliferation and aggregation of PGCs (Jost, 1972b, Jost & Magre, 1988).

The Leydig cells differentiate later, shortly after the testicular cords have formed, and this is correlated with the onset of steroidogenesis (Black & Christensen 1969; Russo & Rosas, 1971; Byskov, 1986). The theory that Sertoli cells induce the development of Leydig cells was first proposed by Kitahara (1923). Jost and Magre (1988) also demonstrated that the differentiation of Leydig cells from mesenchymal precursors is under the influence of Sertoli cells. Precursors migrate from the mesonephric region into the genital ridge before 12.5 dpc and Leydig cells are recognisable at 13.5 dpc in the mouse embryo or in the rat at 15.5 dpc (Wartenberg, 1989a; Jost and Magre, 1993).

The first important step in testicular differentiation is the formation of sex cords, precursors of the future seminiferous tubules. Testicular cords are made up of the primordial Sertoli cells and PGCs; differentiating Sertoli cells organise the position of germ cells within the future testicular cords. The formation of sex cords requires peritubular myoid cells, which migrate from the mesonephric region (Buehr *et al.*, 1993) into the differentiating testis and collaborate with pre-Sertoli cells in the production of a basement membrane (Mackay & Smith, 1989; Tung *et al.*, 1984).

Around the periphery of the developing gonad, a membranous tunica albuginea forms and a prominent blood supply is characteristic of the developing testis (Mackay *et al.*, 1993). Although the tunica albuginea and the interstitial tissue become extensively vascularised, germ cells are isolated from direct contact with blood capillaries by a more or less complex basement membrane and the development of junctional complexes between the Sertoli cells; together these constitute the so-called blood-testis barrier (Dym & Fawcett, 1970).

Differentiation of testicular cords occur in the mouse at 13.5 dpc and in the rat at 14.5 dpc. These cords are disposed in double arcades in planes perpendicular to the length of the gonad (Magre & Jost, 1983). During further development, the interstitial cells become packed together; Sertoli cells migrate to the outer surface of

the cords which remain in double arcades connected with the rete testis. The cords become the future seminiferous tubules once a central lumen develops and a basement membrane has formed (Fig. 1.2). At the same time the seminiferous tubules become convoluted (Clermont & Huckins, 1961; Magre & Jost, 1983).

Differentiation of the Ovary

At the time of testicular differentiation, the ovarian anlage remains morphologically indifferent since female gonads grow more slowly (Jost *et al.*1973). However, the female gonad displays a transient endocrine activity and produces oestradiol at this stage (Johnson & Everitt, 1980). The female gonad can only be recognised as a potential ovary by the absence of testicular cords. Subsequently the PGCs continue to proliferate mitotically, and the primitive sex cords of the female remain irregular and ultimately break up and disappear in the medullary regions. During early stages of ovarian differentiation three main events occur: 1) meiotic prophase is initiated; 2) the diplotene oocyte is enclosed in the germ cell compartment, the follicle; and 3) the steroid-producing cells, the theca cells, and interstitial cells outside the follicle are differentiated (Byskov, 1986; Byskov & Hϕyer, 1994).

In formation of the female gonad, two overall patterns of differentiation may be distinguished, depending on whether the ovarian germ cells enter immediate meiosis (e.g. mouse, human) without previous steroid synthesis by the neighbouring somatic cells, or relatively delayed meiosis (e.g. hamster, rabbit) with steroid synthesis being demonstrable before meiosis begins (Byskov, 1979). Byskov & Hϕyer (1994) believe that cells derived from the mesonephros are the principal contributors to the ovary cell mass. The supporting cells differentiate as pre-follicle or pre-granulosa cells under the influence of oocytes. The processes involved in the formation of the ovarian are well-documented (reviews: Witschi, 1951; Byskov, 1986; Wartenberg, 1983; 1989b; Byskov & Hϕyer, 1994; Hunter, 1995).

During ovarian differentiation, the germ cells become enclosed within elongated cell cords proliferated from the coelomic epithelium which are connected to the mesonephros, although the mesonephric tissue gradually regresses (Byskov, 1986; Byskov & Hϕyer, 1994). Cords of mesonephric cells promote ovarian transformation by colonising the central part of the presumptive gonad and

displacing germ cells towards the ovarian periphery (cortex) (Wartenberg, 1983). Germ cells remaining in the medulla tend to degenerate whilst those in the surrounding cortex continue to proliferate and/or to differentiate (Wartenberg, 1989b). The primitive medullary sex cords degenerate to be replaced by the well-vascularised ovarian stroma. The *tunica albuginea* is formed by mesenchymatous cells. The formation of nests of germ cells distinguishable as oogonia is followed by further development and differentiation so that production of potential gametes - the primary oocytes - is essentially complete by the time of birth in most eutherians (Hunter, 1995).

Oocytes enter meiosis but become arrested at the diplotene stage. During this process, not only does the number of oocytes no longer increase but most of them degenerate and disappear. The oocytes which are maintained and which will be present in the definitive ovary are those surrounded by follicular cells (cells homologous to Sertoli cells in the male) to form primordial follicles (Fig. 1.2) (Jost & Magre, 1993). Extensive atresia of oocytes is a major feature even before the time of birth (Baker, 1972, 1982; Zuckerman & Baker, 1977). Resumption of meiosis is a preliminary to the events of ovulation in the adult (Johnson & Everitt, 1980, Jost & Magre, 1993).

Functional interactions between the theca and granulosa cells and between somatic and germ cells appear important for the endocrine role of the ovary. As to the actual origin of follicular cells, in many eutherians such as the mouse, the granulosa cells are derived from rete cells and originate in the mesonephros. Once such rete cells reach the differentiating ovary, they stop migrating and proliferate actively to accumulate as granulosa cells (Hunter, 1995). In the rat oocytes induce the supporting cell lineage to differentiate as follicle cells rather than as Sertoli cells (McLaren, 1991a). The time at which actual follicles commence to form in different species depends on when the oocytes reach the diplotene stage of the first meiotic prophase, and may occur during fetal life or after birth (Byskov & Hφyer, 1994).

1.1.4. Endocrine differentiation of Gonads

A role for gonadal hormones in sexual differentiation was first proposed by Bouin and Ancel (1903). In subsequent studies, Jost's experiments with the rabbit embryo were clear-cut and established that the development of phenotypic sex in

eutherian mammals is female and that hormone secretion from the fetal testes are essential for male development (Jost, 1947). Development of the female urogenital tract occurs in the absence of gonads (Jost, 1953). This finding facilitated understanding of the scientific principle of sexual differentiation and clinical disorders of human sexual development. Further investigations have been directed towards elucidating the role of hormones secreted by the fetal testes (Jost 1961), the control mechanisms that regulate their rates of secretion during critical periods, and characterising their mechanism of action at the molecular and genetic levels (for review see: Jost & Magre, 1984; 1993; Wilson *et al.*, 1995).

In mammals, all somatic sexual dimorphisms have been assumed to be a consequence of gonadal hormone action. The conventional view of mammalian sexual differentiation has been that a gene or genes on the Y chromosome causes the indifferent gonad to develop into a testis, which then secretes two classes of hormone. Androgens, chiefly in the form of testosterone secreted by the Leydig cell, actively stimulate growth and development of the Wolffian duct system in the male (including the epididymis, vas deferens, seminal vesicles and ejaculatory duct), as demonstrated by Greene *et al.* (1939), Jost (1947) and reviewed by Jost (1953). The polypeptide Müllerian inhibitory substance (MIS) is produced by Sertoli cells and acts locally to cause regression of the adjacent paramesonephric ducts (Müllerian ducts) in genetic males (Josso *et al.*, 1977; Vigier *et al.*, 1983). In the absence of a Y chromosome, the indifferent gonad develops into an ovary which does not produce androgens or MIS, so that the Wolffian duct derivatives regress and the Müllerian ducts persist to form the female reproductive tract (including the Fallopian tubes, uterus and upper vagina) (Jost *et al.*, 1973; Wilson *et al.*, 1981; Short, 1982; Josso & Picard, 1986).

Müllerian inhibiting substance

Müllerian inhibiting substance (also known as Anti- Müllerian hormone) is a large (approximately 140kDa) dimeric glycoprotein molecule and a member of the TGF-β family of growth and differentiation factors. This hormone is synthesised by Sertoli cells within the fetal testis and granulosa cells of the ovary after birth (for review see Cate *et al.*, 1990).

In the male, MIS is the first known molecule to be produced by the developing Sertoli cells (Tran *et al.*, 1977; Tran & Josso, 1982; Josso, 1994) and its activity is expressed at the time of formation of seminiferous tubules, being highest during the period when the Müllerian duct regresses (Tran *et al.*, 1987). Sertoli cells continue to express high levels of MIS after birth, then expression progressively decreases and drops sharply at puberty (Baker *et al.*, 1990; Hutson *et al.* 1990; Josso *et al.*, 1990), after which only traces of MIS are detectable in rat testis fluid (Josso, 1972; 1973; Tran & Josso 1977) and low MIS concentrations are found in human adult serum (Hutson *et al.*, 1990). In the ovary, granulosa cells share many structural and functional characteristics with Sertoli cells. The possibility that MIS was also responsible for the ovarian effect was considered by Jost *et al.* in 1972 but could not be addressed without purified MIS. Not until 1981 was the first indication of ovarian MIS production proposed by Hutson *et al.* who demonstrated anti-Müllerian activity in ovarian tissue of hens. Subsequently, MIS was detected in bovine follicular fluid by bioassay and radioimmuneoassay (Vigier *et al.*, 1984). A low level of MIS is produced at birth by granulosa cells of antral follicles while a much higher level is produced in adult follicles (Takahashi *et al.*, 1986a). However, whatever the degree of follicular maturation or the age of the animal, the production of MIS by granulosa cells is low compared with that of immature Sertoli cells (Picard *et al.*, 1986; Cate *et al.*, 1990).

Taketo *et al.* (1993) investigated the site and timing of anti-Müllerian hormone synthesis in the mouse gonad by immunocytochemical staining. In the XY gonad MIS was first detected in Sertoli cells on day 12 of gestation and staining remained intense until day 4 post partum. MIS was also detectable in the XX gonad in granulosa cells of growing follicles on day 7 post partum and staining remained detectable thereafter.

Müllerian inhibiting substance synthesis begins before the appearance of Leydig cells and has not been demonstrated to require hormonal control. These observations indicate that MIS expression is found in immature Sertoli cells, is probably dependent upon SRY, and is not totally repressed in the mature testis (Taketo *et al.*, 1993). Nonetheless, the Müllerian ducts of developing male embryos are only sensitive to MIS during the early period after gonadal sex differentiation (Josso *et al.*, 1977), that is at the end of the indifferent stage. MIS may have a

physiological role in regulating oogenesis. Takahashi *et al.* (1986b) proposed that MIS may be involved in preventing the oocyte from completing the first meiotic division. Although the role of MIS in the ovary is unknown, studies have identified the stages of follicular development where MIS is expressed.

Testosterone

The second developmental hormone of the fetal testis is testosterone (Jost, 1953). Sexual differentiation of the Wolffian duct derivatives depends on testosterone, secreted by the fetal Leydig cells (Jost, 1963; 1973; Hall, 1994). Although Leydig cells were fully described in 1850 by Leydig, the endocrine function of these cells was first proposed by Bouin and Ancel (1903). In eutherians, at least two populations of Leydig cells have been identified: the fetal Leydig cells are responsible for the primary somatic masculinisation and sex differentiation of the nervous system, while the postnatal population is activated at puberty and remains during adult life (Zirkin and Ewing, 1987; De Kretser & Kerr, 1988). Testosterone formation in the testis begins shortly after the onset of differentiation of the testicular cords and is coincident in eutherians with the histologic differentiation of the fetal Leydig cells (Gondos, 1980). The steroidogenic activity is expressed with two populations of Leydig cells by measuring blood and the activity of the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD).

In the mouse ovary, the first cells to possess organelles characteristic of steroid production appear a few days after birth (Pehleman & Lombard, 1978), when the first follicles start growing (Peters, 1969). This coincides with the expression of 3β-HSD activity, which is initially detectable by day 3 postpartum and increases thereafter (Hϕyer & Byskov, 1981). Furthermore, the first cells to acquire this enzyme activity are the mesonephric-derived intra-ovarian rete cells, which form the first thecal cells. In the human fetal ovary, 3β-HSD activity was also demonstrated at midpregnancy in theca cells of the newly formed follicles (Goldman *et al.*, 1966; Hϕyer, 1980)

1.1.5. Phenotypic Sex

Development of phenotypic sex involves differentiation of both the internal reproductive tract and the external genitalia accompanied by testis descent into the scrotum in the male.

Indifferent Stage

In the early phases of embryonic development, no sex differences in structure can be discerned although the genetic sex has already been determined at fertilisation. The first morphological evidence of sexual differentiation appears much later, at the time when the gonads differentiate. Gonadal ridges develop at the surface of the mesonephros near the root of the dorsal mesentery, appearing as a thickening of the coelomic epithelium and an underlying cellular condensation. The differentiation of the genital tract progresses, subsequently, according to a chronology well determined for each species, but it is at first identical in both sexes, during the so-called indifferent stage (Jost & Magre, 1993).

The internal urogenital tracts in males and females arise from separate anlagen, the Wolffian (mesonephric) and Müllerian (paramesonephric) ducts respectively (Fig. 1.3). The two ducts are present in the early embryonic stages of both sexes (being identical in the two sexes up to 8 weeks in the human and 12 days in the mouse). During development only one pair of ducts in each sex will persist while the other pair regresses as a consequence of gonadal differentiation (Cate & Wilson, 1993; Dyche, 1979). The Wolffian ducts (named after Wolff 1733-1794, who first described it) run in a cranio-caudal direction along the external edge of the mesonephros; this duct is connected anatomically to the indifferent gonad and under the future bladder, to the urogenital sinus. The Müllerian ducts differentiate alongside the Wolffian ducts. Each appears as a funnel of the coelomic epithelium at the top of the mesonephros and is not associated with the gonad. Both types of duct terminate in the urogenital sinus. In the male, the Wolffian ducts give rise to the epididymides, vasa deferentia and seminal vesicles, and the Müllerian ducts disappear (Fig. 1.3). In the female, the Müllerian ducts give rise to the Fallopian tubes, uterus and upper vagina, and the Wolffian ducts either disappear or persist as vestigial remnants (Fig. 1.3).

In contrast, the male and female external genitalia develop from common anlagen (Fig. 1.4): the genital tubercle, genital folds and genital swellings. In the female the system elongates but changes little: the genital tubercle becomes the clitoris; the genital folds become the labia minora; and the genital swellings become the labia majora. In the male fusion and elongation of the genital swellings become the scrotum, and the prostate forms in the wall of the urogenital sinus.

Male development

The development of the male genital ducts involves two different processes (Fig. 1.3): the degeneration of Müllerian ducts and the integration of mesonephros and Wolffian ducts to form the male genital apparatus. The first sign of differentiation in the male urogenital tract is the degeneration of the Müllerian ducts adjacent to the testes, a process which begins just after the formation of the testicular cords. Eventually the Müllerian ducts will regress completely. The transformation of the Wolffian ducts into the male ejaculatory system begins subsequent to the onset of Müllerian duct regression. The portion of the Wolffian duct adjacent to the testis becomes convoluted to form the epididymis; the central portion of the duct becomes the vas deferens. Seminal vesicles develop as buds from the lower portions of the Wolffian ducts just before they enter into the urogenital sinus. The prostatic and membranous portions of the male urethra develop from the pelvic portion of the urogenital sinus (for review see Jost & Magre, 1993; Byskov & Hϕyer, 1994).

The external genitalia of the male (Fig. 1.4) begin to develop shortly after the onset of virilization of the Wolffian ducts and urogenital sinus. The genital tubercle elongates, and the urethral folds begin to fuse over the urethral groove from posterior to anterior so that the urogenital cleft closes to form the penile urethra. The fusion of the urethral folds brings the two genital swellings together to form the scrotum (for review see Jost & Magre, 1993; Frederick & Wilson, 1994).

Two aspects of male phenotypic development take place during the latter phases of gestation: growth of the male phallus and testicular descent. There is little difference in the size of the genital tubercle in the two sexes, but the male phallus grows during the latter phases of fetal development under the influence of

androgens from the fetal testis and by the time of birth is larger than the urogenital tubercle of the female (Frederick & Wilson, 1994).

Testicular descent

In most eutherians, testes can only become fully functional if they have descended from the abdominal cavity to the scrotal sac as spermatogenesis is inhibited at body temperature (Wensing, 1988). Testis descent to the scrotum is described as occurring in two distinct phases (Fig. 1.5)(Wensing & Colenbrander, 1986; Wensing, 1988; Heyns, 1987): 1) the transabdominal phase, an androgen-independent transabdominal migration of the testis to the inguinal region; and 2) the inguinoscrotal phase, involving migration of the testis through the inguinal canal down to the scrotum (Hutson *et al.*, 1990). This phase is regulated by testosterone (Hutson, 1985). In the rat, the transabdominal phase of testicular descent occurs between 16 - 20 d.p.c. (Wensing & Colenbrander, 1986) and the inguinoscrotal phase is completed by the end of the third week after birth.

The mechanics of testicular descent still remain poorly understand, although it is said that the gubernaculum plays a key role (Hunter, 1762). The gubernaculum is a mesenchymal structure extending from the caudal pole of the testis to the region of the inguinal canal in the anterior abdominal wall and into the scrotal swelling. van der Schoot (1993a), based on his observations on the rat, has questioned the concept of two phases of descent and noted that there is no evidence for active testis migration from the posterior abdomen towards the inguinal region during fetal life; rather, the testes remain in place at the base of the abdomen in fetuses of 15-16 days old. Furthermore, van der Schoot (1992) has stated that androgens play no role in the prenatal growth or the postnatal inversion and further development of the gubernacular and cremaster muscles, although in rabbits fetal testes certainly control prenatal growth and differentiation of the gubernacular cones (van der Schoot, 1993b). Anti-Müllerian hormone rather than androgens has been implicated in promoting a first phase of testicular descent though this remains controversial (Hutson, 1985; Hutson *et al.*, 1988; Hutson & Donahoe, 1986).

Female development

The internal reproductive tract of the female is formed from the Müllerian ducts whose cephalic ends are the anlagen for the Fallopian tubes (Fig. 1.3). The caudal portions of the ducts fuse to form the uterus. Meonephros and Wolffian ducts disappear, with the exception of remnants such as the rete ovarii in the ovarian hilum. Contact of the Müllerian ducts with the urogenital sinus induces an intense proliferation of endodermal cells that results in the formation of the uterovaginal plate between the Müllerian ducts and the urogenital sinus (O'Rahilly, 1977). The cells of the uterovaginal plate proliferate, thus increasing the distance between the developing uterus and the urogenital sinus. Later, the plate canalises to form the lumen of the vagina (for review see Frederick & Wilson, 1994).

The vagina forms at the level where Müllerian ducts merge into the urogenital sinus but there are many species variations (for review see Jost & Magre, 1993). In the rabbit, the vagina is formed by the fusion of the posterior parts of the Müllerian ducts. The "Müllerian vagina" opens into the anterior part of the urogenital sinus which will differentiate into a long urethra, the exact homologue of the male prostatic urethra. In the mouse, the fused portion of the Müllerian ducts is short and the vagina derives mainly from the epithelium of the urogenital sinus. Progressively, the vagina separates from the urogenital sinus and opens into an independent orifice on the body surface. In other animal species, the vagina and the female urethra are not so distinctly separate.

In contrast to the male, in which the phallic and pelvic portions of the urogenital sinus are enclosed by fusion of the genital folds, most of the urogenital sinus of the female remains exposed on the surface as a cleft into which the vagina and urethra open. The urogenital tubercle of the female undergoes limited growth and development to form the clitoris. In most species, the genital tubercle and urethral groove are very little modified during differentiation of the female external genitalia (Fig. 1.4) (George & Wilson, 1994).

1.1.6. Role of hormones in sexual differentiation

Development of phenotypic sex results from the action of three hormones (Fig. 1.6): 1) Müllerian inhibiting substance (MIS); 2) testosterone and 3) dihydrotestosterone (DHT). Only the first two are primary products of the testis,

most DHT is formed by 5α - reductase at target tissues (Blecher & Wilkinson, 1989; Josso *et al.*, 1990; Wilson, 1992).

Testosterone promotes male development in two ways: a) by acting directly on the Wolffian duct to stimulate its conversion into epididymis, vas deferens and seminal vesicle; b) by serving as a prohormone for the third fetal hormone dihydrotestosterone which induces both formation of the prostate from the urogenital sinus and formation of the external genitalia (Wilson *et al*, 1981).

Dihydrotestosterone has some 10x the affinity for androgen receptors than testosterone (Grino *et al.*, 1990), enabling the external genitalia to be masculinised by very low concentrations of testosterone. Dihydrotestosterone stimulates the urogenital sinus to form the prostate and the membranous urethra, the urogenital tubercle to form the penis and penile urethra and urogenital swelling to from the scrotum (Wilson *et al.,* 1981). The supporting evidence here is that the external genitalia contain 5α-reductase, the enzyme that converts testosterone to dihydrotestosterone. The fact that the internal ducts do not contain 5α-reductase could act to protect the male embryonic urogenital sinus and external genitalia against high concentrations of progesterone in the blood and amniotic fluid (Hunter, 1995).

The critical role of testosterone in the development of the male urogenital tract can be deduced from three types of embryological and endocrinological evidence. First, the fact that testosterone synthesis immediately precedes the initiation of virilization of the urogenital tract in a variety of species (Lipsett & Tullner, 1965; Wilson & Siiteri, 1973). Second, the administration of androgens to female embryos at an appropriate time in fetal development causes male development of the internal and external genitalia (George & Peterson, 1988). Third, administration of pharmacological agents that specifically inhibit the synthesis or action of androgens impairs male development (Neumann *et al*, 1970).

1.1.7. Summary

During mammalian sex determination and differentiation, the genital ridge arises as a thickening on the medial side of the mesonephros and is bipotential, developing as a testis only if the testis-determining gene on the Y chromosome (Tdy) is active. In the absence of the gene, development follows the default

pathway giving rise to an ovary. In contrast the anlagen of the male and female reproductive tracts, the Wolffian and Müllerian ducts respectively, are both present prior to sex determination. Each anlage is unipotential and the survival and development of one versus the other depends on the type of gonad that differentiates and its products. In the female, the Wolffian duct system degenerates and the Müllerian ducts give rise to the oviducts, uterus and upper vagina. This does not depend on any factors produced by the ovary and so can be considered as a default pathway. In the male, therefore, two processes have to occur. The Wolffian ducts must be maintained and stimulated to differentiate into the male tract and accessory organs by the influence of testosterone produced by Leydig cells in the testis. Additionally, the Müllerian duct system has to regress, due to the action of anti-Müllerian hormone produced by pre-Sertoli cells.

1.2. Sexual differentiation in marsupials

1.2.1. The History of Marsupials

Three sub-classes of living mammals can be distinguished: the monotremes which lay eggs, the marsupials which give birth to relatively undeveloped young and the eutherians which give birth to more developed young. The traditional view is that the marsupial is primitive compared to eutherian mammals, but it is now recognised that they merely represent an alternative path from eutherian evolution. No other function so distinguishes marsupials from the eutherians as the manner of their reproduction.

Fossil evidence has shown that eutherians and marsupials shared a common ancestor and that their divergence occurred more than 100 million years ago. The earliest fossil records which are unquestionably marsupial are 70 - 80 million years old and come from the Milk River formation of Alberta, Canada (Fox, 1971). Thus, it is strongly believed that marsupials originated in Western North America: some time during the Cretaceous they radiated throughout North and South America and then dispersed across the globe by various routes. From North America, one group of didelphids reached Europe, probably via Greenland, and survived there until competition from eutherians led to their demise. Others radiated from South America by island-hopping along the archipelago of West Antarctica, across East

Antarctica and thence into Australia (Butcher, 1995; Tyndale-Biscoe & Renfree, 1987).

The geographical separation of marsupials into Australian and American groups occurred about 70 million years ago (VandeBerg, 1983). There are about 250 living species in 16 families, with approximately two-thirds of them living in Australia. The Americas are inhabited by three families - the Didelphidae, Caenolestidea and Microbiotheriidae of which the Didelphidae is the largest one containing 60 species (Butcher, 1995; Tyndale-Biscoe & Renfree, 1987).

The study of marsupials began in the early part of the 16th Century when Vincente Yanez Pinzon, the Spanish explorer of South America, discovered the opossum during his first voyage to Brazil (Clemens, 1968). Originally, biologists classified the marsupials as a variety of eutherian mammal which showed similar characteristics: opossums, armadillos, hedgehogs, pigs and shrews were grouped together because of their common possession of sharp teeth (Butcher, 1995). It was not until 1816 that de Blainville recognised that despite similarities with the carnivores and rodents, the marsupials could be distinguished from eutherian mammals by the anatomical and physiological features of their reproduction.

As the name implies, most female marsupials possess a pouch (or marsupium) on the ventral abdominal wall that encloses the teats and mammary glands. The most developed form is found in marsupials which climb (phalangers), dig (bandicoots and wombats, in which the pouch opens to the rear), hop (kangaroos) or swim (yapoks). However, in some small terrestrial marsupials such as the South American *Monodelphis*, the pouch is absent (Kirsch, 1977). In certain marsupials the pouch merely consists of folds of skin around the mammae and in many species a pouch is only developed when the mother is suckling.

In 1938, McCrady emphasised an important and useful characteristic of marsupials: the young are born in a underdeveloped state. They are very small and for some time after birth each pup is permanently attached to one teat and has exclusive use of the associated mammary gland, so that the maximum litter size is determined absolutely by the number of functional mammary glands the female possesses. Furthermore, young are most vulnerable not at birth but at the time of emergence from the nest or pouch.

1.2.2. Marsupial Reproductive Anatomy

The unusual reproductive anatomy of marsupials (Fig. 1.7) was discovered over 300 years ago when Tyson dissected Virginia opossums (*Didelphis virginiana*) (1698). Tyson reported that the genital tract was double from the ovaries to the opening of the urethra where the two lateral vaginae joined to form a common urogenital canal. Moreover, there was no direct passage between the uterus (= median vagina) and the common canal. He concluded that the penis of the male might be divided to enter the two vaginae. In the following century, this work was confirmed by his colleague William Cowper (1740) who dissected the same species. These two studies contributed the foundations of reproductive biology in marsupials.

Anatomy of female urogenital tract

Initially, all female marsupials possess two separate uteri, which open by separate cervices into the vaginal culs-de-sac. Two vaginae run lateral to the ureters to open posteriorly into the urogenital sinus (Fig. 1.7); these lateral vaginae are used exclusively for sperm ascent. Birth occurs through a third structure the median vagina or birth canal, which also connects the vaginal culs-de-sac with the urogenital sinus. This median vagina becomes patent at the time of parturition and closes up again afterwards in most species, although it remains open after the first parturition in the macropodids (kangaroos and wallabies) (Tyndale-Biscoe, 1973; Tyndale-Biscoe & Renfree, 1987; Renfree, 1994).

In eutherian mammals, fusion of the posterior end of the paired oviducts forms a single vagina (Fig. 1.7), but the vagina remains paired in marsupials (Fig. 1.7). This is a result of the course of the ureters in marsupials which pass between and not around the developing oviducts, and so fusion into a single vagina is not possible. The retention of the paired lateral vaginae and the development of a third, median birth canal, results in the bizarre, tripartite arrangement of marsupials (for review see Renfree, 1994).

Anatomy of male genitalia

The genital tract consists of paired testes, epididymides and vasa deferentia which open into the anterior end of the large prostate gland. Male marsupials are

unlike eutherians (Fig. 1.7) in that the penis is posterior (caudal) to the scrotum and the vasa deferentia open into the urethra without first having to curve around the ureters. In addition, the pouch, mammary glands and teats are universally absent in all adult males (Tyndale-Biscoe, 1973).

Moreover, in contrast to eutherians, the anal, urinary and genital passages of all marsupials open into a common cloaca with only a single external orifice. The penis of the male protrudes through this when erect. Although the rectum shares this common external orifice, the rectum and the urinogenital openings have separate sphincters (Tyndale-Biscoe & Renfree, 1987; Renfree, 1994).

In marsupial and eutherian mammals, the position of the ureters with respect to the Wolffian ducts differs: the ureters pass medially between the genital ducts in marsupials, and laterally in eutherians to reach the bladder.

1.2.3. Sex Determination and Differentiation

Sex Chromosomes

Marsupials, like eutherians, normally require the presence of a Y chromosome for testicular formation. A male-specific homologue of the putative testis-determining gene SRY (sex-determining region of the Y chromosome) has been identified recently in marsupials (Foster *et al.*, 1992), but the products of this gene have not been isolated. The expression pattern of SRY in tammar differs from that of the mouse where SRY is expressed only in the gonad at the time of testis differentiation. SRY is transcribed not only in the male gonad throughout the period of testicular differentiation, but also at every stage examined, from before genital ridge formation until at least 40 days after birth in both the gonadal ridge, and in a variety of extra-gondadal tissues in males (Harry *et al.*, 1995; Renfree *et al.*, 1997).

Some sexually dimorphic characters such as the scrotum, mammary anlagen, gubernaculum and processes vaginalis appear to be under direct genetic rather than secondary hormonal control. Although the Y chromosome is testis-determining in marsupials as in eutherians, development of a scrotum or pouch appears to be under X chromosome control (Cooper, 1993). Pouched males have an XXY chromosome constitution while females with scrota are either XO or XX/XO in constitution. In the well-studied marsupials such as the tammar wallaby, differentiation of the pouch and scrotum commences before the onset of testosterone synthesis in the testis

(Renfree *et al.*, 1992). McLaren (1991) suggested that direct genetic control may perhaps be determined by the X chromosome, and chromosomal studies indicate that scrotal development in marsupials occurs when only a single X chromosome (XY or XO) is functional, whereas two X chromosomes (XX or (XXY) are necessary for pouch formation (Renfree 1992; Shaw *et al.*, 1990).

Differentiation of Gonads

The early development of the gonad has been described in some species of marsupial: *Dasyurus viverrinus* by Fraser (1919) and Ullmann (1984), *Didelphis virginiana* by Moore (1939) and Morgan (1943), *Perameles nasuta* and *Isoodon macrourus* by Ullmann (1981), *Macropus eugenii* by Alcorn (1975) and in *Monodelphis domestica* by Maitland & Ullmann (1993), Fadem *et al.* (1992), Baker *et al.* (1993; 1990) and Moore & Thurstan (1990). In most these species the gonads are at the indifferent stage at birth but differentiation occurs within a few days and, as in eutherians, the testis is recognisable before the ovary.

As with eutherians, formation of the genital ridge is on the medial side of the mesonephros; unlike eutherians the mesonephros is the functional kidney of the young marsupial (Bentley & Shield, 1962; Tyndale-Biscoe & Renfree, 1987) and is one the most prominent structures in the fetal abdominal cavity. As its function is progressively assumed by the metanephric kidney, the mesonephros starts to regress from the anterior end. Subsequently, the gonad becomes rounded and closely attached to the regressing portion of the mesonephros.

Histologically, differentiation of the testis becomes apparent on day 2 or 3 after birth in *D. virginiana* (McCrady, 1938; Burns, 1939a), *Perameles*, *Isoodon* (Ullmann, 1981) and *Dasyurus viverrinus* (Ullmann, 1984) and between day 3 and 7 in *M. eugenii* (Alcorn, 1975). In genetically male young, the homogeneous blastema becomes organised into a central stroma, in which are the rete cords surrounded by a zone of pale-staining cells, the medullary cords, and an outer layer of fibrous cells and coelomic epithelium (Tyndale-Biscoe & Renfree, 1987)

In all species of marsupial so far studied the ovary can be recognised a few days later than the testis when the blastema differentiates into an inner medulla and an outer cortex in which the germ cells are found. The reason for this probably is

due to the transformation and proliferation of germ cells into spermatogonia and growth of interstitial tissue (Renfree *et al.*, 1996).

Differentiation of the urogenital system

As mentioned above, marsupials are born with functional mesonephroi and the Wolffian ducts are functional urinary ducts in both sexes. The urogenital system, like the gonad, develops from the indifferent stage at birth to the distinctive male and female forms during the course of pouch life. The best-studied species in this respect are *Didelphis virginiana* (Baxter, 1935; McCrady, 1938; Burns, 1939c; Chase, 1939; Moore, 1939; Rubin, 1944) and the tammar wallaby *Macropus eugenii* (Renfree *et al.*, 1996).

Briefly, at the indifferent stage the mesonephric or Wolffian duct is patent and functions as the main urinary duct conveying urine from the mesonephros to the urogenital sinus or proctodaeum until about day 14 after birth in *Didelphis virginiana*. Running parallel and lateral to it is the Müllerian duct, which first appears on day 3 and opens to the coelom near the genital ridge. At day 10 it has made a connection posteriorly to the urogenital sinus near the of the Wolffian duct (Tyndale-Biscoe & Renfree, 1987).

In the tammar wallaby, the Wolffian ducts enlarge and elongate in the male as the testes migrates caudally before descending through the inguinal canal. In the female the Wolffian ducts regress from around day 10 and lose their lumina in the portion between the caudal end of the gonad and the urogenital sinus. However, the distal and proximal portions of the Wolffian duct remain patent until after day 25. The Müllerian ducts in the male begin to regress by about day 7 and around day 21 the mid-portion of the Müllerian ducts, between the gonads and the urogenital sinus, has completely regressed (Renfree *et al.*, 1996).

In male tammars regression of the Müllerian ducts is under the influence of MIS secreted by the testis (Short *et al.* 1988; Hutson *et al.*, 1988). Treatment of neonatal marsupials with oestrogens prevents Müllerian duct regression, presumably by inhibiting MIS production or action. Wolffian duct development is controlled by androgens. Treatment of new-born female pouch young with testosterone prevents regression of the Wolffian duct, which normally disappears within a few weeks of birth (Burns, 1961a & b; Shaw *et al.*, 1988).

Differentiation of Pouch and Scrotum

In *D. virginiana* both pouch and scrotum arise as paired bilateral folds on the ventral surface, anterior to the genital tubercle. Subsequently, the posterior ends fuse to form the scrotum in the male or the posterior lip of the pouch in the female (for review see Tyndale-Biscoe & Renfree, 1987). McCrady (1938) proposed them as homologous structures, as are the scrotum and labia majora of eutherian mammals. However, pouch rudiments have been recorded anterior to the scrotum in the male pouch young of eight species (Beddard, 1891; Bresslau, 1912; Pocock, 1926), and the adult males of a few species retain a well-formed pouch anterior to the scrotum (*Thylacinus*: Beddard, 1891; Pocock, 1926; *Chironectes*: Enders, 1966; Hunsaker, 1977), which suggests that pouch and scrotum arise from different parts of the same anlagen (for review see Tyndale-Biscoe & Renfree, 1987). In *D. virginiana* (McCrady, 1938), *M. eugenii* (Alcorn, 1975) and *T. vulpecula* (Ullmann, 1993) pouch, mammary gland and scrotal bulges are histogically distinct at birth.

In all marsupial species which have been studied so far the scrotal anlagen only develop in genetic males, and in most species primordia of the mammary glands only develop in genetic females: both structures differentiate several days before birth (Alcorn, 1975; O *et al.*, 1988). These early sexual dimorphisms appear to be under direct genetic control determined by X-linked genes (see: O *et al*, 1988; Shaw *et al.*, 1988, 1995; Renfree and Short 1988; Renfree *et al.*, 1995). However the mammary anlagen are present in male neonates of American didelphid marsupials *Didelphis virginiana* and *Monodelphis domestica* (Renfree *et al.*, 1990).

Role of Hormones

Burns (1945) studied the effects of hormone administration on development of the sexual phenotype in new-born opossums (*Didelphis virginiana*). Because of their sexual immaturity at the time of birth, the pouch young are accessible for experimental manipulation after they attach to the nipple. Administration of oestrogen caused some feminization of the urogenital tract of the male opossum, and treatment of female pouch young with androgens caused virilization of the urogenital tract. However, no hormone or combination of hormones caused reversal of the basic phenotype or influenced the basic secondary sex characteristics such as development of the pouch in the female or the scrotum in the male (Burns, 1945;

1961). As a consequence, these studies in the opossum cast doubt on the theory that hormones play a primary role in mammalian sexual differentiation.

The formation of a testis ensures virilisation only because testicular hormones act to elaborate a male genital system and male secondary sexual characteristics (Jost, 1953). Whilst this is the accepted sequence for eutherian mammals, O *et al.* (1988) have noted an apparent and important exception in marsupials in that scrotal bulges and mammary anlagen can be identified in the fetus before birth, and pouch development is the first evidence of sexual dimorphism in marsupials which can be identified at the gross level after birth. Somatic sexual differentiation was deduced to be under primary genetic rather than endocrine control, since the gubernaculum and scrotum undergo a significant phase of differentiation whilst the gonads are still sexually indifferent, as do the mammary anlagen and pouch in females (Renfree *et al.*, 1992, Ullmann, 1993). The gonadal steroids in the marsupial pouch young, as in pubertal mammals, appear to act as modifiers rather than as inducers of development (Wilson, 1994). These evidences imply that the differentiation of the scrotum and the pouch in the marsupial is controlled by nonhormonal processes. Thus, phenotypic development in marsupials differs in several important respects from the process in eutherian mammals.

Müllerian-inhibiting substance injected into a marsupial ovary, that of the tammar wallaby, even in quite massive amounts, fails to bring about masculinisation (Short *et al.*, 1988). Whilst the timing of this experimental approach may need to be modified, it is possible that the structure of the marsupial MIS molecule differs very slightly from that of eutherians or that there is a rigorous form of genetic control over gonadal determination that is resistant to this particular endocrine intervention (for review see Hunter, 1995).

The time scale of virilisation of the urogenital tract of the tammar wallaby differs from that in eutherian mammals, being much delayed. Testosterone is the androgen found in the neonatal testis and 5α-reductase activity is present in the urogenital sinus and phallus at the time of their virilisation (Renfree *et al.*, 1992a). This would suggest comparable endocrine function in marsupials and eutherians promoting full differentiation of the tract.

In the tammar wallaby, *Macropus eugenii,* O *et al.* (1988) reported that differentiation of the gubernaculum is hormone independent, highlighting the fact

that there are sexual dimorphisms in marsupials which precede morphological differentiation of the gonads, suggesting a precocious influence of one or more sex-linked gene.

1.2.4. The importance of studying sexual differentiation in marsupials.

All marsupial species investigated (a mere handful of the 249 extant species) show many differences in respect to eutherian species: a) it has proved difficult to sex the offspring at birth by external phenotypic characteristics; b) the young are born at the indifferent stage (development comparable to embryonic stages of eutherian species) of sexual differentiation, so that the process is readily accessible for experimental investigation; c) gonadal differentiation continues after birth, and is followed by differentiation of the Wolffian (mesonephric) and Müllerian ducts; d) testicular descent is not complete until some weeks after birth. This allows for postnatal research into reproductive development to be undertaken without having to kill the mother. Studies of fetal and neonatal stages of gonadal and genital differentiation in marsupials can contribute to our understanding of how sexual dimorphisms arise. e) The marsupial neonate is not under the influence of any placental hormones whilst it is in the pouch, the location at which gonadal differentiation is completed and this relative independence suggests that it might be possible to modify the gonads by administration of sex steroids to induce a complete, functional gonadal sex-reversal; f) structural features of the reproductive tracts differ from eutherians and g) the low number and large size of the chromosomes has enabled these to be identified more readily when compared with most eutherian species.

Evidence in marsupials indicates that some sexually dimorphic structures, such as the scrotum, pouch, teat and mammary gland, are more likely to be under direct genetic than hormonal control. How genetic sex is translated into phenotypic sex remains one of the most intriguing unanswered questions in mammalian sex determination and differentiation today and marsupials may prove to be the experimental animals of choice for solving this problem. Marsupials, therefore represent an important model system for studying sexual differentiation.

1.3. The grey short-tailed opossum, *Monodelphis domestica*

1.3.1. History

The American grey short-tailed opossum (*Monodelphis domestica*) (Fig. 1.8) is a member of the most ancient marsupial family Didelphidae, which separated from Australian marsupials about 70 million years ago (VandeBerg, 1983; 1990). *Monodelphis domestica* is one of seventeen species of the pouchless *Monodelphis* genus (Burnett, 1830) distributed from eastern Panama southward to central Argentina. This species is omnivorous, nocturnal in nature and gains its specific name from its tendency to occupy human habitation, (often reducing the numbers of both invertebrate and vertebrate pests) (Fadem *et al.*, 1982).

Fig. 1.8. The female grey sort-tailed opossum (*Monodelphis domestica*) with young

The word, Didelphis, used by Linneaus in his "system Naturae" is derived from the Greek "didelphys" meaning "with two uteri", i. e. the internal uterus and the pouch which was looked upon as an external uterus. This term 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 or external uterus".

Although several American marsupials such as the Virginia opossum (*Didelphis virginiana*: Jurgelski, 1974; Jurgelski *et al.*, 1974; Harder and Fleming 1982), the mouse opossums (*Marmosa elegans* and *Marmosa mitis*: Barnes, 1968a, b; Barnes and Barthold 1969; *Marmosa robinsoni*: Godfrey 1975); and grey four-eyed opossum (*Caluromys derbianus* & Philander opossum: Barnes 1968b) have been kept and bred under captive conditions, none of these appears to be well suited for captive breeding. These species show low reproductive and survival rates, aggressive behaviour, cannibalism and health problems.

In 1978 the representatives of the National Zoological Park in Washington D. C., captured four male and five female *M. domestica*, from the state of Pernambuco, in Brazil. Unlike other marsupial species, these survived and bred prolifically in the laboratory. In 1979 the National Zoological Park donated 20 animals to the Southwest Foundation for the exploration of this species as a suitable experimental laboratory marsupial.

1.3.2. Genetics

The chromosome number of *M. domestica* is 18 (2n), and the sex chromosome is easily distinguished by its small size relative to the autosomes.

1.3.3. Reproductive Biology

Sexual maturity is reached at 4-5 months in both sexes (Fadem & Rayve, 1985) and animals breed throughout the year (Fadem *et al.* 1982; VandeBerg, 1983). Females of up to 28 months of age have produced litters and males of 39 months have sired young but the majority of animals become reproductively senescent at an earlier age than this.

1.3.4. Gestation, Behaviour and Early Life

The gestation period in *Monodelphis domestica* is 14-15 days (Fadem et al., 1982, Fadem & Rayve, 1985; VandeBerg, 1983). Litters range in size from 2 -14 with an average of 7 pups in laboratory conditions and 8.5 pups per litter in wild

populations (Cothran *et al.*, 1985); the females do not have a pouch, but a roughly circular mammary area on their abdomen.

Like all marsupials, the young are born in a very immature state of development - each one measuring approximately 1.5 - 2 cm in length, and weighing 97.9 ± 12.6 mg (Fig. 1.9). They attach to a nipple within minutes or even seconds of birth and cannot release it until about 14 days of age, usually in the nest, at which time they weigh 840.8 ± 49.8 mg (for review see VandeBerg, 1990).

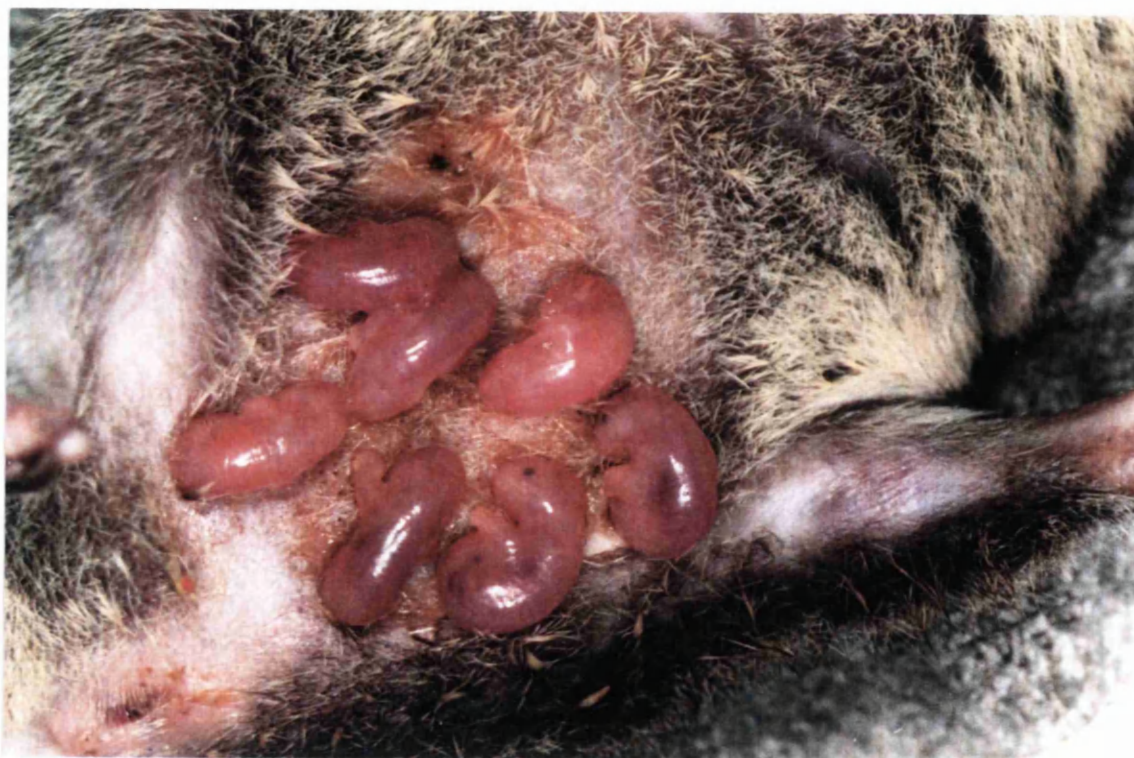


Fig. 1.9. Newborn *Monodelphis domestica* attached to the teats

Nest-building is performed by both sexes and an intact nest appears to be important for females to maintain the body temperature of young pups since disruption of a nursing female's nest can cause the loss of the litter. Homoiothermy is not developed until day 25 days after birth (Fadem *et al.*, 1988). Gould (1984) observed that whilst still attached to the nipple, pups emit ultrasonic sounds which presumably emerge through the nostrils. These ultrasonic calls are of high intensity and have a repetition rate exceeding that observed in rodents.

Between 18 - 25 days of age the pups can open their mouths and emit a more intense vocalisation, the lower frequencies of which can be heard by humans. By about 3 weeks of age the pups are fully furred and at 4-5 weeks their eyes are open and they begin to eat solid food. At this stage they are often seen out of the nest riding on the mother's back (Fig. 1.8). The pups are frequently present on the nipples for up to 3 weeks of age, whether the mother is resting or active, and they continue to suckle until they are separated from their mother or lactation ceases naturally (9-11 weeks post partum). By 7 - 8 weeks the juveniles can be weaned, separated from their mother and housed individually, at which time they weigh 20.2 ± 3.3g. If the young are not removed from their mother's cage, lactation can continue for as long as 75 days postpartum (Crisp *et al.*, 1989). Rapid growth continues until about 200-250 days when it begins to plateau (Cothran *et al.*, 1985). An adult weighs between 80 to 155g and generally males are heavier than females (Fadem *et al.*, 1982). The size of *Monodelphis* is intermediate between a mouse and a rat.

1.3.5. Opossum (*Monodelphis domestica*): a successful laboratory animals

The grey short-tailed opossum, *Monodelphis domestica* is increasingly used as a laboratory species; this is because of its small size, a short gestation period (14 days), tractability, easy maintenance and high fecundity (litter number 3-14) under normal laboratory conditions. This species is therefore becoming the first practical marsupial of choice for laboratory research (Adam *et al.* 1988; Fadem, 1985; Fadem, *et al.* 1992; Fadem & Rayve, 1985; Moore & Thurstan, 1990).

1.4. Objectives of this study

Since this species has recently become the marsupial of choice for laboratory research, it is important to establish basic information on its reproductive biology. During the eighteen years of its captivity, *Monodelphis* has been intensively studied in many biological aspects. These studies include the physiological characteristics (Kraus & Fadem, 1987), sexual behaviour and oestrus (Trupin & Fadem, 1982; Fadem, 1985; Fadem & Rayve, 1985; Baggott *et al.*, 1987); clinical chemical and hematological characteristics (VandeBerg *et al.*, 1986); biochemistry and genetics (Sinha *et al.*, 1972; Sinha & Kakati, 1976; VandeBerg *et al.*, 1987; Merry *et al.*,

1983; Hayman, 1990) and endocrinology (Etgen & Fadem, 1987; Fadem & Erskine, 1987; Schwanzel-Fukuda *et al.*, 1988) of the species. In some cases the studies have direct medical significance. For example, adult *Monodelphis domestica* resemble humans in expressing both photorepair and excision repair pathways and consequently have been established as a model mammalian organism for research on photorepair mechanisms (Ley, 1987a; 1987b; Ley *et al.*, 1987). Due to the advantageous general biological characteristics of *Monodelphis* when compared with other marsupials, it is an ideal candidate for obtaining valuable knowledge. However, despite the substantial amount of work conducted, sexual differentiation of the reproductive system has been neglected. The aims of this study of *Monodelphis domestica* are therefore:

a.) to examine differentiation of the testis from the indifferent gonad primordium;

b.) to examine the subsequent development of the testis at various stages;

c.) to characterise the development of Leydig cells morphologically (to ascertain whether there are separately identifiable neonatal and adult populations of Leydig cells);

d.) to characterise Leydig cells functionally by immunocytochemical identification of the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD);

e.) to study development of the reproductive tract and testis descent;

f.) to study steroid hormone synthesis by measurement of testosterone levels in the gonads, adrenal glands and peripheral plasma in both sexes using radioimmunoasssay;

g.) to study Müllerian Inhibiting Substance production.

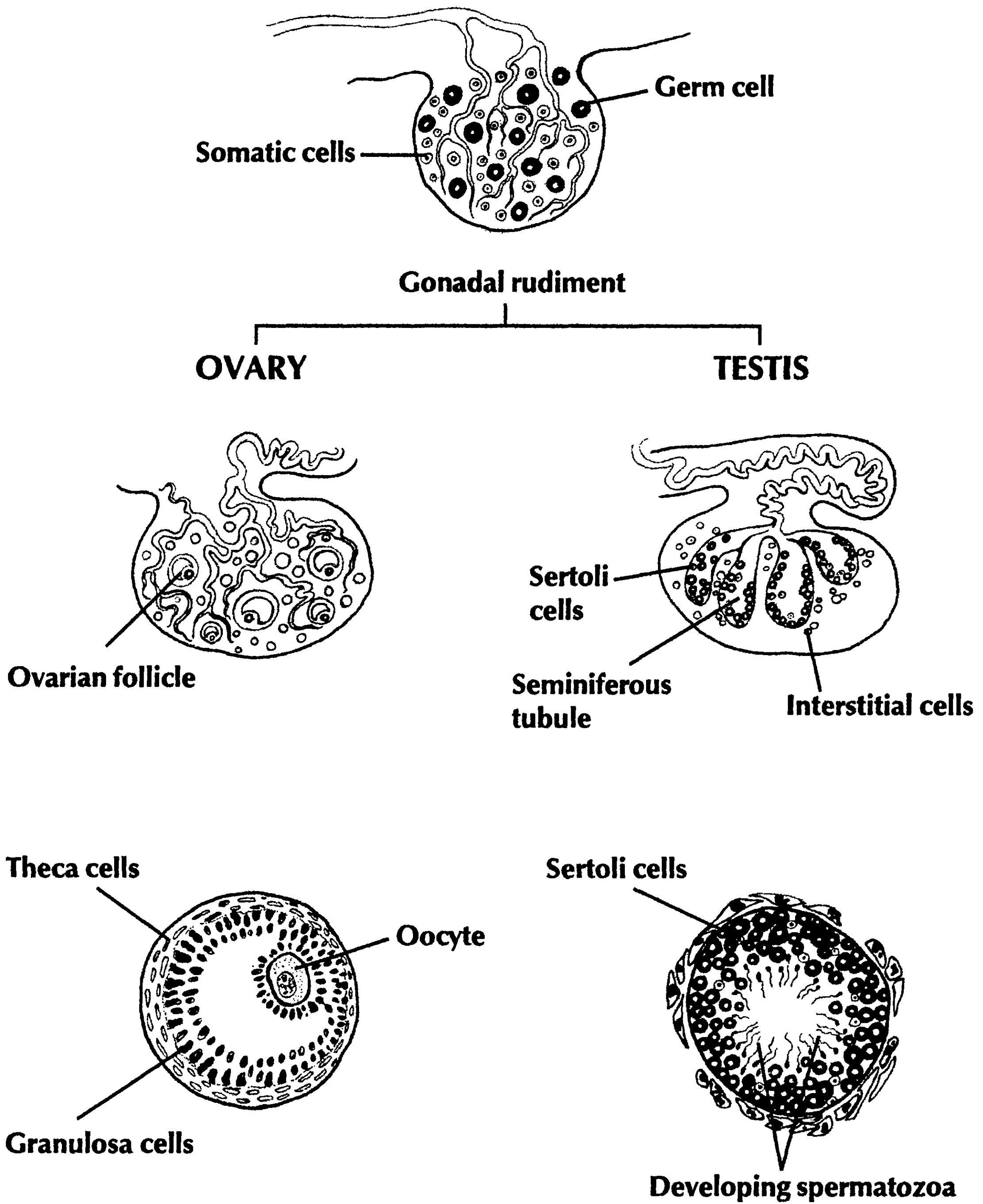
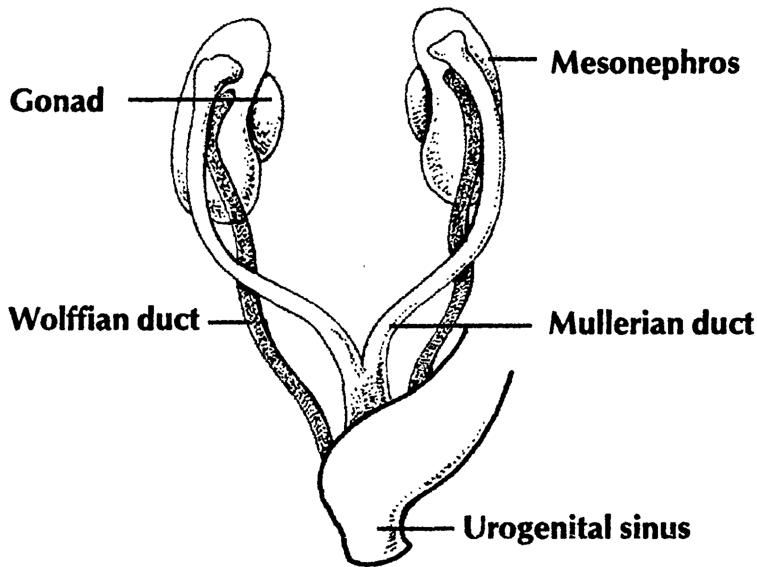


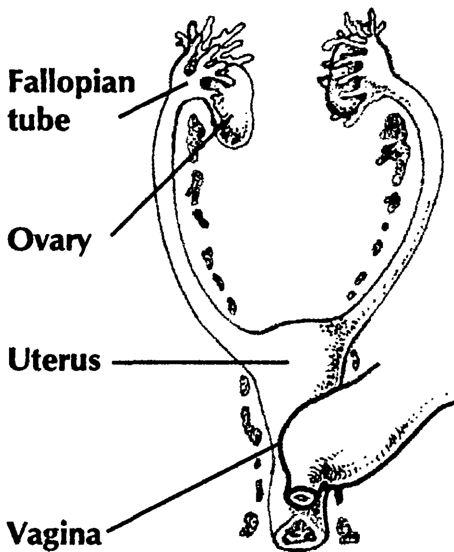
Fig. 1.2. Developing ovary and testis.
(Modified from Byskov & Hoyer, 1994)

INDIFFERENT STAGE



ADULT FEMALE

ADULT MALE



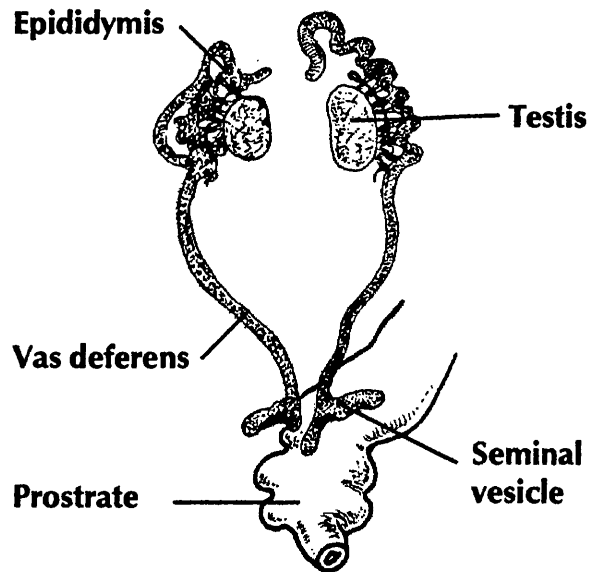
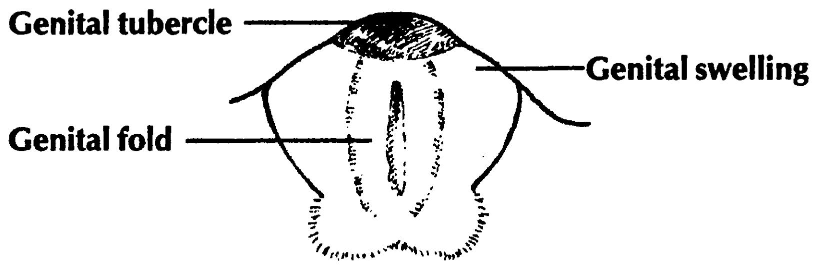


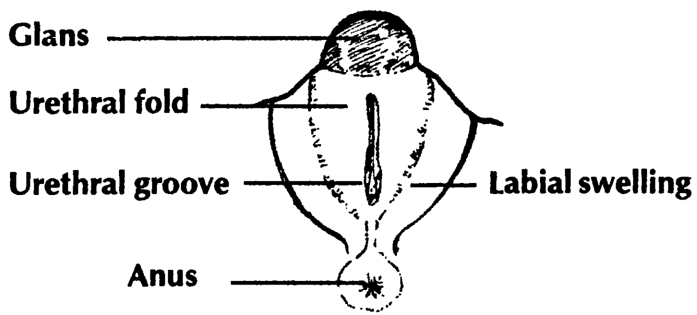
Fig. 1.3. The development of internal genitalia in eutherians.

(Modified from George & Wilson, 1994)

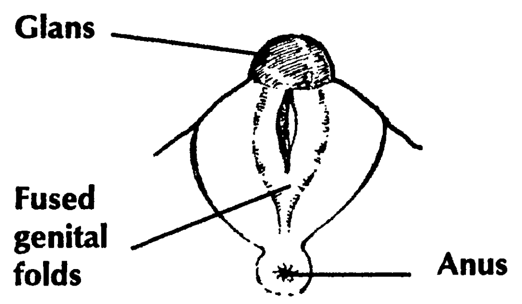
INDIFFERENT STAGE

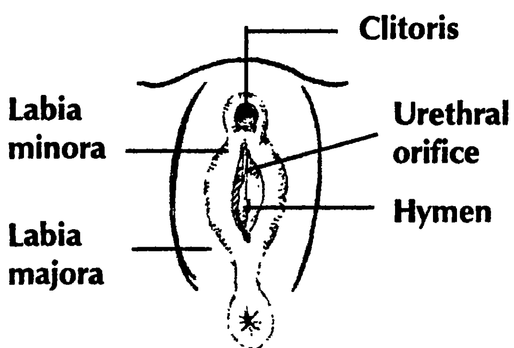


FEMALE



MALE





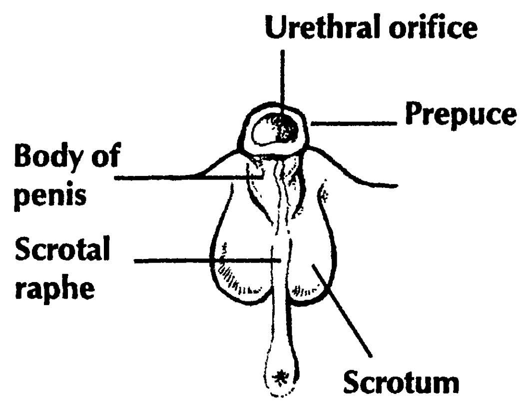


Fig 1.4. The development of the external genitalia from the indifferent stages to the formation of male and female organs.
(Modified from George & Wilson, 1994)

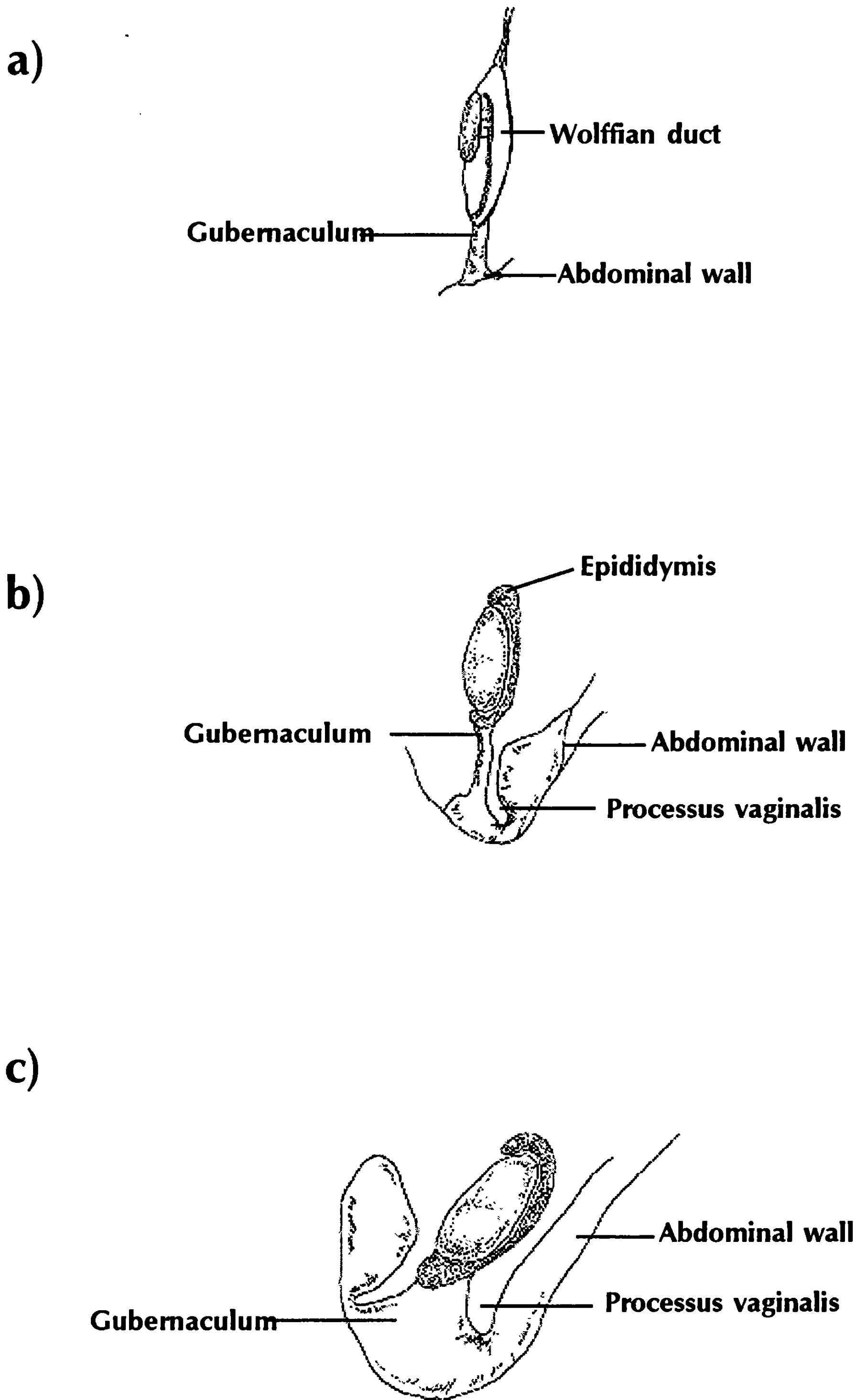


Fig. 1.5. Testicular descent. a) :Before transabdominal descent; b) : Transdominal descent; c) :Inguinal descent.
(Modified from George and Wilson, 1994)

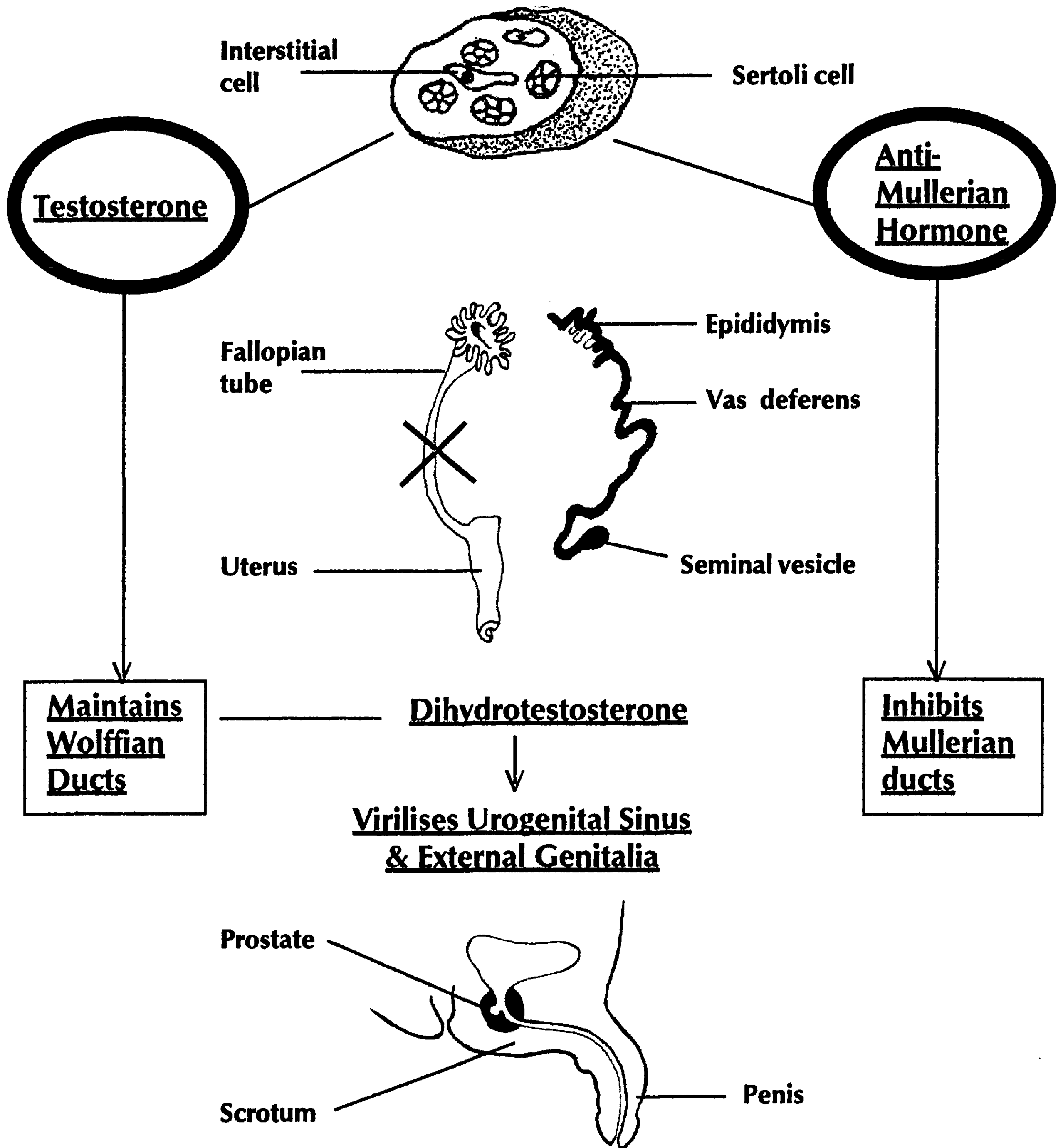
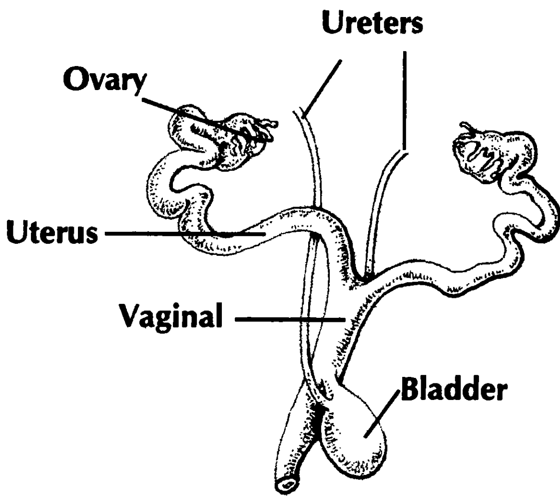
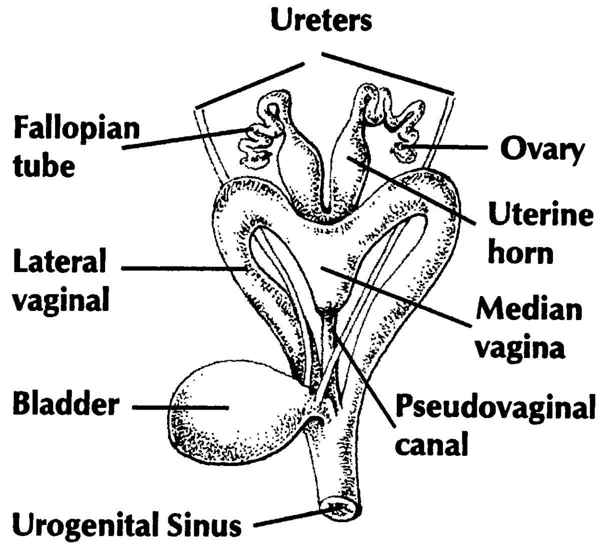


Fig. 1.6. The role of hormones in sexual differentiation.
(Modified from Hunter, 1995)

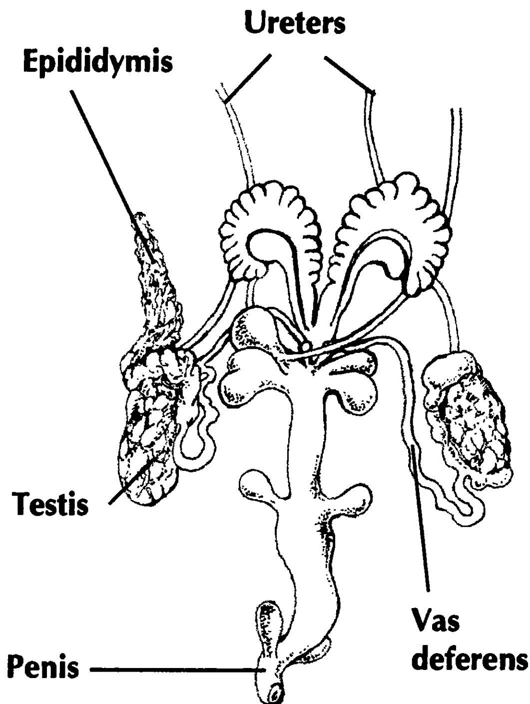
EUTHERIANS **MARSUPIALS**

Adult Female





Adult Male



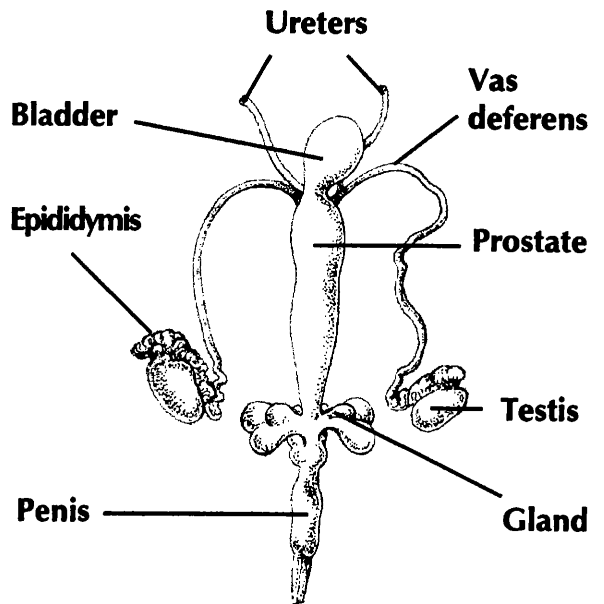


Fig. 1.7. Reproductive system of eutherians and marsupials.
(Modified from McKeever,1970; Renfree *et al.*, 1994)

Chapter 2: Testis Development in the Opossum *Monodelphis domestica*

2.1. Introduction

One of the ways in which marsupials may differ from eutherians is in the mechanism of sexual differentiation. In eutherians the female phenotype will develop by default unless the testis-determining gene on the Y chromosome switches gonadal development to form a testis (see chapter 1 p20), whose hormones then control masculinisation of the reproductive tract, external genitalia and brain (George & Wilson, 1994; Short, 1982). The sex determining gene, SRY (human) and Sry (mouse), is thought to act to control a hierarchy of secondary regulatory genes: triggering of differentiation of the somatic cell line along the Sertoli cell pathway is a key event (Hunter, 1995). In marsupials, however, some sexually dimorphic structures - the scrotal and mammary gland primordia - are said to develop prior to gonadal differentiation (O *et al.*, 1988; Renfree *et al.*, 1992; Renfree & Short, 1988; Ullmann, 1993) and, as such, appear to be independent of androgens secreted by the Leydig cells. Since sexual differentiation of the testis in *Monodelphis domestica* has variously been reported as a prenatal event (Baker *et al.* 1990, 1993; Maitland & Ullmann, 1993) or a postnatal one (Moore & Thurstan, 1990; Fadem *et al.* 1992) a systematic study is timely.

In widely-documented species such as the laboratory mouse, testis differentiation occurs in a step-wise manner. At about 12.5 days *post coitum* (dpc) the testis is first distinguishable morphologically both by its greater vascularity and by the appearance of testicular cords (Sertoli and germ cells) at its cranial end. The *tunica albuginea* develops by 15 dpc; two days later a basal lamina and peritubular cells surround each cord and Leydig cells are identifiable morphologically. The ovary remains indifferent until 14 dpc, when clusters of germ cells begin to form indistinct ovigerous cords (Mackay & Smith, 1989). A similar sequence of events is seen in the rat, although it begins about a day later reflecting the longer gestation in this species (Jost & Magre, 1988).

Fetal and postnatal populations of Leydig cells have been distinguished on ultrastructural criteria in the rat (Zirkin & Ewing, 1987). The transient fetal population is responsible for the prenatal masculinisation of the reproductive and nervous systems, while the adult population is activated at puberty. The fetal

population arises from precursors which probably originate from mesonephric mesenchyme (De Kretser & Kerr, 1988). They were thought to disappear during postnatal life as a result of either cell death or cells in the adult testis where their physiological role remains enigmatic. The fetal Leydig cells dedifferentiate (Gondos *et al.*, 1974), but Kerr and Knell (1988) have reported their persistence as a small, distinct population of cells which initially regress during the perinatal period. Subsequently phase of prepubertal growth is reinitiated before a further phase of regression occurs during the second week *post partum.* Coincident with terminal regression of the fetal Leydig cells is the development of adult-type Leydig cells which are believed to differentiate *de novo* from primitive interstitial fibroblasts (or mesenchymal cells) rather than to transdifferentiate from fetal cells. In rats, therefore, there appear to be three consecutive waves of Leydig cell development: fetal, early juvenile and juvenile-adult; a similar developmental sequence pattern has been reported for the pig (De Kretser & Kerr, 1988).

The main objectives of this chapter are 1) to examine early differentiation of the testes; 2) to characterise testis development morphologically; 3) to ascertain whether there are separately identifiable neonatal and adult populations of Leydig cells.

2.1. Materials and Methods

2.1.1. Animals

The animals used in the present study were bred at Glasgow University were derived from 19 animals (10 males, 9 females) acquired from Manchester University in 1989, 23 females from Southampton University in 1992 and an additional 15 animals in 1993 (4 males, 11 females) and 10 females in 1996 from Manchester University.

The opossums were housed in plastic rat cages, measuring 56 x 38 x 18 cm (RC1/F, North Kent Plastics), with wood shaving for floor coverage and shredded tissue paper supplied for bedding. The breeding system was applied whereby the female was placed directly in to the male's cage and the pair left together for 14 days. Following separation from a mate, the female was returned to her own cage, given a nest box (20 x 16 x 8 cm) and plenty of nesting material.

The animal house was maintained at 24°C with a cycle of 14 hours light, 10 hours dark throughout the year; and a humidity was approximately 35%. Animals were checked daily for litters. Animals were fed with a reconstituted cat food (SDS Powdered Carnivore Meat). Twice weekly the veterinary vitamin supplement SA-37 (Intervet Labs. Ltd.) and fresh fruit, such as bananas, were given. Fresh water was available from sipper tube bottles.

A total of 190 opossums of the following ages were used: 0,1, 2, 3,4 5, 8, 16, 24 days after birth (the day of birth being designated as day 0), 7 - 9 weeks. Animals aged twelve weeks (pre-pubertal) 4 months (pubertal) and 1-3 years (adult) were also collected. Details of animals used at each age point are shown in Table 2.1. Young animals (from days 0 - 24) were killed by inhalation of CO_2 or Halothane gas and testes were immersion fixed. Older animals (over 4 weeks of age) were anaesthetized by an intraperitoneal injection of 4% sodium pentabarbitone and fixed by perfusion with phosphate buffered-fixative (see below).

2.2.2. Karyotyping

Karyotyping was carried out according to the method of Evans (1987) (Appendix 1). The liver was removed, minced and cultured in Williams E medium (GIBCO) containing colchicine (0.4 mg/ml) for 1 hour at 37°C and then transferred to hypotonic 0.56% KCl for 10 minutes. The tissue was then fixed in methanol: glacial acetic acid (3:1 V/V) and spread onto slides. Chromosomes were stained with Giemsa's solution in 0.1M phosphate buffer at pH 6.8 for 15 minutes (Appendix 1).

2.2.3. Tissue Preparation

Testes were perfusion- or immersion-fixed with a primary fixative (modification of Karnovsky, 1965), composed of 3% glutaraldehyde + 1% formaldehyde in 0.1 M phosphate buffer (pH 7.2-7.4) for 24 hours at room temperature (20°C) then, after a buffer wash, tissue was post-fixed with 1% OsO_4 in 0.1 M phosphate buffer for 30-60 minutes. Subsequently, specimens were dehydrated through an ascending ethanol series before being embedded in Araldite resin (Appendix 2).

Table 2.1. Details of animals used

Age	Number of Litters	Number of Animals
Day 0	4	27
Day 1	2	12
Day 2	4	23
Day 3	2	14
Day 4	3	15
Day 5	2	13
Day 8	1	8
Day 16	2	15
Day 24	1	11
4 weeks		4
7 weeks		4
8 weeks		3
9 weeks		3
12 weeks		4
4 months		9
1-3 years		25

*From days 0 - 24 at least 3 animals of each sex were used. Beyond that only males were investigated.

2.2.4. Light Microscopy

For light microscopy, semi-thin (1-2 μm) sections were cut on a Reichert-Jung Ultracut with glass knives, then stained with Mayer's haemalum and eosin (Appendix 3). All photographs ware taken on a Leitz Vario - Orthomat photomicroscope.

2.2.5. Transmission Electron Microscopy

For transmission electron microscopy, ultra-thin sections were cut on a Reichert-Jung Ultracut E with a diamond knife (Diatome-MP3455) and double post-stained with both saturated uranyl acetate solution and Reynolds' lead citrate (Reynolds, 1963) (Appendix 3). Specimens were examined in a Philips - CM 100 transmission electron microscope.

2.2.6. Scanning Electron Microscopy

After primary fixation, testes for scanning electron microscopy were cut on a vibratome (200-300 µm) and postfixed in 1% OsO_4 in 0.1 M phosphate butter for 30-60 minutes. Dehydration was carried out using a graded series of acetone solutions; material was then critical point dried (Polaron E300 critical point drying apparatus) and finally coated with gold in a sputter coater (Polaron E5000) (Appendix 4). All specimens were examined under a Jeol JSM-T300 scanning electron microscope.

2.3. Results

2.3.1. Light Microscopical Observations

New born and 1 day old specimens were karyotyped and semi-thin sections of gonads examined. The diploid chromosome number for *Monodelphis domestica* is 18; the karyotypes of male and female opossums are shown in Figure 2.1a & 2.1b. The sex chromosome pair is the smallest, the Y being particularly small.

At birth (day 0) the gonads in both sexes are elongate in shape, extending along the medial aspect of the mesonephroi. At this stage some male gonads remain undifferentiated (Fig. 2.2a), resembling those of females of the same age (Fig. 2.2b). In others testicular cord development appears to be beginning (Fig. 2.2c). The prospective testis is surrounded by a *tunica albuginea* of flattened fibroblasts 4-5 cells in thickness. In developing testicular cords several cell types are already distinguishable: large pale staining cells with sub-spherical nuclei and fibroblast-like cells. At this stage the larger pale cells are either found scattered in small groups or aggregated into cord-like structures (Fig. 2.2c). The latter presumably include the pre-Sertoli cells, but at this stage it is difficult to distinguish between these and the primordial germ cells at LM level. In immersion-fixed material, small capillaries with nucleated erythrocytes are seen occasionally (Fig. 2.2c).

By day 1 the testis primordium is clearly distinguishable in all male specimens. As can be seen from Figure 2.3. a *tunica albuginea* 4-8 cells deep now completely surrounds the organ. It is now possible to distinguish between the 2 cell types: primordial germ cells are large pale cells and the pre-Sertoli cells have darkly stained nuclei. Both cell types are now all gathered into testicular cords located in a broad zone below the *tunica albuginea*. Peritubular cells are beginning to surround

the cords. The central region of the gonad is occupied by a stroma consisting of undifferentiated fibroblast-like cells.

By days 2 - 5 *post partum*, the testis is becoming rounded, in contrast to the ovary which remains elongated. In 4 animals measured on day 5, mean testis length is 0.4 ± 0.03 mm (mean ± SEM). The *tunica albuginea* becomes somewhat reduced in thickness as the cords grow. The 1-2 testicular cords seen per section are located peripherally and are composed of primordial germ cells and pre-Sertoli cells surrounded by 3-4 layers of peritubular cells (Fig. 2.4). Mitotic figures occur in both germ cells and pre-Sertoli cells. Spindle-shaped Leydig cells are observed between the cords in the enlarging interstitial spaces and are identifiable by their intense basophilia.

By day 16 in 5 animals measured, mean testis length has increased to 0.99 ± 0.02 mm and each section now reveals several cord profiles (Fig. 2.5). Leydig cells increase in both size and number and occupy a major proportion of the interstitial tissue; their irregular granular nuclei are very obvious (Fig. 2.5). Mitotic figures are present in all cell types within the testis. The testis continues to grow, mean length measuring 1.5 ± 0.02 mm by day 24 (3 animals) and by the end of week four, the testicular cords dominate the organ (Fig. 2.6).

Between 7-12 weeks after birth the testis length increases from 3.7 ± 0.2 to 6.5 ± 0.8 mm (5 animals used per stage). Flattened peritubular cells are reduced to a single layer and the interstitial tissue is becoming less cellular (Fig. 2.7). Mitosis is still occurring in both germ cells (Fig. 2.7) and Sertoli cells. Blood vessels and lymphatics in the interstitial tissue are readily identifiable by the lymphoprotein content of the latter. In addition, the interstitial tissue contains macrophages which can be distinguished from Leydig cells by their inclusions and reduced basophilia. At 12 weeks the testicular cords are not yet patent and mature sperm are still absent (Fig. 2.7).

By 4 months, testis length is 7.4 ± 0.39 mm (9 animals) and rises to 8.5 ± 0.78 mm (18 animals) in adults (1-3 years). Each testicular cord possesses a lumen and spermatogenic stages up to and including mature sperm are visible, so it is appropriate now to refer to them as seminiferous tubules (Fig. 2.8 & 2.9). The peritubular cell layer has become further flattened (Fig. 2.8). Mature Sertoli cells

are columnar in shape and their cytoplasmic processes are associated with late spermatids (Fig. 2.8). Leydig cells now greatly outnumber other interstitial tissue cell types and in the adult can be seen closely packed around blood vessels (Fig. 2.9).

2.3.2. Transmission Electron Microscopy

Pre-Sertoli and Sertoli Cells

Pre-Sertoli cells can first be distinguished from primordial germ cells in the postnatal period (days 0 - 5) by the high electron density of their nuclei (Fig. 2.10). At both 12 weeks and 4 months, Sertoli cells proper are readily seen and have apical and lateral cytoplasm processes which make contact with germ cell surfaces (Fig. 2.11a & 2.12a). Sertoli cells are tall, irregularly columnar in shape and rest on the basal lamina of the seminiferous epithelium. Their nuclei are large and irregular in shape with numerous clumps of heterochromatin. One or two nucleoli with granular and other components of varying electron density are found in each nucleus. The Sertoli cell cytoplasm contains many tubular mitochondria and numerous electron dense lipid inclusions. Both rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER) are present, the latter being more abundant. Microtubules and microfilaments are occasionally found in the cytoplasm. By 4 months well-defined tight junctions form between the processes of adjacent Sertoli cells with many parallel lines of fusion of the apposed membranes (Fig. 2.12b) and the gap junctions are found between Sertoli cells and germ cells (Fig. 2.12a).

Germ Cells

Primordial germ cells are pale, relatively large and rounded in shape with predominantly rounded nuclei. At juvenile stages (up to 8 weeks), spermatogonia are common within the testicular cords and neighbouring cells are connected by intercellular bridges. Spermatogonial cytoplasm shows numerous mitochondria with tubular cristae, profiles of both SER and RER are present, as are abundant free ribosomes. By 12 weeks spermatocytes with synaptonemal complexes appear (Fig. 2.11a & 2.11b). From 4 months, all stages of germ cell development may be seen in the testis (Fig. 2.12a). In general, spermatogonia lie against the basement membrane and primary spermatocytes are found towards the periphery of the tubule wall while

other cell types such as spermatids and mature spermatozoan are located closer to the lumen. Primary spermatocytes are characterised by a large spherical nucleus; when present, synaptonemal complexes are evidence that the pachytene stage of meiosis has been reached (Fig. 2.12b). Mitochondria are ovoid and occasionally aggregated into groups of two or three. Well-defined Golgi bodies appear as large complexes of several lamellae and associated vesicles. Immature spermatids are rounded and smaller in size than spermatocytes. The spermatid has a centrally placed spherical nucleus with homogeneous chromatin, a well developed Golgi complex, and an adjacent centriole; the first signs of acrosome development are seen (Fig. 2.12a). Late spermatids are elongated in shape and their nuclei are condensed and the acrosome is more obvious. During spermiogensesis the nuclear shape changes and the mature spermatozoon nucleus becomes "U"-shaped. A fully formed acrosome is present at the head-end of the mature spermatozoon and axial filaments are found in the tail.

Leydig Cells

Leydig cells are not detectable at birth, but are first seen on day 3 (Fig. 2.13) when they can be readily distinguished from fibroblasts by their highly electron dense nuclei and cytoplasm. Over the course of the next few days they develop the expected ultrastructural features of steroidogenically active cells, namely a variable amount of elongate profiles and vesicles of SER; mitochondria with tubular cristae; abundant lipid droplets of high electron density clustered in the cytoplasm (Figs. 2.13 & 2.14). By this stage SER is extensive and occasionally RER and Golgi bodies are visible; nuclei are irregular in shape and the nucleus:cytoplasm ratio is approximately 1.5:1. Subsequently Leydig cells are surrounded by envelope cells.

From 7 - 12 weeks Leydig cells still form clusters (Fig. 2.15a). SER has increased in amount and forms parallel profiles (Fig. 2.15b). At this stage lipid inclusions are still present but reduced in number compared to previous stages. Leydig cell nuclei are irregular in shape and the nucleus:cytoplasm ratio is 1:1 as a result of an increase in the amount of cytoplasm.

At 4 months Leydig cells remain closely packed but are no longer surrounded by envelope cells. Masses of SER now form highly organised parallel arrays which were not seen at earlier stages (Fig. 2.16) and lipid inclusions are

further reduced. Nuclei are round or irregularly ovoid with finely dispersed chromatin; and the nucleus:cytoplasm ratio is 1:3 as the amount of cytoplasm is now further increased.

In the adult, Leydig cells reach their greatest size and their morphological features resemble those seen at 4 months, except that lipid inclusions are now sparse (Fig. 2.17). With ageing (2-3 years), masses of SER are present but disordered (Fig. 2.18). Small amounts of RER and some secondary lysosomes are visible. Nuclei are irregular in shape and position and some heterochromatic nuclei can be seen at this stage.

2.3.3. Scanning Electron Microscopy

By 3.5 months fractured testes show seminiferous tubules, with various stages of germ cells, surrounded by peritubular myoid cells (Fig. 2.19a & b). At this stage (3-3.5 months) although the lumen is well developed, mature sperm are still absent. The interstitial tissue contains Leydig cells and abundant blood vessels; the Leydig cells are irregular in shape (Fig. 2.20). These cells, covered by numerous short microvilli and blebs are seen at 4 months (Fig. 2.21).

During the period between puberty (4 months) and adulthood, elongate early spermatids and mature spermatids with sperm tails are present in the seminiferous tubules (Fig. 2.22). Head-head pairing sperm are found in the epididymis (Fig. 2.23). Leydig cells appear to be round or oval in shape and retain numerous small microvilli.

2.4. Discussion

While gonadal differentiation occurs in eutherians before birth (George & Wilson, 1994; Hunter, 1995), the limited observations on marsupials indicate that this process is a perinatal event. In bandicoots (Ullmann, 1981) the testis can be initially identified on the first day of pouch life, while in the marsupial native cat (Ullmann, 1984) and brown marsupial mouse (Taggart *et al.*, 1993a) it is not distinguishable until day 3 *post partum.* In the brush tail possum gonadal differentiation occurs perinatally (Ullmann, 1993). Tammar wallaby gonads were initially described as indifferent at birth (O *et al.*, 1988) but, more recently, subtle

ultrastructural differences between the sexes have allowed prenatal identification of the testis (Renfree *et al.*, 1992).

Among american marsupials, McCrady (1938) reported testicular cord formation in the Virginia opossum as a prenatal event; but early observations on testis development in *Monodelphis* suggested that the gonads were undifferentiated at birth. Thus Fadem *et al.* (1992) first recorded testis development on day 4 *post partum*, while Moore & Thurstan (1990) observed this on day 3. However Baker *et al.* (1990; 1993) and Maitland & Ullmann (1993) described recognisable testes at birth, indicating that testis differentiation is a prenatal event in this species. In the present study about half the karyotyped males were found to have histologically differentiated testes at birth. It thus appears that in *Monodelphis,* (as in the brush tail possum) testis differentiation is a truly perinatal event.

Fadem & Rayve (1985) and Adam *et al.* (1988) have already reported that puberty occurs at 4 months, but it is unclear whether these authors used morphological, endocrinological or behavioural criteria to calculate the onset of puberty. This study provides evidence firstly that, at 3 months, the seminiferous tubules have not yet developed a lumen and sperm are absent - from which it can be deduce that this stage is pre-pubertal. Secondly, by 4 months the seminiferous tubules are patent, various spermatogenic stages, including sperm, can be seen and Leydig cells greatly outnumber other interstitial cell types. Thus the morphological features associated with the onset of puberty in *Monodelphis* appear at 4 months.

The Sertoli cell is regarded as a principal regulator of materials delivered to the germ cells and also secretes fluid into the tubular lumen, the direction of fluid secretion being determined by the presence of the Sertoli cell barrier (George & Wilson, 1994). The latter is widely presumed to have a marked influence on spermatogenesis. Several studies have demonstrated its morphological and functional development in which inter-Sertoli junctional specialisations realign in order to provide an effective barrier (De Kretser & Kerr, 1988). Russell *et al.* (1989) described its postnatal development in the rat as on days 15 - 16 and its completion in all tubules prior to day 18. Cavicchia & Sacerdote (1991) indicated that the appearance of the blood-testis barrier takes place earlier, (days 13 - 20) with the appearance of zygotene-pachytene spermatocytes. In *Monodelphis*, the development of the blood-testis barrier probably occurs before puberty with the

appearance of inter-Sertoli cell tight junctions at 12 weeks. In addition, synaptonemal complexes are found in some primary spermatocytes at this stage indicating that the pachytene stage of meiosis has been reached. By 4 months (puberty), the number of inter-Sertoli cell tight junctions (perpendicular to the basal lamina and parallel to each other) are abundant, suggesting the morphological substrate of the blood testis barrier is now fully developed.

Rod-like crystalloid inclusions occur in Sertoli cells of the koala (Harding *et al.*, 1982) and the Virginia opossum (Duesberg, 1919), often located close to the nucleus and the basal lamina. However, such inclusions are absent in *Monodelphis*.

The spermatogenic cells of *Monodelphis* present few unusual characteristics. The present study indicates that the nucleus is oval in spermatocytes, spherical in immature spermatids and U-shaped in mature sperm. This confirms a study based on immunostaining of suspensions of dissociated cells in *Monodelphis* by Olson & Winfrey (1991); similar findings were reported by Rattner (1972) in *Didelphis virginiana* and the mouse opossum *Marmosa mitus*. Pairing of spermatozoa in the epididymis probably occurs in all American marsupials (Rodger, 1982; Taggart *et al.*, 1993b) and the observations of this phenomenon have presented in *Monodelphis* confirm those of Taggart *et al.* (1993b).

The ultrastructural study confirms the earlier tentative identification of Leydig cells in *Monodelphis* on day 3 (Moore & Thurstan, 1990), by which time they possess the machinery for steroidogenesis. In the rat, in which Leydig cells have been most intensively studied, fetal Leydig cells are characterised by numerous lipid inclusions and clusters surrounded by envelope cells (Kerr & Knell, 1988; Kuopio *et al.*, 1989). In the adult testis Leydig cells generally lack lipid inclusions and are not surrounded by envelope cells (Kerr & Knell, 1988). In *Monodelphis* though the clusters of Leydig cells found in immature animals are more loosely organised than in the rat, nevertheless the delicate processes of envelope cells can be demonstrated around them at the ultrastructural level. Moreover, the lipid inclusions which occur in these cells at early stages (day 3 onwards) are rapidly reduced at the onset of puberty (4 months).

The adult structure of Leydig cells is attained by 4 months and is characterised by the extraordinary abundance of SER which is highly organised into masses of parallel arrays and occupies the major part of the cytoplasm giving it great

density. Christensen & Fawcett (1961) also demonstrated an unusually abundant SER in Virginia opossum Leydig cells, but this species lacks parallel arrays. Leydig cells may possess species-specific characteristics such as the crystals of Reinke in the human (De Kretser & Kerr, 1988), glycogen granules in the fetal rat (Kerr & Knell, 1988)) but such inclusions, are not found in *Monodelphis*.

Adult type Leydig cells in the rat are believed to differentiate from mesenchymal cells, after the disappearance of the fetal type cells (Kerr & Knell, 1988; Kuopio *et al.*, 1989). According to these authors, some fetal Leydig cells persist in the adult rat testis. Christensen and Fawcett (1961) also reported the persistence of relatively undifferentiated mesenchymal cells in the testis of the adult Virginia opossum that are believed to be capable of developing into adult-typical Leydig cells. In *Monodelphis* neither identify mesenchymal cells nor fetal-type Leydig cells could be identified in the mature testis, and there was no a period, subsequent to their appearance on day 3, when Leydig cells were absent. It is, therefore hypothesize that, in *Monodelphis,* the fetal-type Leydig cells give rise directly to the adult cells. Associated with this transformation is a 2-3 fold increase in the amount of cytoplasm, the remarkable development of the SER and the loss of envelope cells. Scanning electron microscopy reveals curious blebs on the surface of the Leydig cells: their function is unknown.

To summarise, this study suggests firstly that testicular differentiation in *Monodelphis* is a truly perinatal phenomenon since only about 50% of the animals show gonadal sex differentiation at birth. Secondly, although a morphological difference is evident in the Leydig cells of immature and mature animals (which equate to the fetal- and adult-type cells described in eutherians), two separate and distinct populations could not be recognised; neither could a period in postnatal development be identified in which Leydig cells were absent. Since neither degenerating Leydig cells nor mesenchymal cells were evident in the testis, it would suggest that fetal-type Leydig cells transform directly into adult-type cells by loss of lipid inclusions, loss of surrounding envelope cells and the development of highly organised arrays of SER. Thirdly, the appearance of patent seminiferous tubules with mature sperm has been demonstrated by four months, the period when peripheral testosterone begins to rise to adult levels (see chapter 4). On the evidence

of this data it is concluded that, in *Monodelphis*, the pubertal period is reached 4 months after birth.

Fig. 2.1. Chromosome spread from new born opossums (day 0):

a.) Chromosomes of a female opossum. The X chromosome pair (arrows) is the smallest.
Magnification: x 2000

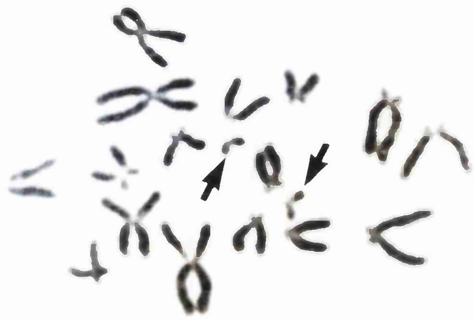
b.) Chromosomes of a male opossum. The X chromosome (arrow) is small and the Y chromosome (arrowhead) is minute.
Magnification: x 2600

Fig. 2.2. Longitudinal sections of XY and XX gonads of neonatal opossums (day 0):

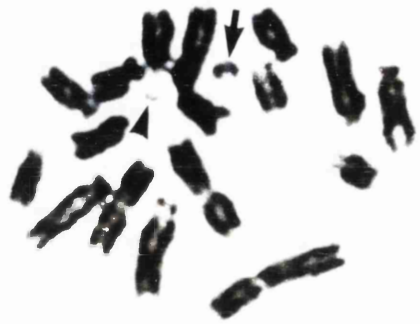
a.) An XY gonad lacking testicular cords.
Magnification: x 200

b.) The XX gonad shows no obvious cord formation.
Magnification: x 220

c.) The XY gonad is surrounded by a *tunica albuginea* (Ta) of 3-4 cells thickness. Note initial formation of testicular cords (Tc) with large pale cells (arrows). A small capillary with nucleated cells is seen (arrowhead).
Magnification: x 200



1a



1b

2a

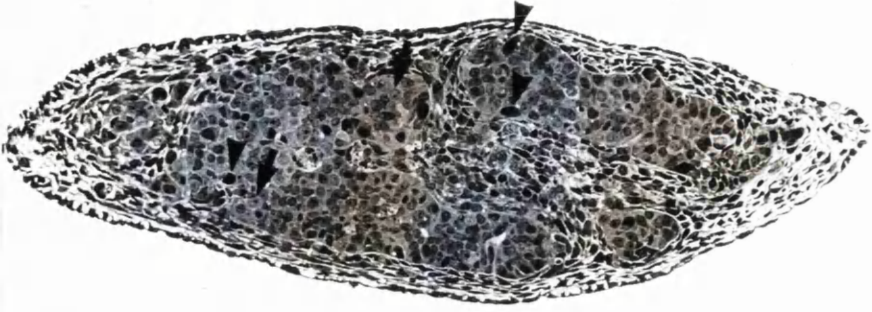
2b

2c

Fig. 2.3. Longitudinal section through the male gonad (day 1), showing the 4-8 cells deep *tunica albuginea* (Ta). The sex cords are well-defined and could easily be distinguished from interstitial tissue. Note primordial germ cells (arrows) and pre-Sertoli cells (arrowheads).
Magnification: x 200

Fig. 2.4. Transverse section through the male gonad on day 3. Note Leydig cells (arrows) and peritubular cells (arrowheads).
Magnification: x 200

Fig. 2.5. Transverse section through the male gonad (day 16), showing both germ cells and Sertoli cells. Note mitotic figures (arrows), 3-4 layers of peritubular cells (arrowheads) and well-defined Leydig cells (Lc).
Magnification: x 200



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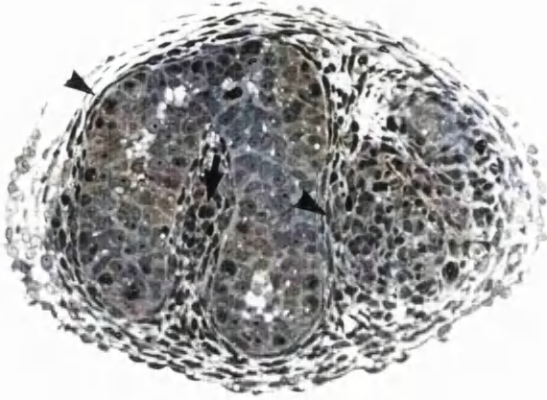
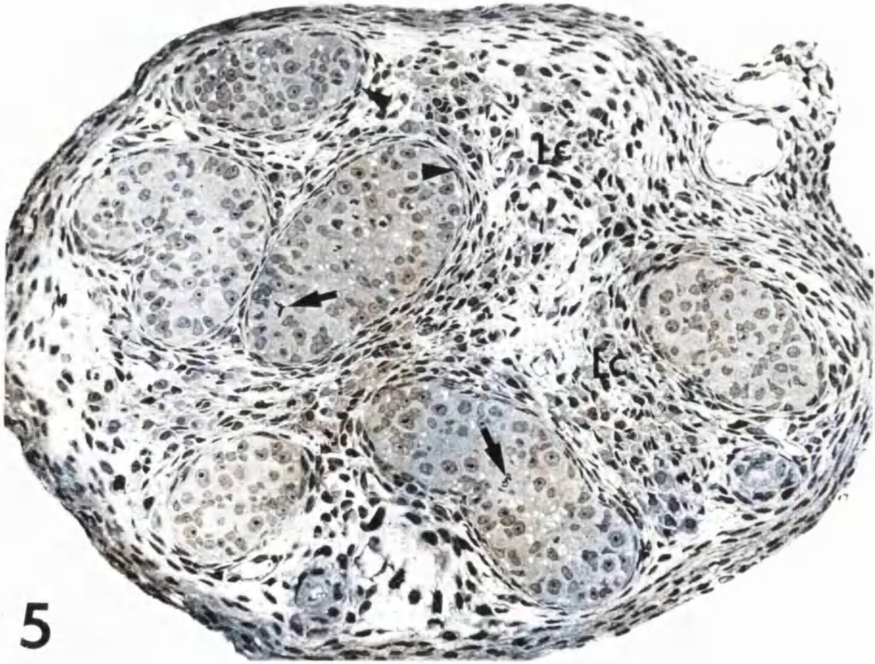


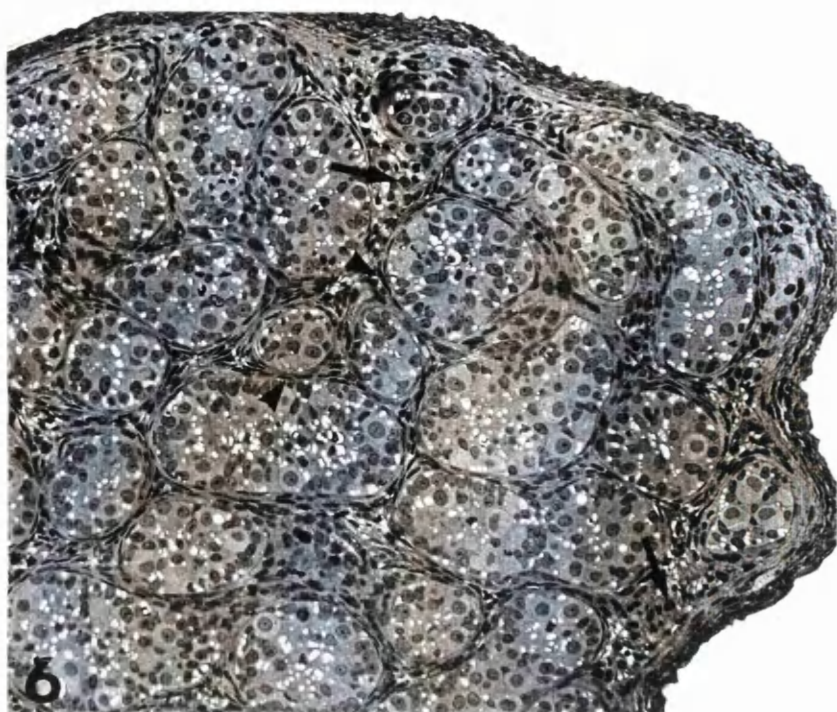
4



5

Fig. 2.6. Transverse section through the male gonad (day 24). The testicular cords predominate the gonad. Note Leydig cells (arrows) and 2-3 layers of peritubular cells (arrowheads).
Magnification: x 200

Fig. 2.7. Transverse section through the pre-pubertal testis (12 weeks), showing the interstitial tissue becoming less cellular. Note a single layer of peritubular cells (Pc), germ cells in mitosis (arrows) and abundant Sertoli cells (arrowheads) with extending cytoplasm. There are extensive capillaries and lymphatics in the interstitium.
Magnification: x 200.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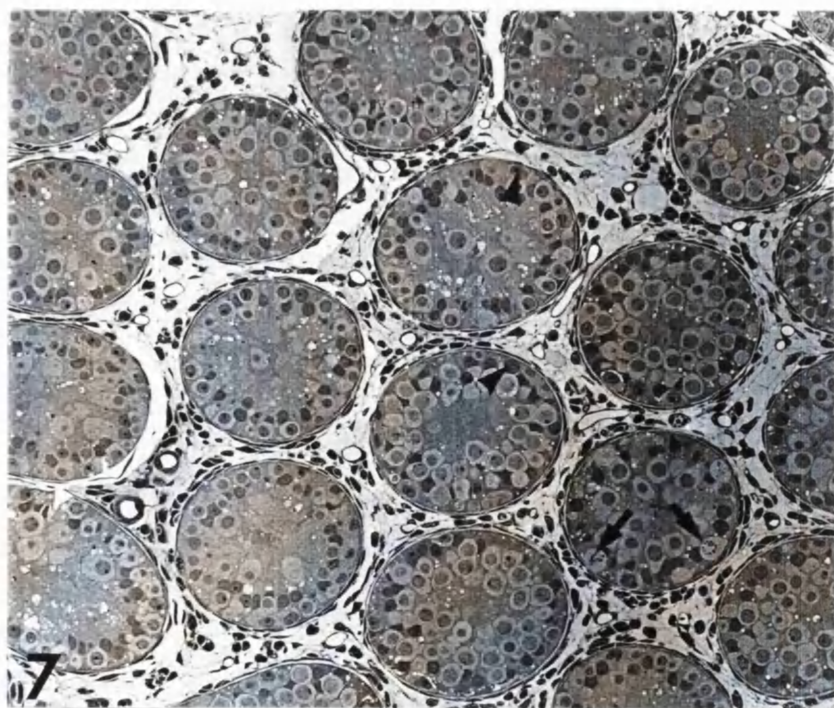

7

Fig. 2.8. Transverse section through the pubertal testis (4 months), showing various stages of germ cell development in the seminiferous tubules and closely-packed Leydig cells (Lc) around blood vessels in the interstitial tissue. Note spermatids (Sp), sperm tails (St). This is the first stage at which the seminiferous tubules are patent.
Magnification: x 200

Fig. 2.9. Transverse section through the adult testis (1 year). Note abundant Leydig cells (Lc) and numerous sperm (S). * The apparent absence of a lumen in this tubule is an effect of the plane of section.
Magnification: x 200

Sp
Sp
St
8

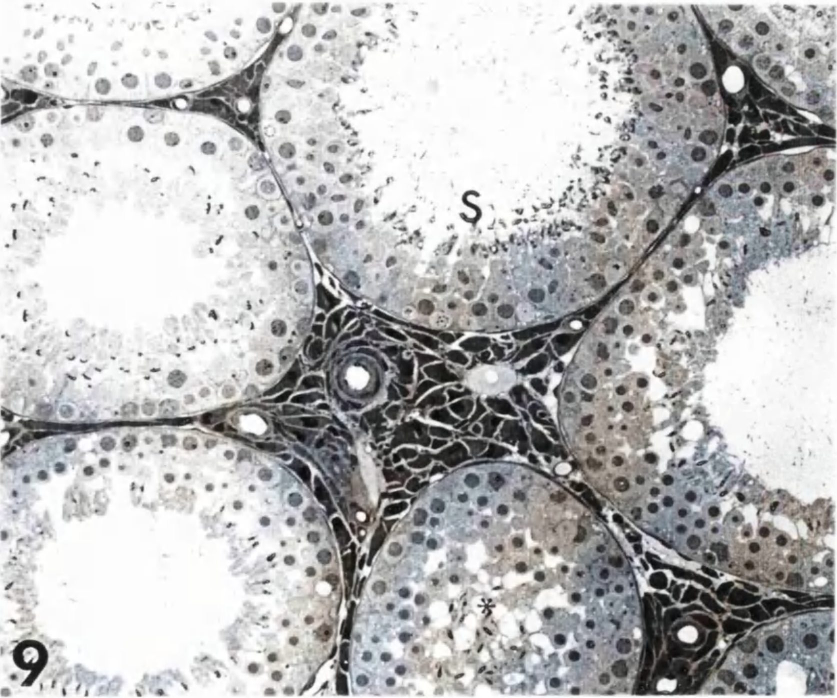

S
*
9

Fig. 2.10. Electron micrograph of a neonatal male gonad (day 0), showing pre-Sertoli cells (Psc), primordial germ cells (Pgc) and a basal lamina (arrows).

Bar = 10μm.

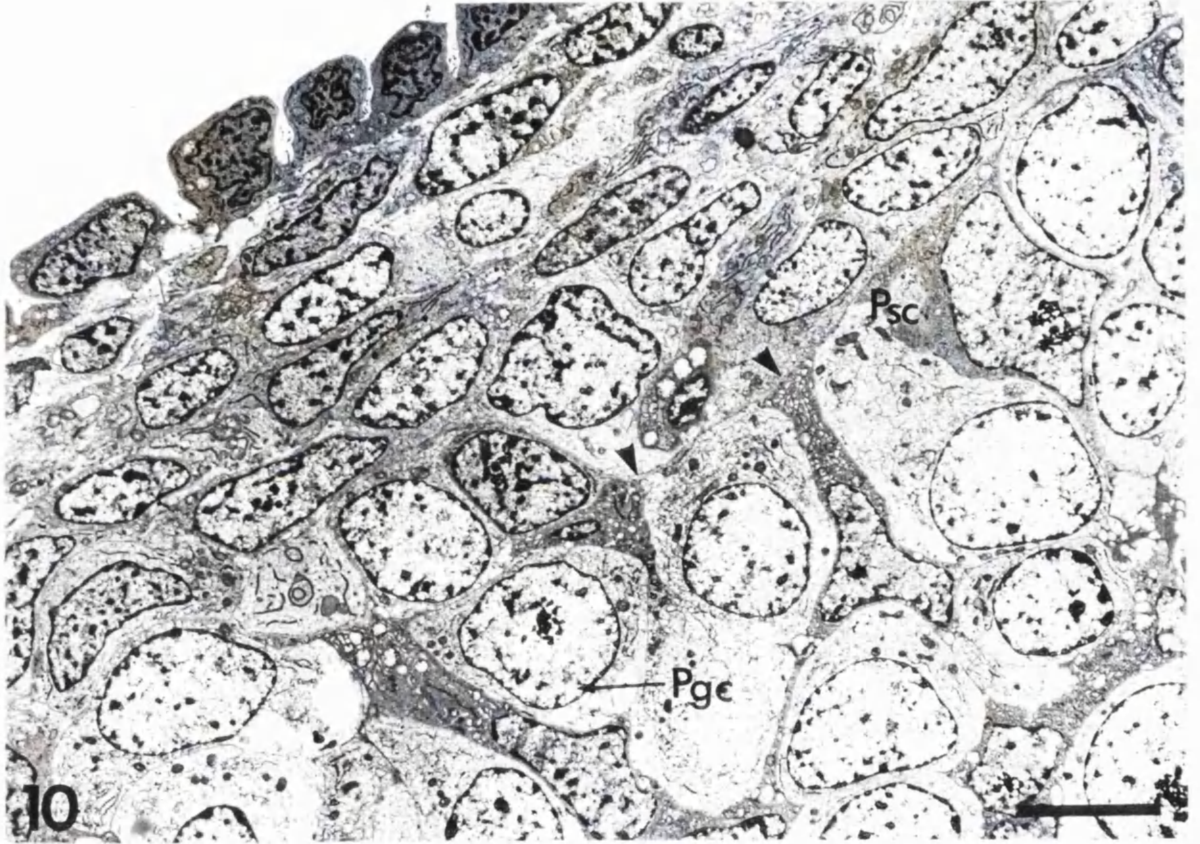
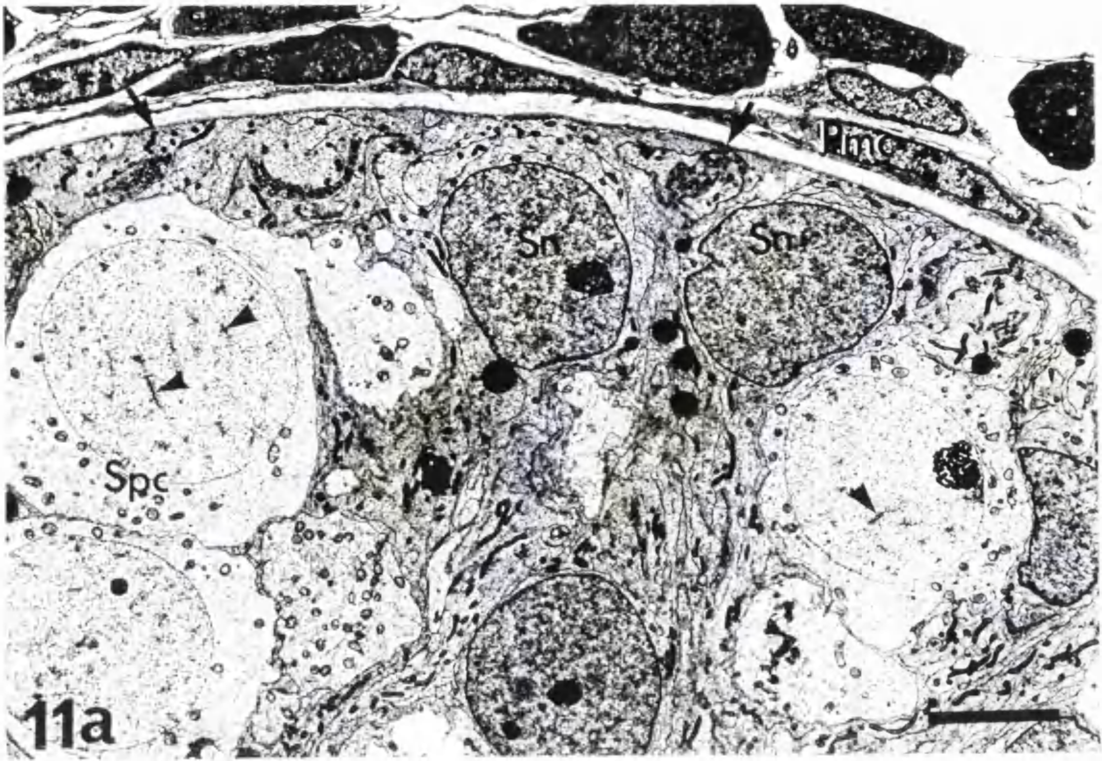

Psc
Pge
10

Fig. 2.11. Electron micrograph of pre-pubertal testis (12 weeks):

a.) Lower power micrograph of testicular cords showing following features: peritubular myoid cells (Pmc), basement membrane (arrow), spermatocytes (Spc) with synaptonemal complexes (arrowheads), Sertoli cell nuclei (Sn).
Bar = 5µm.

b.) Spermatocytes with synaptonemal complexes (arrowheads) and nuclear pores (arrowheads). Note well-developed Golgi complex (G).
Bar = 5µm.


Pmc
Sn
Sn
Spc
11a

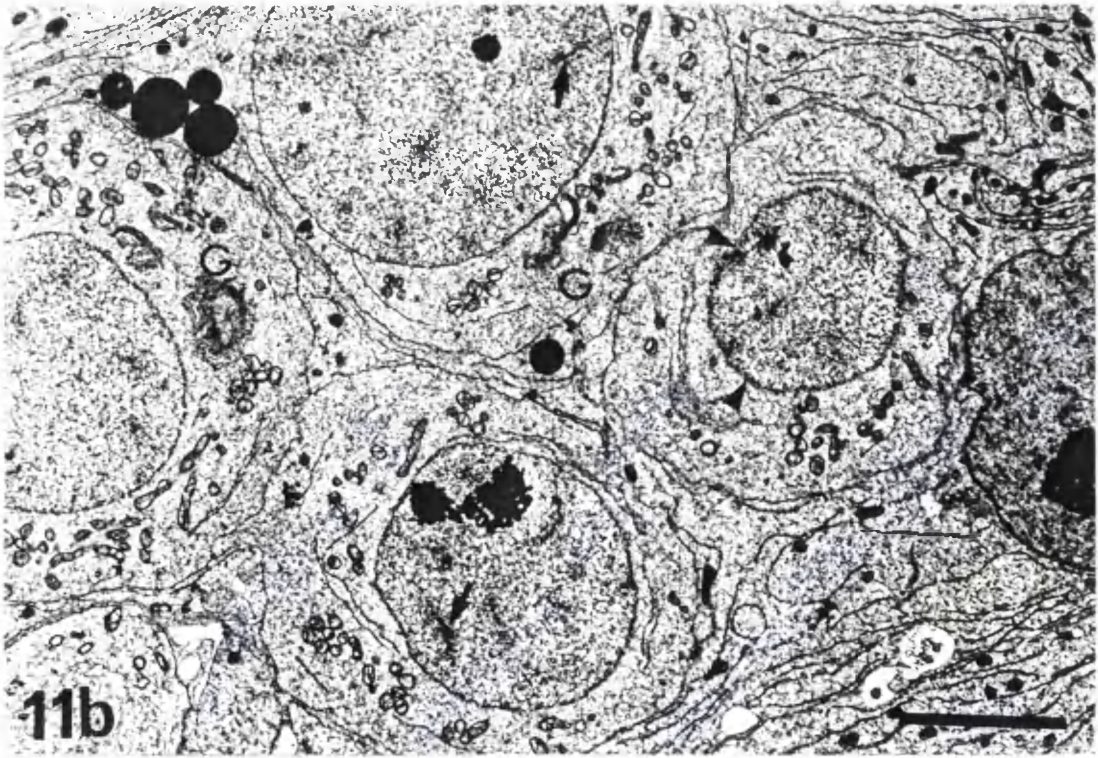

G
G
11b

Fig. 2.12. Electron micrograph of pubertal testis (4 month):

a.) Low-power electron micrograph of seminiferous tubule (4 month) showing the following features: spermatogonia (Spg), primary spermatocytes (Psc), spermatids (Sp), acrosome (arrowheads) and Sertoli cell nuclei (Sn).
Bar = 10µm.

b.) Well-defined tight junction showing the parallel lines of fusion of the apposed membranes and cisternae of endoplasmic reticulum (arrowheads) Bar = 0.1µm.

Psc
Sn
Spg
Sp
12a

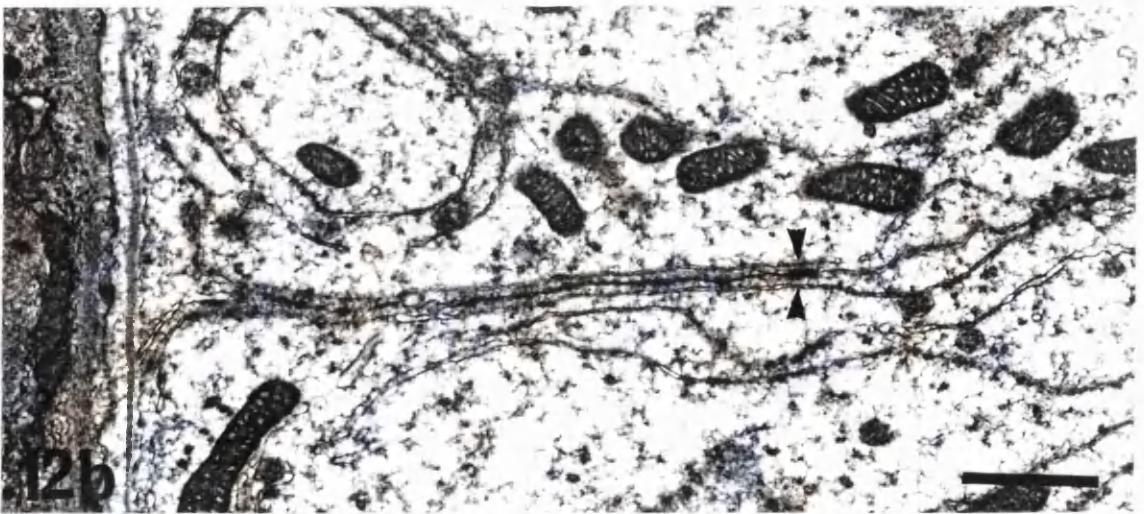
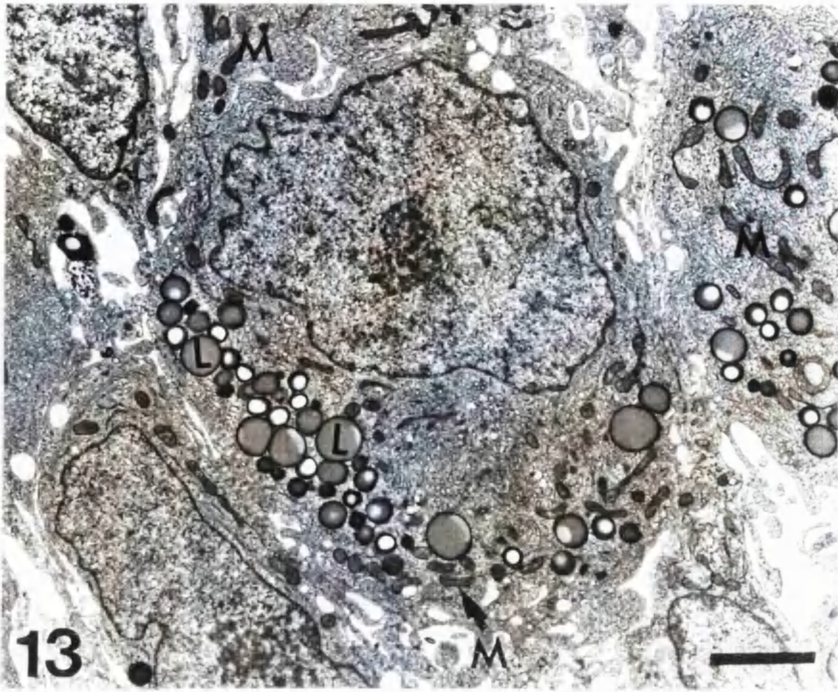

12b

Fig. 2.13. Day 3 Leydig cell showing vesicles of SER; mitochondria (M) and abundant lipid inclusions (L).

Bar = 2μm.

Fig. 2.14. Day 8 Leydig cell showing elongate profiles and vesicles of SER (arrowheads) and mitochondria with tubular cristae (M).

Bar = 2μm.


M
M
L
L
M
13

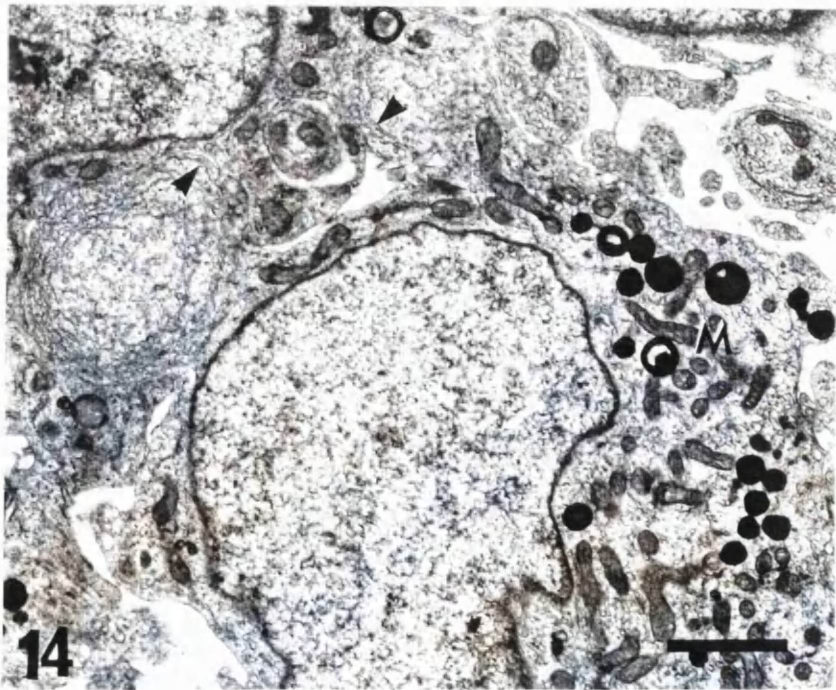
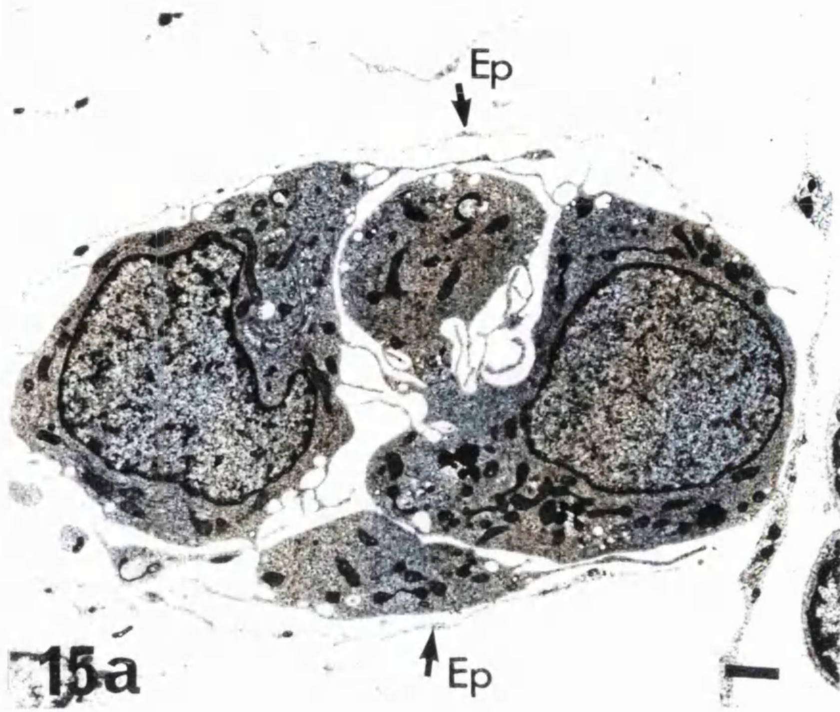

M
14

Fig. 2.15. Electron micrograph of pre-pubertal testis (12 weeks):

a.) Leydig cells clustered by surrounding envelope cells (12 weeks). Note envelope cell processes (arrows).
Bar = 2μm.

b.) Portion of Leydig cell showing parallel alignment of SER profiles (arrows). Note RER (arrowheads) and mitochondria (M).
Bar = 1μm.


Ep
Ep
15a

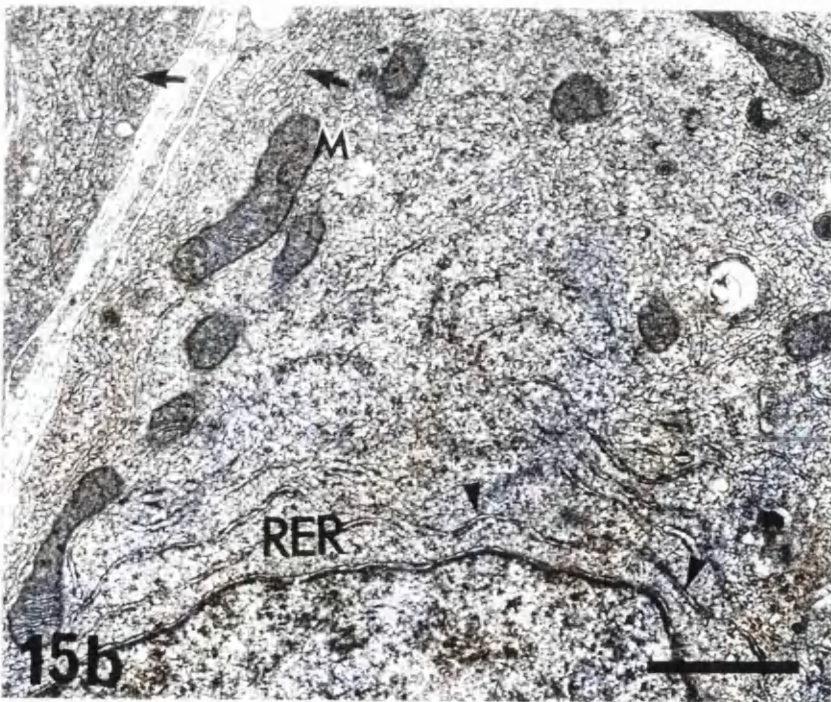
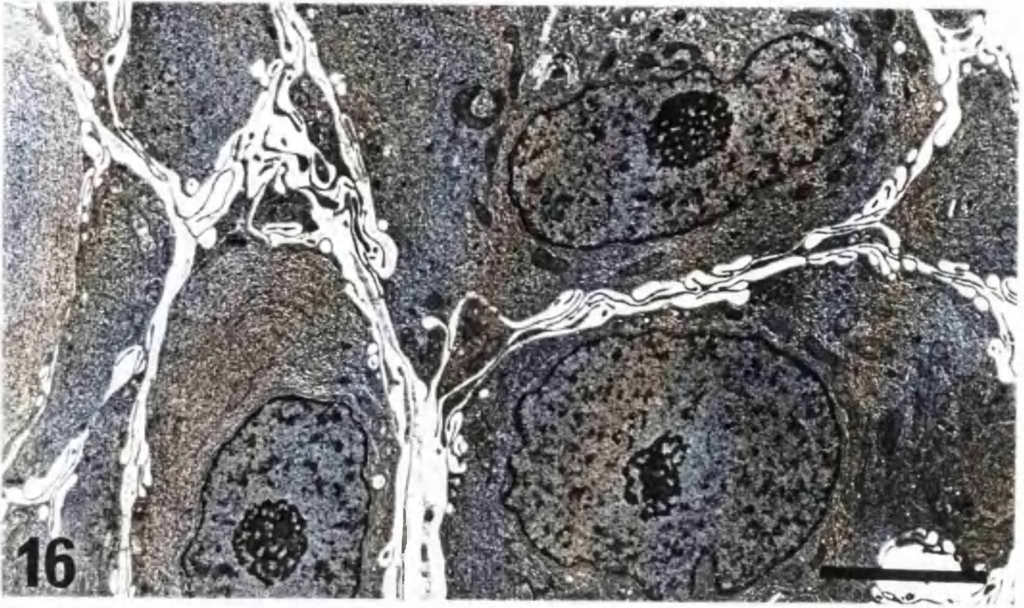

M
RER
15b

Fig. 2.16. Electron micrograph of pubertal testis showing closely-packed Leydig cells.
Bar = 10μm.

Fig. 2.17. Electron micrograph of adult testis (1 year) showing part of a Leydig cell. The cytoplasm shows an abundance of SER highly organised into masses of parallel arrays.
Bar = 1μm.

Fig. 2.18. Electron micrograph of ageing testis (3 year) showing masses of SER, but disorganised.
Bar = 2μm.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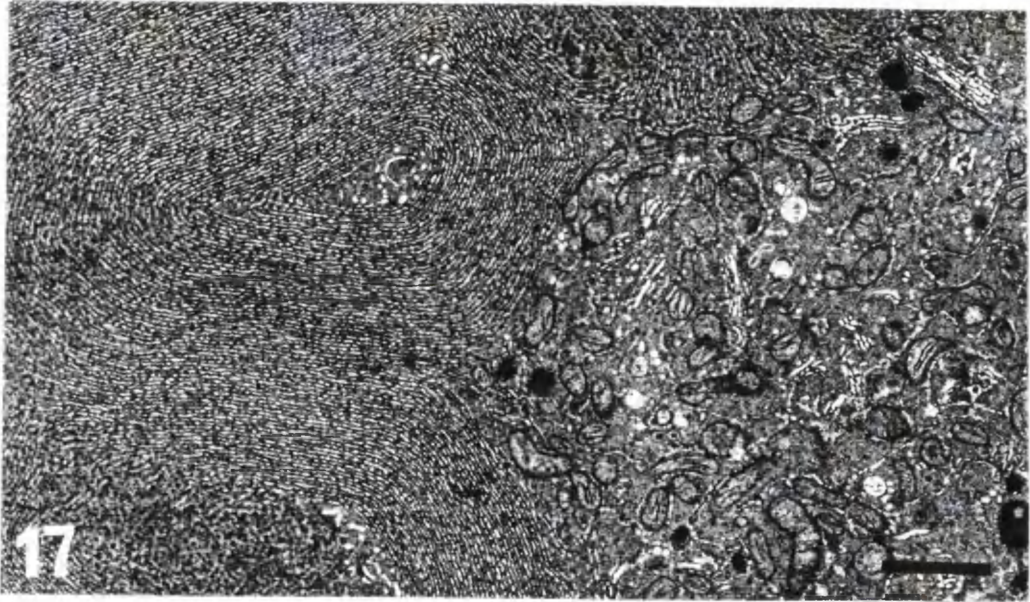
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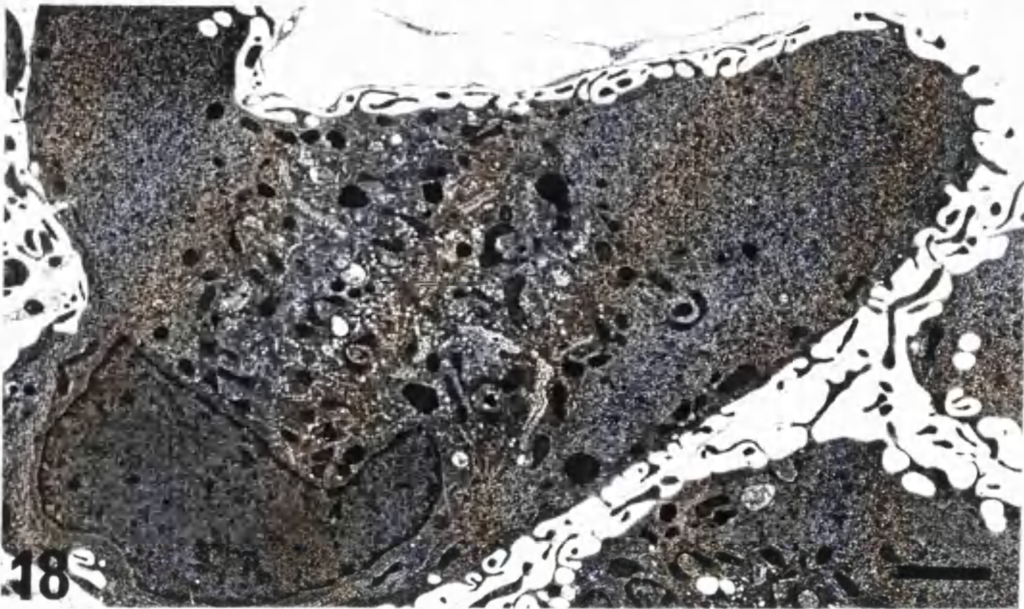
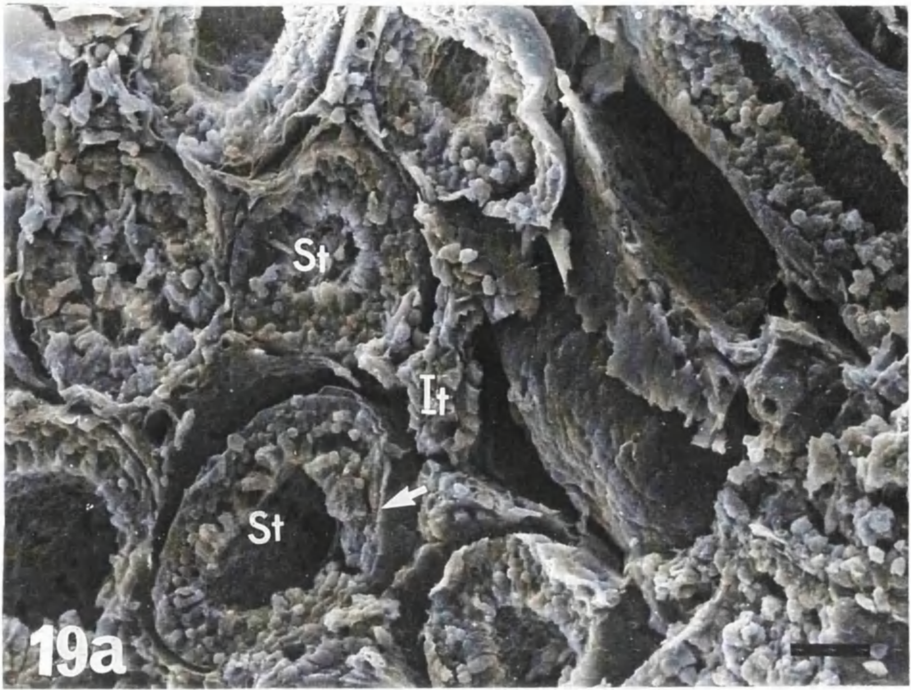

18

Fig. 2.19. Scanning micrograph of fractured testis (3.5 months):

a.) Low power micrograph showing the relation between seminiferous tubules (St) and interstitial tissue (It). Note peritubular myoid cells (arrow). Bar = 500μm.

b.) High power micrograph showing Sertoli cells with extending cytoplasm (Scp) and germ cells (arrows). Note patent seminiferous tubule (St). Bar = 200μm.


St
It
St
19a

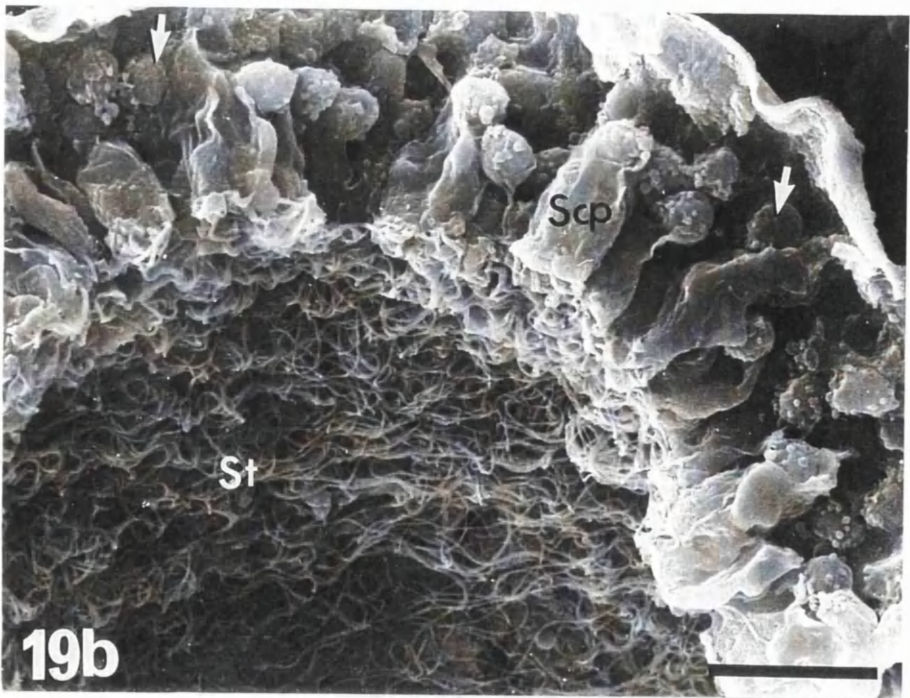
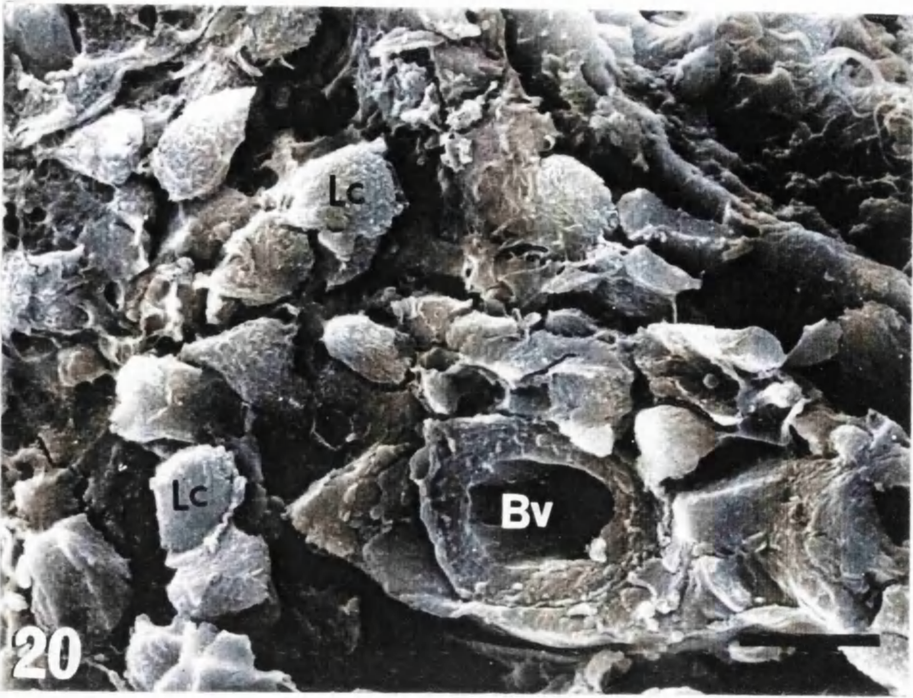

Scp
St
19b

Fig. 2.20 Scanning micrograph of fractured testis (3.5 months) showing Leydig cells (Lc) surrounding a blood vessel (Bv).

Bar = 200μm.

Fig. 2.21. Scanning micrograph of fractured pubertal testis (4 months) showing mature Leydig cells covered by numerous short microvilli and blebs (arrowheads).

Bar = 100μm.


Lc
Lc
Bv
20

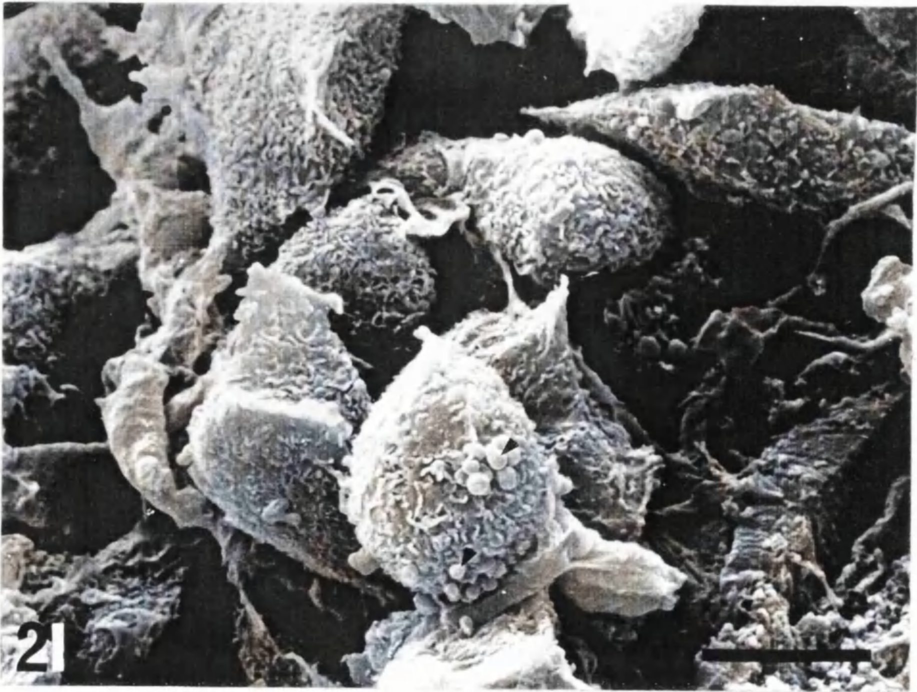

21

Fig. 2.22. Scanning micrograph of fractured adult testis (1 year) showing mature sperm.
Bar = 100μm.

Fig. 2.23. Scanning micrograph of fractured adult epididymis showing head-head paired sperm.
Bar = 20μm.

22

23

Chapter 3: The Early Postnatal Development of the Reproductive Tract of *Monodelphis domestica.*

3.1. Introduction

The modern interpretation of phenotypic sexual differentiation, based upon Jost (1947), views establishment of the gonad as a genetically programmed, while the subsequent differentiation of the reproductive tract, genitalia and nervous system depend upon physiological factors during development. Thus, reproductive tract development involves two sets of primitive genital tracts, the mesonephric (Wolffian) and paramesonephric (Müllerian) ducts (see chapter 1 p11-12). Androgens produced by the testis maintain the Wolffian duct which gives rise to much of the adult male reproductive system while MIS causes regression of the Müllerian duct. The lack of androgens causes degeneration of the Wolffian duct while the lack of MIS permits the Müllerian duct to persist and give rise to much of the female reproductive system (see chapter 1 p8-11). Müllerian inhibiting substance is produced by granulosa cells as well as Sertoli cells (Cate *et al.*, 1990), it may have additional actions on gonadal differentiation and testicular descent (Munsterberg & Lovell-Badge, 1991; Hutson *et al.*, 1988) though these involvements are unconfirmed at present.

Marsupials pose a number of challenges to views on differentiation obtained from studies of eutherian mammals. Firstly, there is disparity in the timing and exclusivity of differentiation. Thus, development of the gonads and reproductive system generally occurs postnatally; mammary anlagen develop in both sexes of South American marsupials but not in Australasian species (Renfree *et al.*, 1987; 1990; Robinson *et al.*, 1991) and both sexes possess large cremaster muscles as adults (Tyndale-Biscoe & Renfree, 1987; van der Schoot *et al.*, 1996). Secondly, the process of differentiation leads to anatomical endpoints which are strikingly different from eutherians (Diagram. 3.1) e.g. i) a female system with two separate uteri, two lateral vaginae and one median vagina/birth canal or ii) a male system in which the scrotum is located anterior to a bifid penis (Cowper, 1740; Renfree, 1994). Thirdly, and most importantly, the development in marsupials of several sexually dimorphic structures such as the pouch, scrotum, mammary primordium, gubernaculum and processus vaginalis appears to occur prior to differentiation of the gonad and may, therefore, be independent of hormones (O *et al.*, 1988; Shaw *et al.*, 1988; 1995; Renfree & Short 1988; Renfree *et al.*, 1995). Despite these differences, there are

sufficient similarities to suggest that common processes may also operate during differentiation. Thus, as in eutherians, differentiation of the gonad depends upon a testis-determining gene SRY on the Y chromosome (Foster *et al.*, 1992). Moreover, the testes of marsupials produce both testosterone (George *et al.*, 1985; Fadem & Harder, 1992; Renfree *et al.*, 1992a) and MIS (Hutson *et al.*, 1988) during development and some aspects of differentiation appear to depend on these (Hutson *et al.*, 1988; Shaw *et al.*, 1988).

Although earlier reports suggested that gonadal differentiation in *Monodelphis* did not occur until 3-4 days after birth (Fadem *et al.*, 1992; Moore & Thurstan, 1990), the evidence using karyotyped neonates has shown that 50% of testes are differentiated on the day of birth itself, and all by the next day (see Chapter 2). Leydig cells can be identified morphologically by day 3 (see Chapter 2) but 3β-hydroxysteroid dehydrogenase activity is not detectable before day 16 and both testicular and adrenal testosterone levels are uniformly low until 4 weeks (see Chapter 4). In this chapter: a) the developmental time course of reproductive tract formation and gonad descent; b) MIS production in *Monodelphis* will be documented.

3.2. Materials and Methods

3.2.1. Animals

The opossums were maintained as described in chapter 2 and pups of both sexes were examined at 0, 1, 2, 4, 5, 6, 8, 10, 12, 14, 15, 16, 20, 24, 28 days after birth (birth = day 0). Female animals from 5 - 7 weeks and adults of both sexes were also examined. Details of animals used at each age point are shown in Table 3.1 and Table 3.2. Animals were killed by rapid decapitation and immersion-fixed with 3% glutaraldehyde + 1% formaldehyde in 0.1 M phosphate buffer (pH 7.2-7.4) for semi-thin resin histology, transmission and scanning electron microscopy (Appendix 1). For paraffin histology, specimens were fixed with 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2-4) (Appendix 1).

For co-culture, the CBA mice used in the present study were from an inbred colony (Glasgow University, Laboratory of Human Anatomy), and maintained on a reversed lighting regime. Males were placed with females for 4 hours, between 12

noon and 4pm, during the dark phase. Those females with a vaginal plug at the end the period were taken to be at day 0 of pregnancy.

Table 3.1. Details of Animals Used for Light Microscopy, Scanning and Transmission Electron Microscopy*

Age	No. of Female opossums	No. of Male opossums
day 0	4	3
day 1	3	3
day 2	3	3
day 4	2	2
day 5	2	3
day 6	2	2
day 8	3	4
day 10	3	2
day 11	1	2
day 12	2	4
day 14	2	2
day 15	3	5
day 16	3	4
day 17	2	4
day 19	2	2
day 20	3	3
day 24	4	5
day 28	2	5
day 35	2	
7 weeks	2	
adult	3	3

*From day 0-2 animals were sexed by karyotyping. Beyond that point, animals can be sexed from external genitalia.

At specific stages of pregnancy, animals were sacrificed by inhalation with CO_2. The uterine horns were dissected out and placed in a petri-dish and the fetuses, with amniotic membranes intact, were transferred into a solution of Hanks buffer in order to maintain an isotonic equilibrium. All dissections were carried out in a laminar flow cabinet and surrounding membranes were removed using fine needles.

Fetuses of gestational age E 13.5 day were utilized, the developmental stage being determined with reference to Theiler (1972). Prior to E 12.5 day, the gonads are

indifferent. Following male sexual differentiation (E 13.5 day), the presence of testicular cords and distinct blood vessels allows easy identification under the dissecting microscope when compared with the relatively homogeneous ovary so that sexing can be made on morphological criteria.

3.2.2. Karyotyping

Accurate sexing of newborn and day 1 opossums was achieved by karyotyping as described in chapter 2 (Appendix 1).

3.2.3. Tissue Preparation

Dissected mesonephroi or reproductive tracts for light (semi-thin section), TEM and SEM were fixed with a primary fixative, composed of 3% glutaraldehyde + 1% formaldehyde in 0.1 M phosphate buffer (pH 7.2-7.4) for 24 hours at room temperature (20°C) (Appendix 2).

For histology, specimens were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer and rinsed in buffer for 10-30 minutes after fixation, then dehydrated through an ascending ethanol series and embedded in paraffin at 57°C (Appendix 2).

3.2.4. Co-culture

Opossum testes (Table 3.2) and female mouse urogenital ridges were dissected in Hank's buffer and then incubated in a co-culture system to detect MIS biological activity (Hutson *et al.* 1988). The number of animals used at various age point are shown in Table 3.2. The standard MIS bioassay was used female E13.5 mouse urogenital ridges co-cultured with developing opossum testes for 72 and 120 hours on a collagen disc over 0.6 ml of CMRL-100 medium (Gibco) containing 10% fetal calf serum (Gibco) and 0.1% gentamycin (Gibco). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The specimens were processed for light and transmission electron microscopy as in chapter 2 (Appendix 2).

The criteria for regression are adapted from a 5 point scale described by Hutson *et al.* (1988). Grade 1: Early regression (duct smaller than normal, basement membrane beginning to dissolve and mesenchyme around duct forming a loose

whorl); Grade 2: Increasing regression (duct about half the normal size); Grade 3: Further regression (lumen decreases in size); Grade 4: Further regression (lumen obliterated) and Grade 5: Total regression (duct-has disappeared).

Table 3.2. Details of Animals Used for Co-culture

Age	No. of Male Opossums
day 4	3
day 6-8	3
day 9-10	3
day 11	2
day 12	3
day 14	2
day 15	2
day 16	4

3.2.5. Light Microscopy

Semi-thin (1 - 2 µm) resin sections were stained with Toluidine blue (Appendix 3) and paraffin sections (5 - 7 µm) were stained with Mayer's haemalum and eosin (Appendix 3). All photographs were taken on a Leitz Vario - Orthomat photomicroscope.

3.2.6. Scanning Electron Microscopy

Animals were dissected to display the developing reproductive tract and then fixed for SEM (Appendix 4). Tissue were processed and examined as in chapter 2.

3.2.7. Transmission Electron Microscopy

Ultra-thin transverse sections through the mesonephros were double post-stained with both saturated uranyl acetate solution and Reynolds' lead citrate as described in chapter 2 (Appendix 2). Specimens were examined in a Philips - CM 100 transmission electron microscope.

3.3. Results

3.3.1. Mesonephros, Wolffian and Müllerian Ducts

At birth the mesonephros is fully developed and functional with convoluted mesonephric tubules (Fig. 3.1a & b, Fig. 3.2a & b); while the metanephros is smaller until day 5 (Fig. 3.3a & b). Degeneration of the mesonephros begins around day 10 and is almost completed by day 19 in the male; however, the female mesonephros is still relatively large at day 14. Cranial to the ovary, the mesonephros does not regress as much as in the male although it too has almost disappeared by day 19.

Well-developed Wolffian ducts are seen in both sexes at birth (Fig. 3.4a & b), extending to the urogenital sinus. These ducts are patent with pale-staining cuboidal epithelium and continue to develop in both sexes (Fig. 3.4c & d) until about day 10. In the female the Wolffian ducts is larger than the Müllerian ducts until day 14 (Fig. 3.4f) when the Wolffian ducts begin to regress at its cranial end. By day 15 the duct lumen is obliterated (Fig. 3.4h). Wolffian ducts show further regression by day 16 and have almost disappeared by day 17 (Fig. 3.4j).

Müllerian ducts are clearly seen on day 1 and they grow in a caudal direction and reach the urogenital sinus on day 6. At this stage, Müllerian ducts are still not patent (Fig. 3.4c-d); it is easily distinguished from the Wolffian ducts by the deep staining of its pseudostratified epithelium and its lateral position. Epithelial cells of the Müllerian ducts are supported by a basement membrane and surrounded by mesenchymal cells (Fig. 3.4c1-2). Prior to day 9 the Müllerian ducts continue to increase in size in both sexes.

In the male the Müllerian ducts begin to regress at its cranial end by day 10, when concentric whorls of mesenchymal cells surround the duct (a sign of early regression), this regression extends throughout the mesonephros by day 12. Increasing regression is clearly seen in the Müllerian ducts at day 14 and the mesenchymal cells forming whorls around the duct become flatter (Fig. 3.4e). At the ultrastructural level the breakdown of the basement membrane is evident (Fig. 4e1-2). By day 15 the Müllerian ducts lumen has completely disappeared (Fig. 3.4g); further regression is seen throughout the mesonephros by day 16 and the Müllerian ducts have almost disappeared by day 17 (Fig. 3.4i).

In the female the Müllerian ducts are smaller than Wolffian ducts until day 14 (Fig. 3.4f). By day 15 well-developed Müllerian ducts are patent (Fig. 3.4h) and their

lumen begin to enlarge at day 16. Its differentiation into a cranial, narrow, convoluted part with a transport and secretary function, the oviducts and a more dilated caudal part (the uterus) begin when the ducts converge at the urogenital sinus (Fig. 3.5a) and the ostium of the oviducts appear (Fig. 3.5a). The Müllerian ducts do not fuse to form a single median vagina or uterus. Instead, the uteri and vaginae remain separate: the paired but separate uteri are referred to as uterine 'horns'. The developing Müllerian ducts fuse medially at the junction of the developing uterus and vaginal sections to form an anterior vaginal expansion, from which the two lateral vaginae extend, looping around the ureters as they pass down to the urogenital sinus. By day 20 separation into the narrower cranial oviduct primordium and a broader caudal uterus are clearly seen (Fig. 3.6a). At the same time the vaginal culs-de-sac start to form (Fig. 3.6a). The cranial portion of the Müllerian ducts further elongate by day 24 and little further change is observed at day 28 although the oviduct has begun to coil. By day 35 the Müllerian ducts have further narrowed and become substantially convoluted to form the Fallopian tube (Fig. 3.7). Further uterine elongation is evident by 7 weeks.

Differentiation of the male Wolffian ducts to form the epididymis and vas deferens has also begun by day 16 (Fig. 3.5b & Fig. 3.13a). Further development of epididymis is seen on day 20 (Fig. 3.6b). At day 24 the Müllerian duct is further elongated and convluted to form the epididymis (Fig. 3.8). The epididymis is fully developed by day 28 when the testis has finally descented into the scrotum (Fig. 3.9a & b).

The reproductive tracts are fully functional in both sexes after 4 months. The adult male genital tracts consist of epididymides and vasa deferentia which open into the anterior end of the large prostate gland (Fig. 3.10a). In the female, the paired uteri are separate and open by separate cervices, each leading into a vaginal cul de sac and then to separate lateral vaginae (Fig. 3.10b).

3.3.2. Gubernaculum and Gonadal Descent

The gubernaculum in *Monodelphis* consists of the gubernacular cord (the cranial part) which is an inconspicuous strand of mesenchyme attached to the caudal end of the gonad and mesonephros and the gubernacular bulb connected with the ventral abdominal wall. In *Monodelphis* the gubernaculum is not developed at birth

(Fig. 1a & b), being first identifiable by day 2 in both sexes as a peritoneal fold with a bulbar enlargement at the caudal end. Sex differences are apparent by day 3.5 (Fig. 3.11a & b) when the male gubernacular cord is significantly longer than the female; the gubernacular bulb connects with the ventral abdominal wall in both sexes. In the male the gubernaculum elongates and extends through the inguinal canal to reach the scrotum. By day 15 it has reached the neck of the scrotum and shows developing myofibres and dense mesenchyme (Fig. 3.12a - d). The cremaster muscle, which takes origin on the iliac spine, inserts on the *tunica vaginalis* of the testis in males and in the female inserts into the substance of the mammary gland (Tyndale-Biscoe & Renfree, 1987). A differentiated cremaster muscle is seen at the periphery of the inguinal part of the gubernacular bulb at day 16 (Fig. 3.13a & b). At this time the gubernaculum has extended through the developing body wall into the scrotal bulges, with the apex of the processus vaginalis within the gubernaculum extending from the inguinal canal to the neck of scrotal bulges. By day 24 the when testes have reached the neck of the scrotum the gubernacular cord has almost disappeared (Fig. 3.8).

In the female, the gubernacular bulb remains as in the male, but the gubernacular cord does not grow much in length during development (Fig. 3.14a-d). The gubernacular bulb continues growing until day 20 (Fig. 3.14d), but it starts to decrease in size by day 28 (Fig. 3.14e) and a further reduction is seen on day 35 (Fig. 3.14f). At this time the gubernaculum contains some pigmented cells. During development the gubercular bulb is retained into adult life as the "uterine round body" (van de Schoot *et al.*, 1996). In contrast to the male, the female gubernaculum never penetrates deeper into the inguinal ring.

At birth gonads in both sexes are elongate in shape (Fig. 3.1a & b). From day 3 onwards, the testes become more rounded than the ovary (Fig. 3.3a & b). At this time gonads in both sexes are initially attached to the large mesonephroi in the abdominal cavity (Fig. 3.1-3). Around day 10-12 the testes and the ovaries start their caudal migration together with the mesonephros. At day 13 the testes begin their transabdominal descent, reaching the inguinal canal within another two days. The lower part of the testis and the caudal end of the mesonephros are attached to the inguinal region by the gubernacular cord. Inguinal descent begins at day 16 (Fig. 3.5b, Fig. 3.13a); at this time the testis and the mesonephros have both completely descended into the inguinal canal (Fig. 3.15a & b). By day 24 testes have reached the

scrotal sac (Fig. 3.8) and achieved their final position at the base of the scrotum by day 28 (Fig. 3.9a & b). In the female at day 16 the mesonephros cranial to the ovary is still relatively larger than in the male (Fig. 5a & 5b). The Müllerian ducts after turning medially fuse with the anterior part of the urogenital sinus. During development ovaries migrate caudally but remain in the abdominal cavity.

3.3.3. MIS Detection

Müllerian inhibiting substance activity is not detectable during early development by co-culture bioassay, in that there is no observed regression of the Müllerian duct (Fig. 3.16a) in the female mouse urogenital complex when compared with control experiments (Fig. 3.16b). MIS can first be detected on day 15 when the testis causes Müllerian duct regression and also stimulates the Wolffian duct (Fig. 3.16c). In control experiments, no regression of the Müllerian duct is found on day 15 (Fig. 3.16d).

3.4. Discussion

Genital tract development of marsupials and eutherians differs in the timing of key events relative to birth (Tyndale-Biscoe & Renfree, 1987; Renfree *et al.*, 1996). *Monodelphis,* like other marsupial species, is born with a fully functional mesonephros and the urogenital system at a sexually indifferent stage (Diagram 3.1): the Wolffian ducts are patent to the urogenital sinus and elongated gonads are found in both sexes. Differentiation of the Wolffian and Müllerian ducts follows the normal eutherian pattern, and takes place after gonadal differentiation under the influence of gonadal hormones. In the male, Müllerian duct regression occurs between day 12-16 after birth; the onset of MIS production presumably occurs prior to this period. Testicular androgen production probably occurs on day 15, since Wolffian duct regression in the female begins at this time. This is confirmed by the first detection of the enzyme 3β-HSD in Leydig cells at day 16 (Xie *et al.*, submitted). Furthermore, Müllerian duct regression in the male occurs earlier than the corresponding regression of Wolffian ducts in the female.

The gross anatomy of the adult female reproductive system of marsupials is different to that of eutherian mammals, due to a difference in the migration of the embryonic urinary and genital ducts (Shaw *et al.*, 1988; Tyndale-Biscoe & Renfree,

1987). In *Monodelphis*, as in other marsupials, the ureters pass medial to the Wolffian and Müllerian ducts, rather than laterally to them, as in eutherians. As a result, the female Müllerian ducts do not fuse to form a single median vagina or uterus. Instead, the uteri and vaginae remain separate. The developing Müllerian ducts fuse medially at the junction of the developing uterus and vaginal sections to form an anterior vaginal expansion, from which the two lateral vaginae develop, looping around the ureters as they pass down to the urogenital sinus.

Hunter (1762) proposed that the gubernaculum plays a key role in testicular descent and undergoes specific changes that are absent in the female (Backhouse, 1964). Wensing (1973) reported that during transabdominal migration of the testis, the caudal end of the gubernaculum initially enlarges, only to regress again during testicular descent into the scrotum. This initial gubernacular swelling may be controlled by MIS, since it is abolished in male mice fetuses exposed to diethylstilboestrol (DES), which also prevents regression of their Müllerian ducts (Raynaud, 1958). Although in recent years the gubernaculum has received much attention there is still much confusion regarding this structure.

It is generally agreed in most eutherians (Wensing & Colenbrander 1986) that the gubernaculum is a column of mesenchyme initially connecting the gonad on the urogenital ridge with the anterior abdominal wall at the future inguinal region. The extraperitoneal end is embedded in the abdominal wall as the abdominal muscles develop around it, leaving a mesenchymal defect that will become the inguinal canal. An annular peritoneal diverticulum grows into the distal gubernaculum to form the processus vaginalis. Apart from a narrow connecting 'mesorchium' posteriorly, the processus vaginalis divides the gubernaculum into three parts: the central column or gubernaculum proper; the parietal or vaginal part surrounding the processus vaginalis and the origin of the developing cremaster muscle; and the infravaginal solid tip of the gubernaculum distal to the processus vaginalis.

However, van der Schoot (1996b) claimed that the strand which runs down from the lower end of the testis to the scrotum is a ligament, rather than the gubernacular cord. The structure termed the gubernaculum by van der Schoot (1992; 1996a) in the fetal rat emerges as a part of the developing ventral/inguinal abdominal wall and differentiates from the inguinal fold of the mesonephric mesentery. The postnatal gubernaculum develops from the fetal gubernaculum and consists of the

bulge of dense connective tissue on the dorsomedial aspect of the base of the cremasteric sac; the cremaster muscles and the covering sheath of the processus vaginalis.

In marsupials, the male gubernaculum resembles that of the most eutherians (O *et al.*, 1988; Griffiths *et al.*, 1993a; Renfree *et al.*, 1996) as demonstrated by Wensing & Colenbrander (1986). Gubernacular structures have been studied exclusively as part of male genital development (review see van der Schoot, 1996b), but have been neglected in females. In the female gubernaculum becomes the uterine round ligaments and round bodies (Burns, 1939a, 1945, 1961a, Finkel, 1945) following a poorly understood process. The gubernaculum in the tammar wallaby develops as a cord of connective tissue, from the nephrogenic cord at the posterior end of the gonad and mesonephros; it connects with the anterior abdominal wall, is first identified in day-22 embryos and no sex difference in its structure is apparent until the day before birth (day 25 of gestation) (Renfree *et al.*, 1996).

A function of the female gubernaculum as a suspensory support of the internal genitalia has been proposed in *Monodelphis* (van de Schoot *et al.*, 1996). Present studies indicate that gubernacular differentiation in *Monodelphis* is a postnatal event since it is first identified on day 2 in both sexes. No sex differences prior to day 3.5 are observed.

Morphological steps in testis descent to the scrotum fall into two distinct phases (Heyns, 1987; Hutson *et al.*, 1990): transabdominal migration and inguinoscrotal descent. Although complete testicular descent into the scrotum occurs in most eutherians (such as the rat: Wensing, 1986; 1988) and marsupials (such as the tammar wallaby: Hutson *et al.*, 1988; Griffiths *et al.*, 1993a), there are significant differences in scrotal anatomy between them. In the rat, the scrotum is located a significant distance from the inguinal canal posteroinferior to the phallus, and consists of soft thin skin. The gubernaculum must migrate from the inguinal canal to the scrotum during the second phase of descent, then its tip or infravaginal part remains free as it does in humans (Heyns, 1987). However, in marsupials the scrotum is anterior to phallus and the scrotum has a hard scrotal 'shell' overlying the inguinal canal cranial to the phallus (Tyndale-Biscoe & Renfree, 1987; Griffiths *et al.*, 1993b). In *Monodelphis*, as in the tammar, the distance for migration is therefore

reduced and gubernaculum becomes densely adherent to the inside of the scrotum throughout the process.

The ontogeny of testicular caudal migration and scrotal descent in *Monodelphis* seem to be a more continuous process than in many eutherian species. Around day 10-12 both testis and ovary become more posteriorly located relative to the kidney due to differential growth and mesonephric regression. Transabdominal migration begins at day 13; inguinoscrotal descent starts at day 16 and descent into the scrotal sac is completed by day 28.

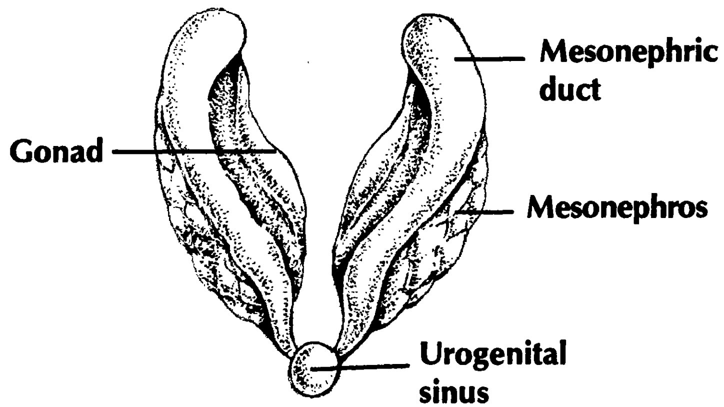
In eutherians, testicular descent is under separate hormonal control (Hutson, 1985). Transabdominal migration from its original abdominal position to the site of the future internal inguinal ring does not require androgen (Hutson & Donahoe, 1986). Müllerian inhibiting substance has been proposed as a possible regulator of this phase (Josso & Tran; 1977). Inguinoscrotal descent is regulated by androgens, as the testes remain at the inguinal ring in testicular feminisation (Hutson & Donahoe, 1986). In *Monodelphis*, the transabdominal phase of testicular descent starts at day 13 and inguinoscrotal descent begins on day 16, which coincides with the timing of MIS and testosterone production. This would be consistent with its proposed role in the control of testicular descent phase.

Müllerian inhibiting substance in eutherian mammals such as the rat, calf and human causes regression of the Müllerian (or paramesonephric) duct, the anlage of the Fallopian tube, uterus cervix and upper vagina (Josso & Picard, 1986, Josso *et al.*, 1993). Müllerian inhibitory substance of testicular origin can transform rat ovaries into testes *in vitro* (Vigier *et al.* 1987). Picon (1969) devised an *in vitro* organ culture assay to detect Müllerian inhibiting substance using the 14 day rat gonadal urogenital ridge. If cultured for 3 days with embryonic mammalian testis, regression of the Müllerian duct could be detected histologically. This co-culture bioassay technique has also been used in the human (Josso, 1972, 1973; Blanchard & Josso, 1974). In marsupials, Hutson *et al.* (1988) employed a modified MIS bioassay co-culturing 13.5 day fetal mouse urogenital ridges with developing wallaby testes. The results show MIS activity in pouch young 2 to 85 days old. In *Monodelphis*, the male Müllerian duct starts regressing between days 12-16, which presumably indicates that the MIS production occurs prior to this period although MIS activity cannot be detected until day 15 using bioassay techniques. Lack of an effect may be a result of

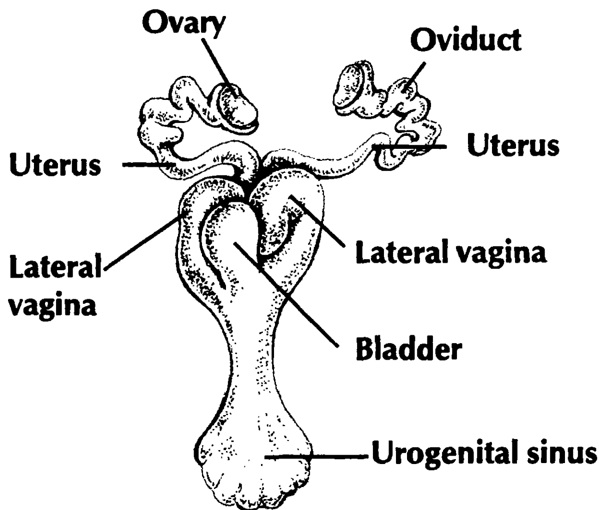
small size of gonads in *Monodelphis* when compared with those of the wallaby, causes the concentration of MIS in the co-culture medium to be too low.

To summarise, the development of reproductive tract and gonadal descent in *Monodelpis* follows the eutherian pattern and occurs under the influence of gonadal hormones. This study suggests that the onset of MIS production occurs prior to day 12 and testicular androgen secretion begins at day 15. This is consistent with the role of these hormones in controlling the two phases of testicular descent.

INDIFFERENT STAGE



ADULT FEMALE



ADULT MALE

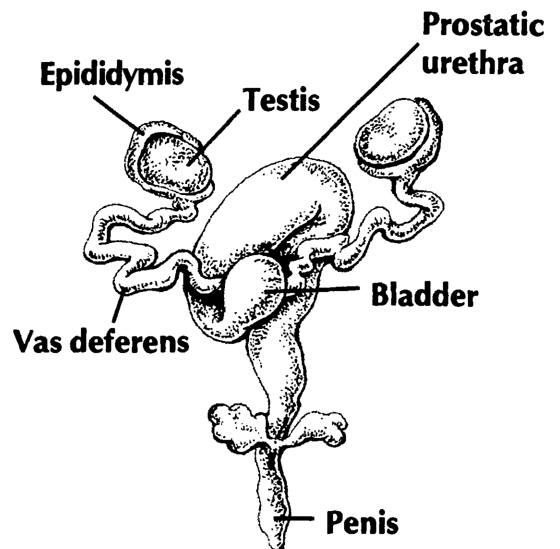


Diagram. 3.1 Reproductive tract of the opossum.

(Modified from Fadem & Tesoriero, 1986)

Fig. 3.1a-b. Newborn opossums sexed by karyotyping:

Scanning electron micrographs of female (**a**) and male (**b**) opossums show elongated gonads attached to the large mesonephros (Ms) in both sexes.

Note testis (T), ovary (O), metanephros (Mt), colon (Co) mesonephic ducts (arrows).

Magnification: x 58

♀
Mt
Ms
O
Ia

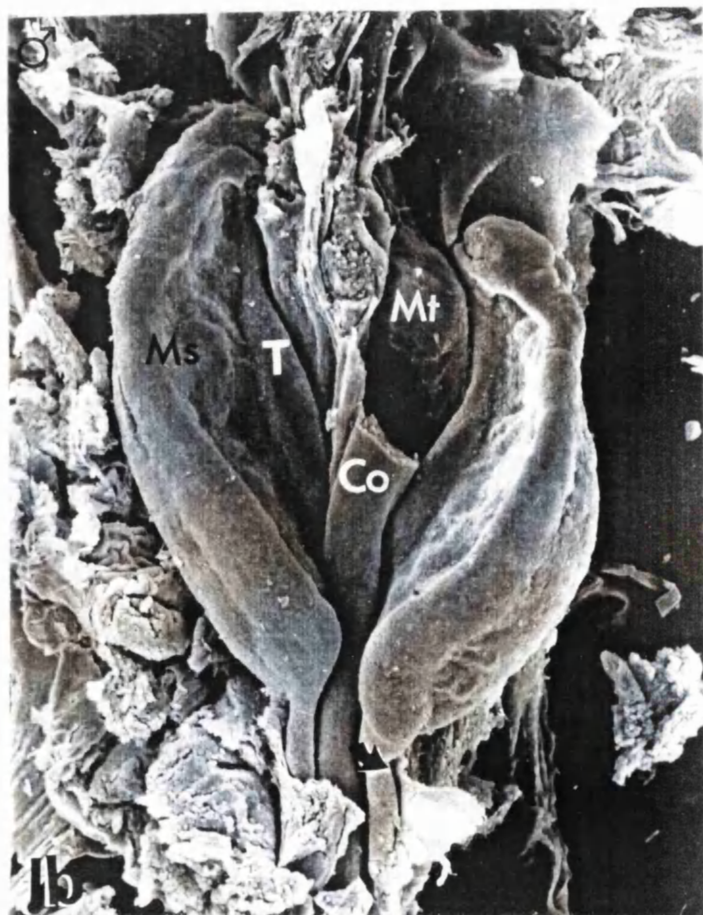
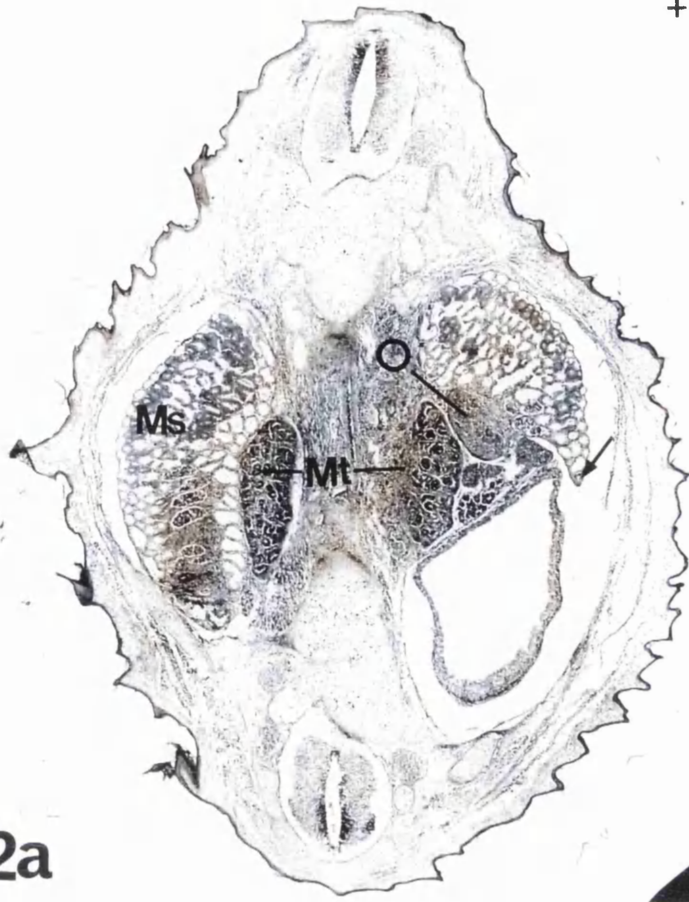

♂
Mt
Ms
T
Co
Ib

Fig. 3.2a-b. Histological transverse sections of opossums on day 0:

Female (**a**) and male (**b**) opossums both showing the large mesonephros (Ms) with patent mesonephric tubules and the first sign of paramesonephric (Müllerian) ducts (arrows) at the caudal end.

Note testis (T), ovary (O), metanephros (Mt) and intestine (It).

Magnification: x 25


♀
O
Ms
Mt
2a

♂
Ms
T
Mt
It
2b

Fig. 3.3a-b. Scanning electron micrographs of the opossum urogenital system on day 5:

Female (a) and male (b) opossums showing elongated ovary (O) and rounded testis (T) attached to the mesonephros (Ms).

Note metanephros (Mt), adrenal (A), gubernaculum (G) colon (Co), bladder (B) and inguinal canal (arrows).

Magnification: x 58

A
K
O
Co
G
B
3a

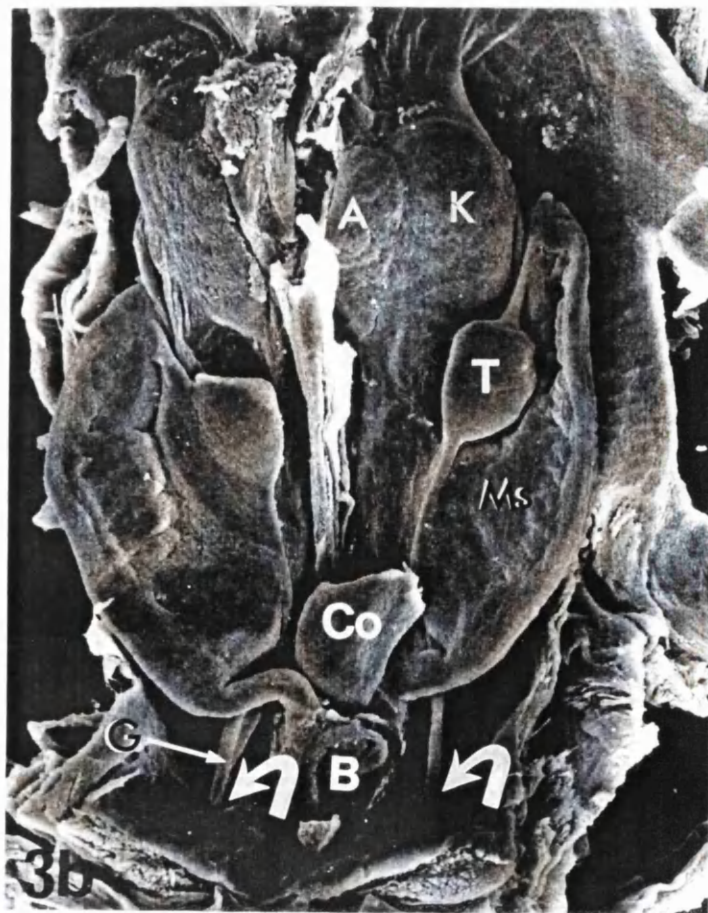
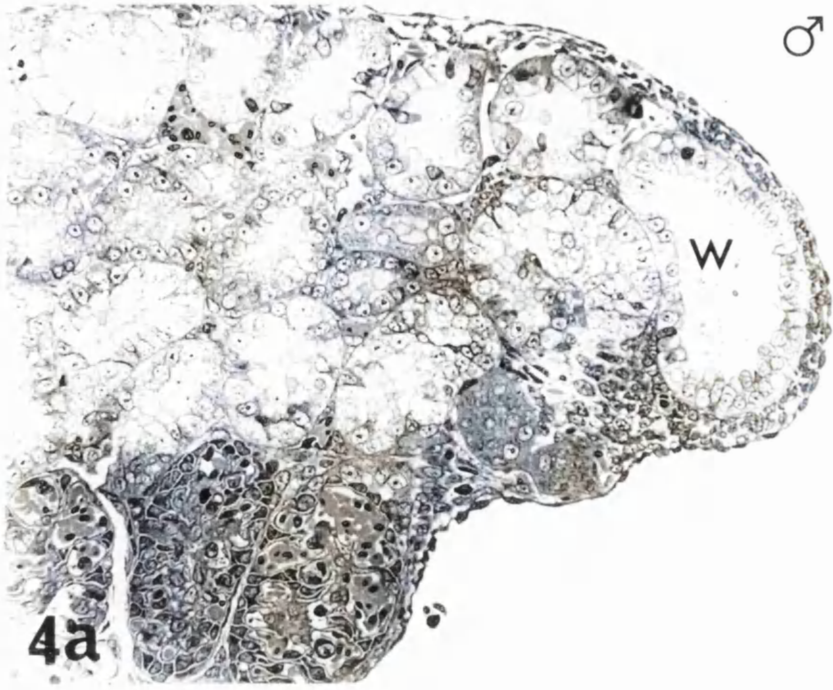

A
K
T
Ms
Co
G
B
3b

Fig. 3.4a & b. Transverse sections through the opossum mesonephros on day 0:

Karyotyped male (**a**) and female (**b**) newbron opossums showing fully developed Wolffian ducts (W).

<u>Magnification: x 240</u>


♂
W
4a

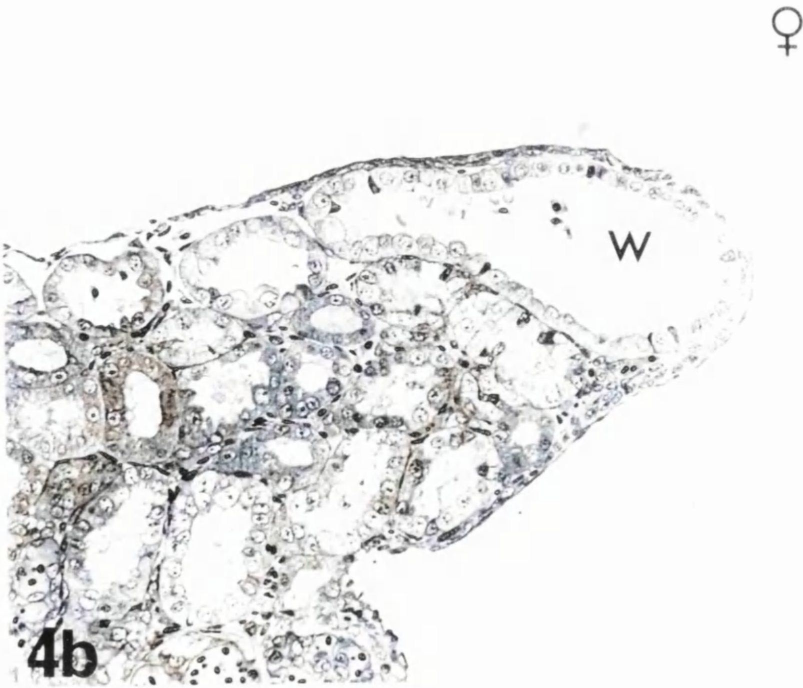
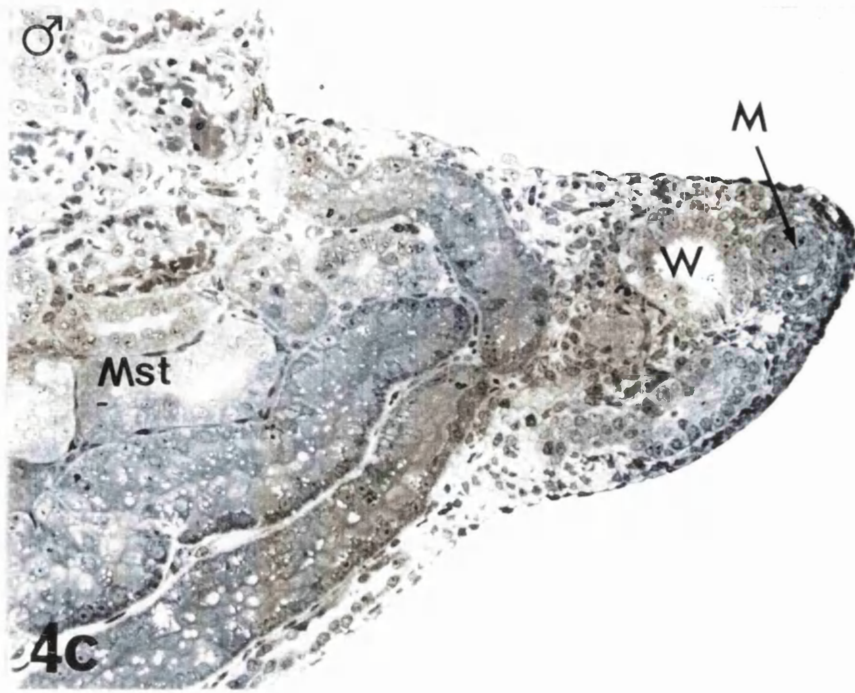

♀
W
4b

Fig. 3.4c & d. Transverse sections through the opossum mesonephros on day 6:

Male (c) and female (d) both showing well-developed Müllerian ducts(M).

Note Wolffian ducts (W) and mesonephric tubules (Mst).

Magnification: x 240


♂
M
W
Mst
4c

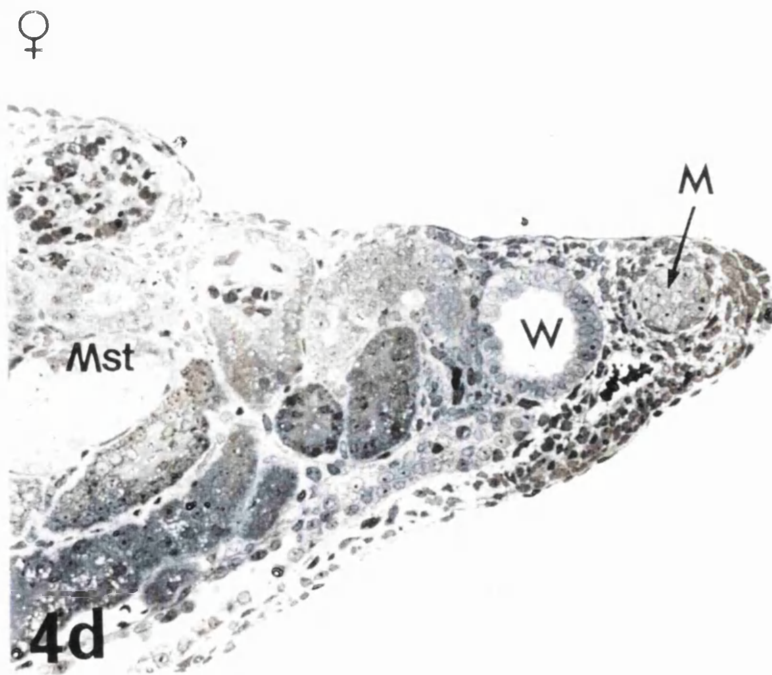
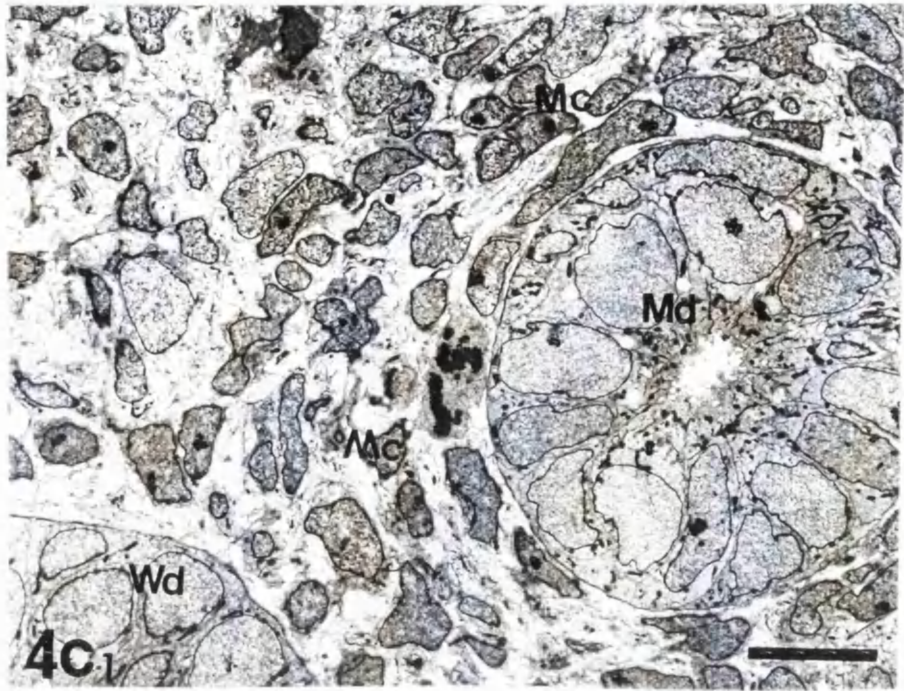

♀
M
W
Mst
4d

Fig. 3.4c1-2. Transmission electron micrographs of transverse sections through the male opossum mesonephros on day 6:

c1.) Low power of micrograph shows the Müllerian duct (Md) are surrounded by mesenchymal cells (Mc).
Bar = 100μm.

c2.) High power of micrograph showing the Müllerian duct epithelium and well-developed basement membrane (arrow).
Bar = 100μm.


Mc
Md
Mc
Wd
4c1

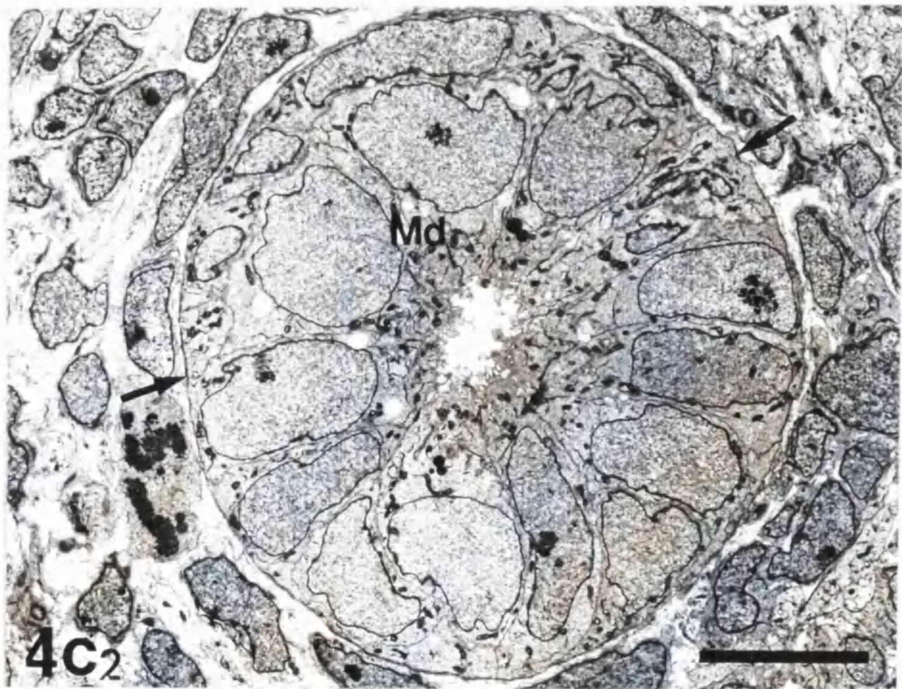
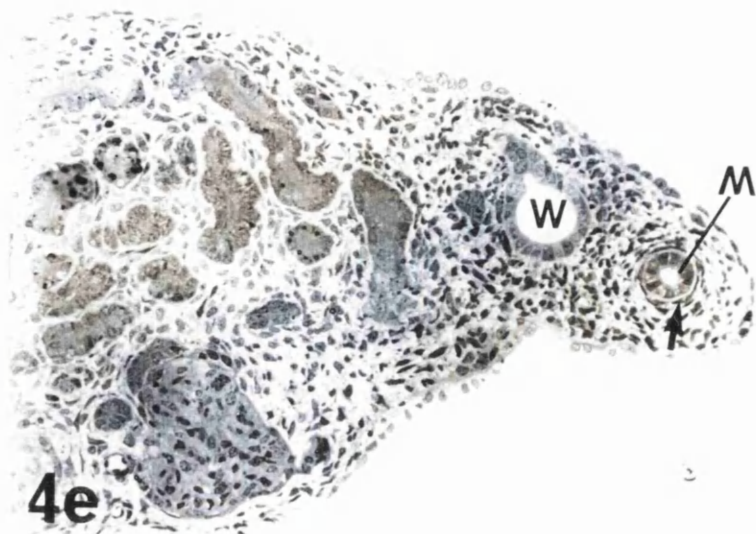

Md
4c2

Fig. 3.4e-f. Transverse sections through the opossum mesonephros on day 14:

Light micrographs show although evidence of Müllerian duct (M) regression is seen in the male (**e**): whorls of mesenchymal cells are aggregated around the duct (arrows), Müllerian ducts are still relatively large in the female (**f**).

Magnification: x 240

♂


W
M
4e

♀

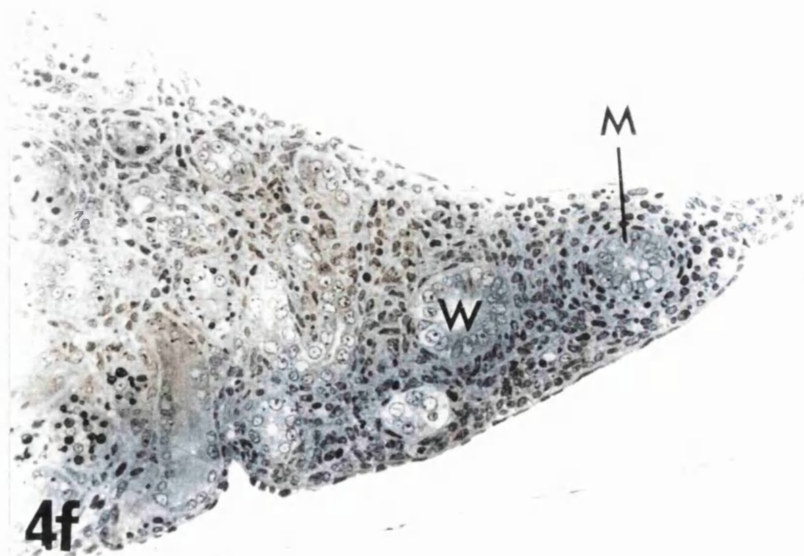
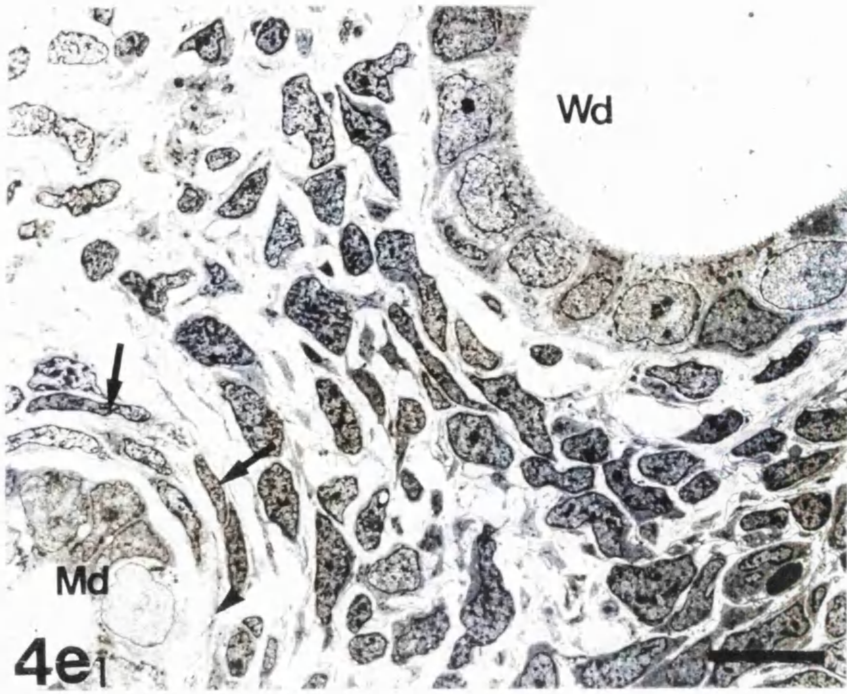

M
W
4f

Fig. 3.4e1-2. Transmission electron micrographs of transverse sections through male mesonephros:

a.) Low power micrograph showing flattened mesenchymal cells (arrowhead) around the Müllerian duct.
Bar = 100μm.

b.) High power micrograph showing breakdown of the basement membrane (arrow).
Bar = 20μm.

Note Wolffian (Wd) and Müllerian duct (Md).


Wd
Md
4e1

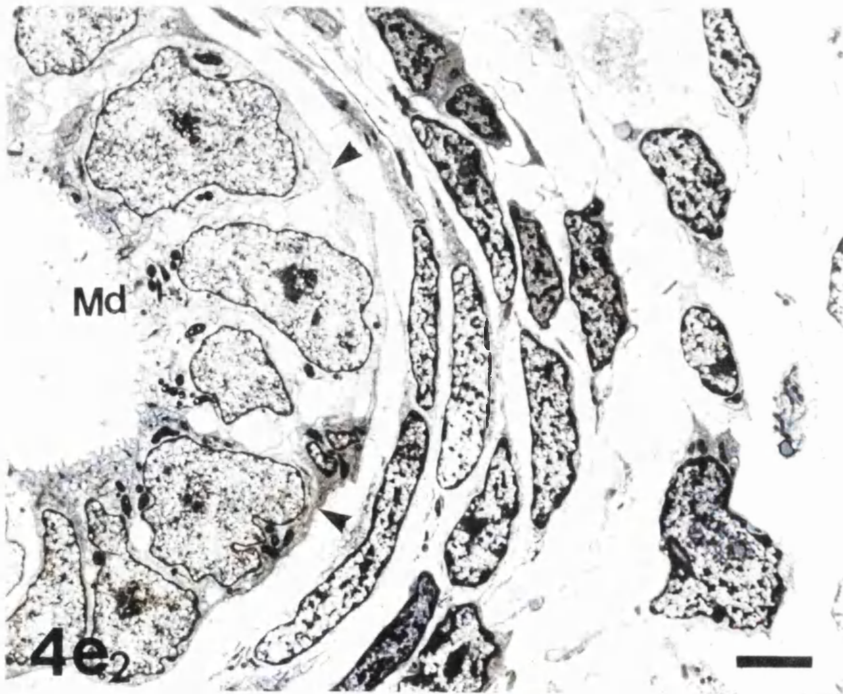

Md
4e2

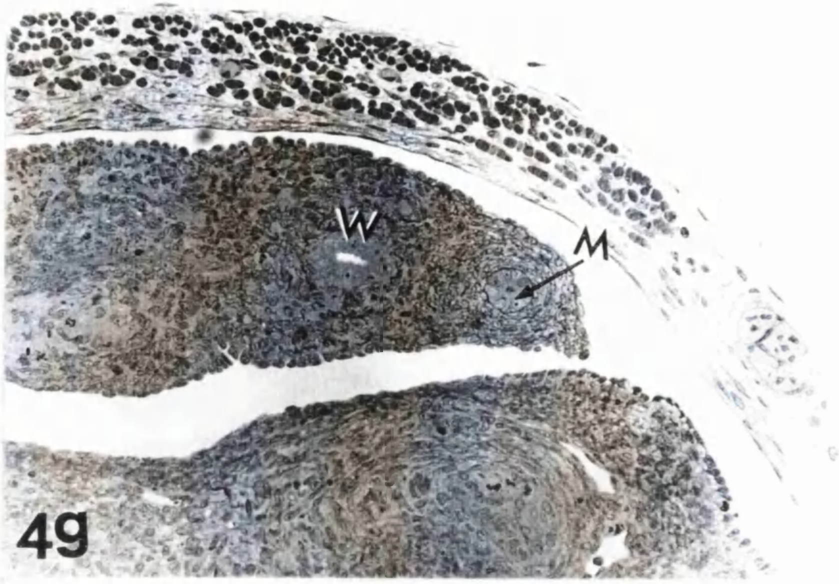
Fig. 3.4g-h. Transverse sections through the opossum mesonephros on day 15:

g.) Male showing further regression of the Müllerian duct which now lacks a lumen.

h.) Female showing regression of the Wolffian duct (W) with obliterated lumen (W) and well-developed Müllerian duct (M).

Magnification: x 240

♂


W
M
4g

♀

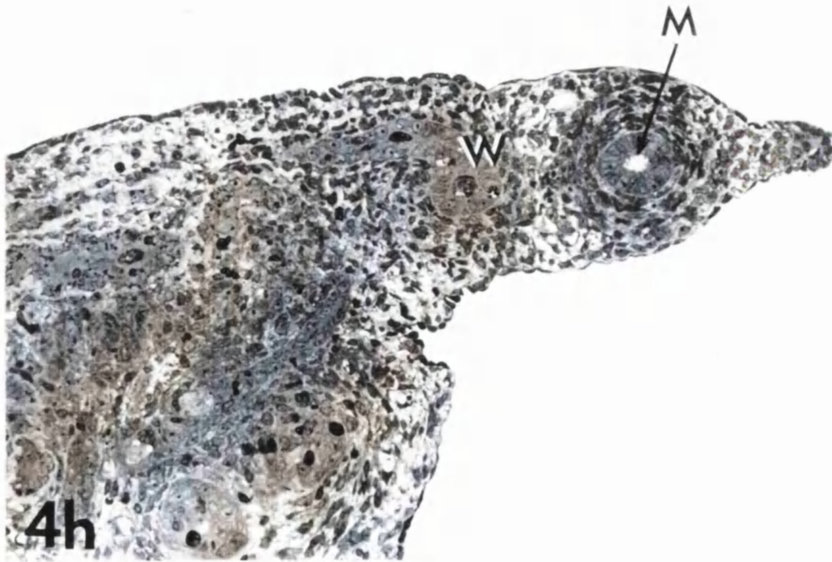
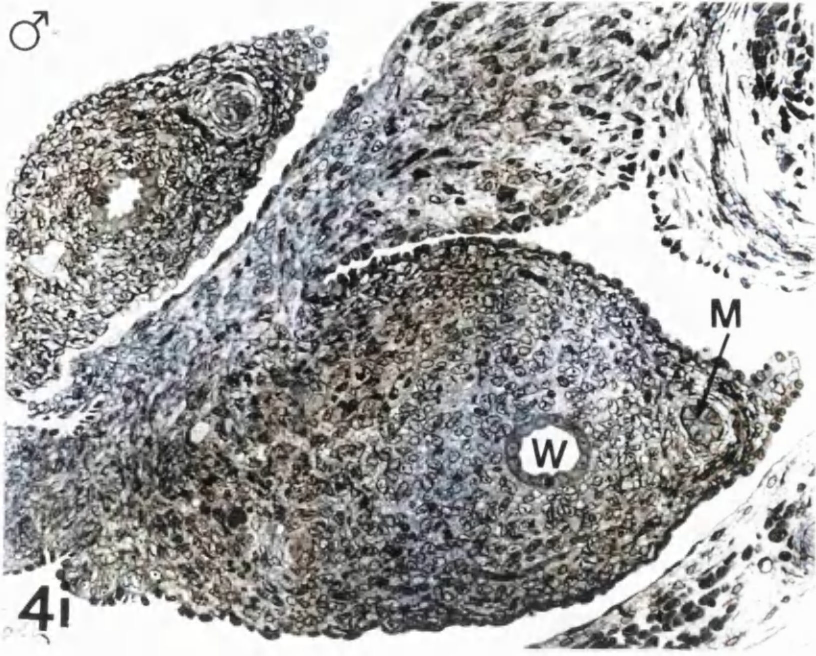
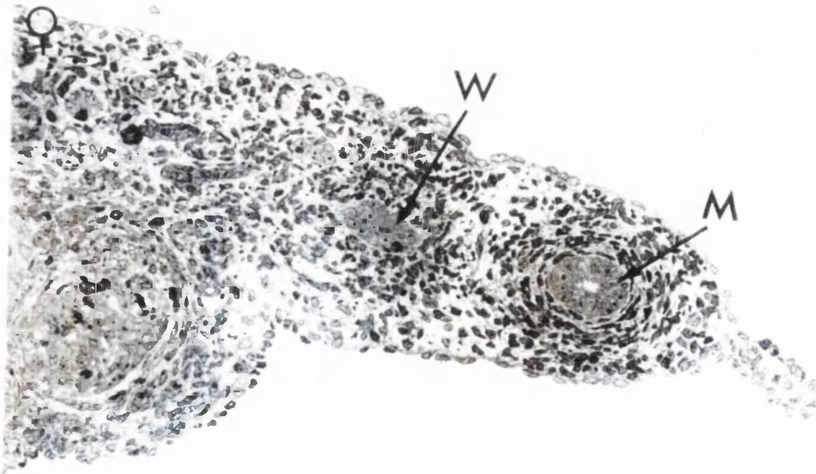

M
W
4h

Fig. 3.4i-j. Transverse sections through the opossum mesonephros on day 17:

The Müllerian duct (M) has almost disappeared in the male (**i**) and as has the Wolffian duct (W) in the female (**j**).

Magnification: x 240





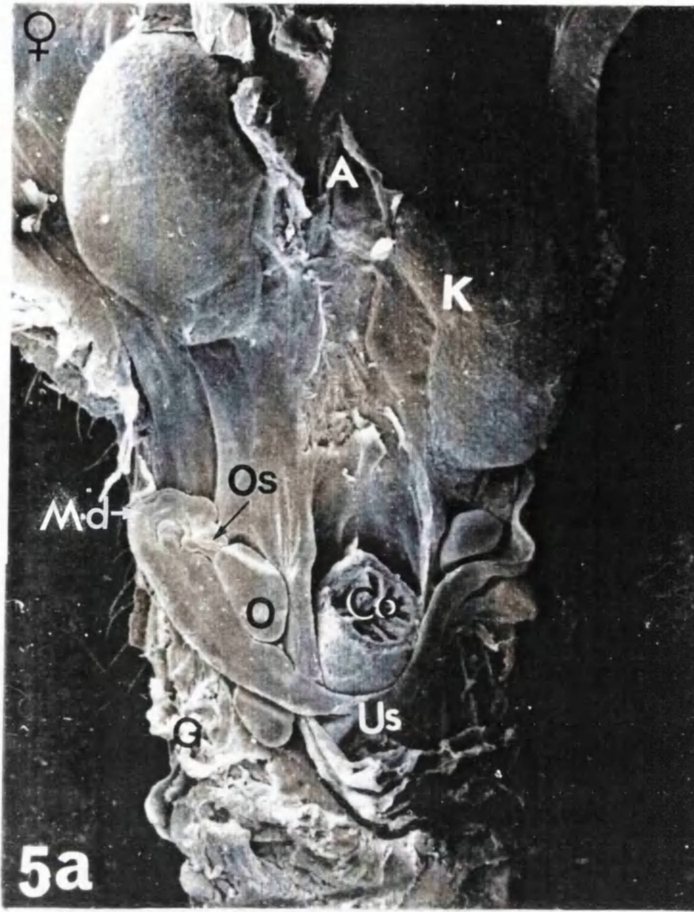
4j

Fig. 3.5a-b. Scanning electron micrographs of the opossum urogenital system on day 16:

Female (**a**) shows differentiation of the Müllerian duct (Md). Male (**b**) shows testis (T) now close to the entrance of the inguinal canal.

Note kidney (K), adrenal (A), colon (Co), urogenital sinus (Us), bladder (B), ovary (O), ostium of the oviduct (Os), scrotum (S), epididymis (Ep), intact testis (*) and inguinal canal (arrow).

Magnification: x 25.



♀
A
K
Os
Md
O
Co
Us
G
5a

♂
A
K
Ep
T
B
*
S
5b

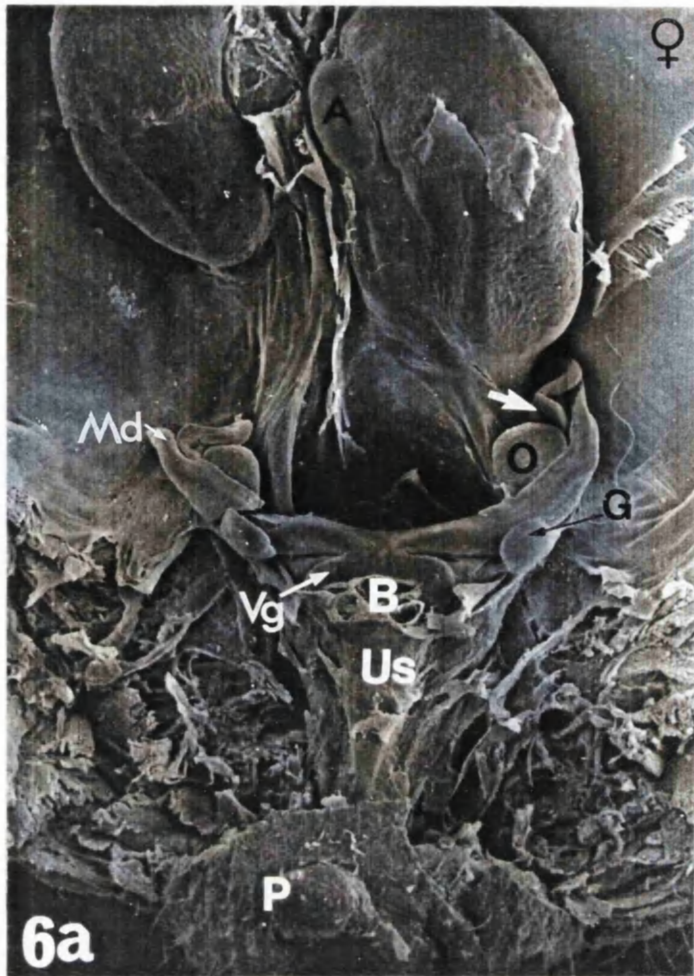
Fig. 3.6a-b. Scanning electron micrographs of the opossum urogenital system on day 20:

a.) The female opossum shows the Müllerian duct differentiated into a narrower cranial (Md) and a broader caudal region where the vagina (Vg) is forming by the junction of the two sides.

b.) The male opossum shows further development of epididymis (Ep) and vas deferens.

Note kidney (K), adrenal (A), bladder (B), ureter (Ur), testis (T), intact testis (*), ovary (O), urogenital sinus (Us), gubernaculum (G), scrotum (S) and phallus (P).

Magnification: x 18.


♀
A
Md
O
G
Vg
B
Us
P
6a

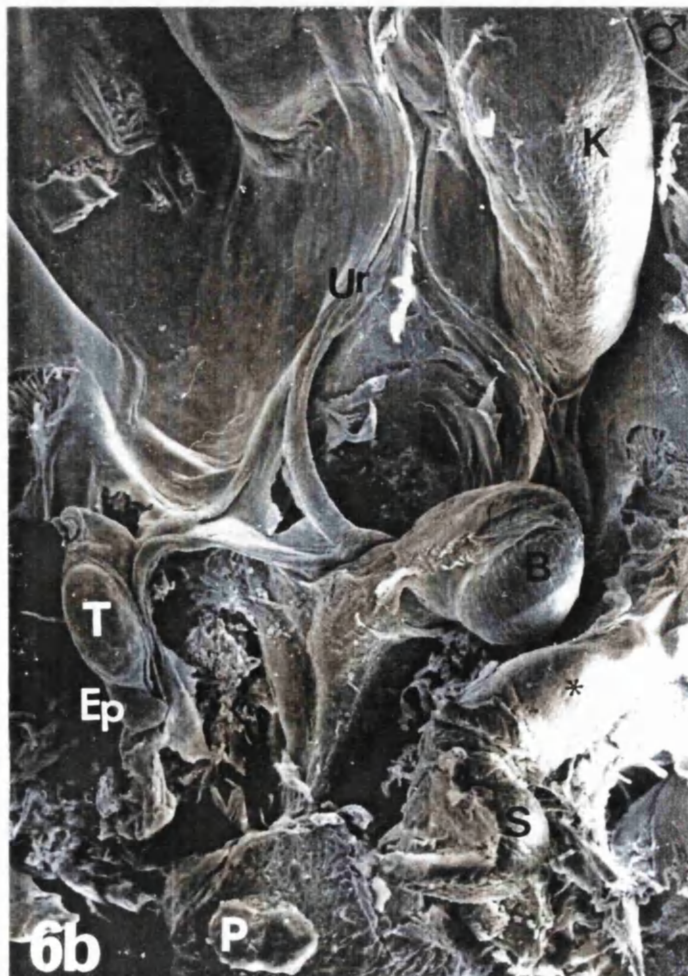

♂
K
Ur
B
T
Ep
*
S
P
6b

Fig. 3.7. Scanning electron micrographs of female urogenital system on day 35:

The Müllerian duct becomes convoluted to form the Fallopian tube (arrow). The uterus (U) and lateral vagina (Lv) are clearly seen.

Note kidney (K), adrenal (A), gubernaculum (arrowheads) and urogenital sinus (Us).

Magnification: x 12

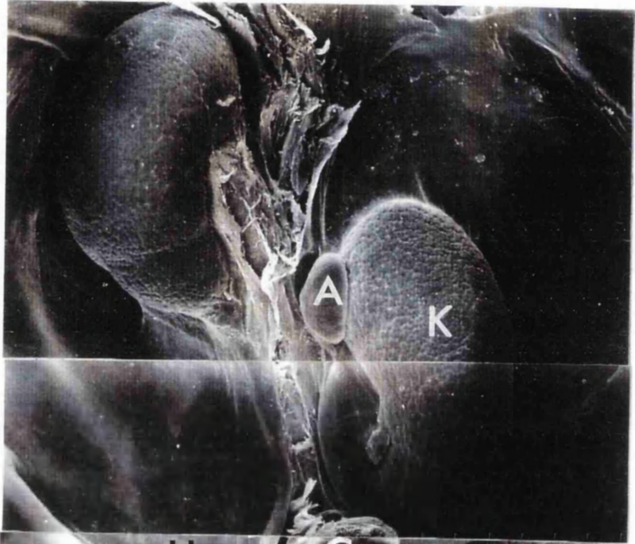
A
K

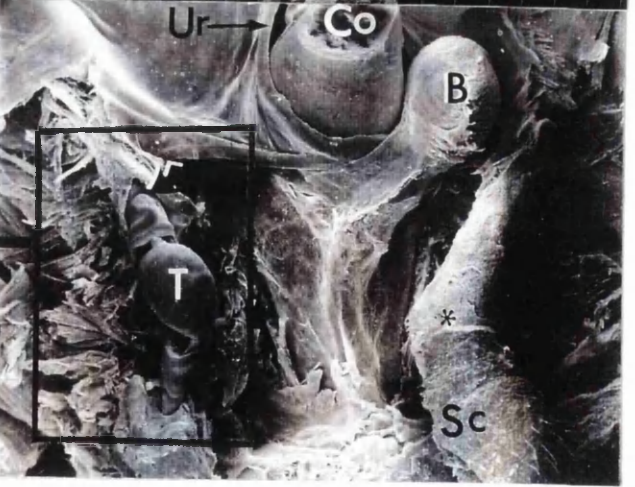
U
Lv
Us
7

Fig. 3.8. Scanning electron micrographs of the male opossum urogenital system on day 24 shows testes (T) have moved from their intra-abdominal position through the inguinal canal (arrows) into the extra-abdominal spece and reached to scrotum sac (Sc). Well-developed epididymis (Ep) are clear seen.

Note kidney (K), adrenal (A), bladder (B), colon (Co), intact testis (*), ureter (Ur), vas deferens (Vd) and gubernaculum (G).

<u>Magnification: x 12</u>


A
K


Ur
Co
B
T
*
Sc

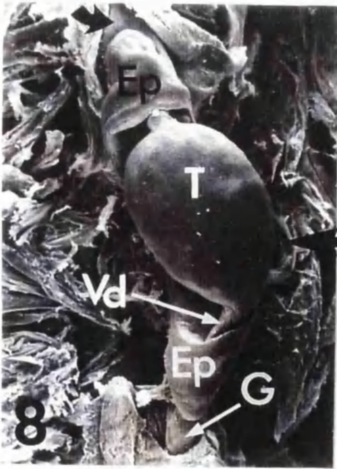

Ep
T
Vd
Ep
G
8

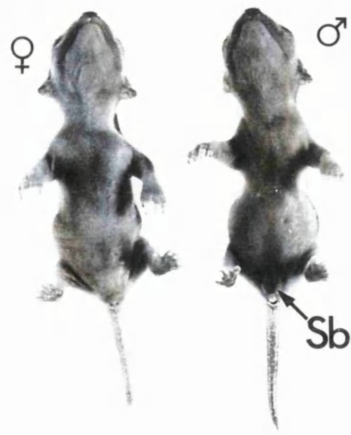
Fig. 3.9a-b. Young opossum at day 28:

a.) The external genitalia of female and male opossums shows scrotal bulges (Sb) in the male, but absent in the female.

b.) Scanning electron micrographs of the male opossum urogenital system shows testes (T) descended into their final position at the base of the scrotum and a fully developed epididymis (Ep).

Note kidney (K), adrenal (A), bladder (B), colon (Co). intact testis and scrotum (*) and vas deferens (Vd).

Magnification: x 12


♀
♂
Sb
9a

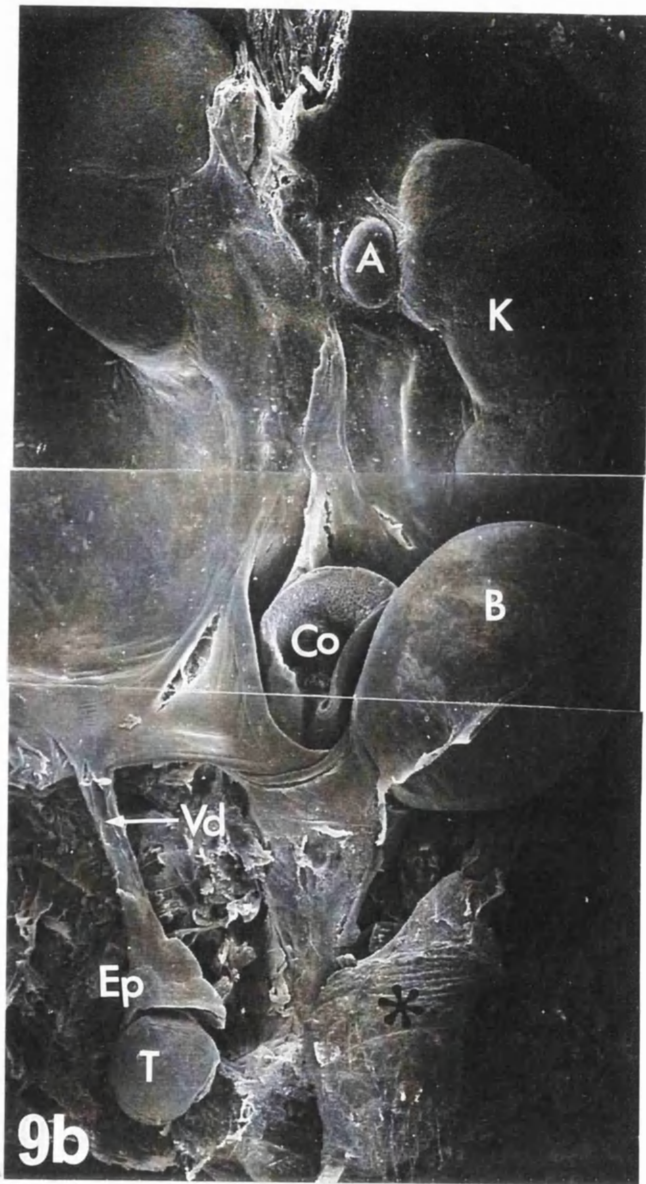
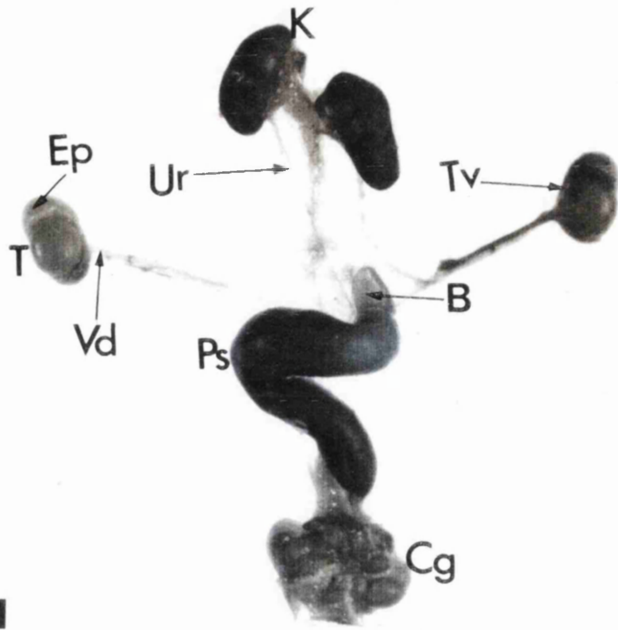

A
K
B
Co
Vd
Ep
T
*
9b

Fig. 3.10a-b. The gross anatomy of the adult male and female opossum reproductive system:

a.) Male opossum shows the characteristic large prostate (Ps) and Cowper glands (Cg).

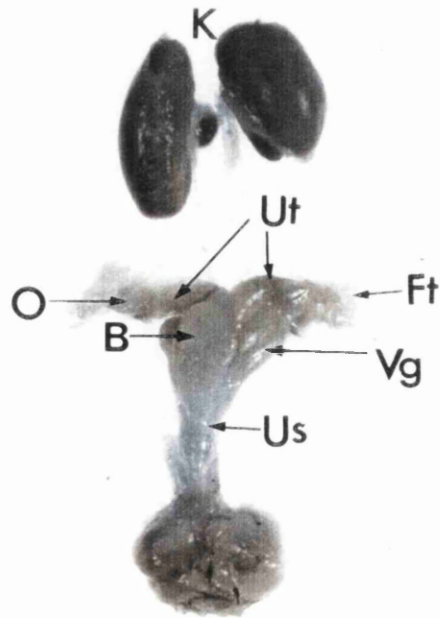
b.) Female opossum shows two uteri (Ut) and two vaginae (Vg).

Note kidney (K), bladder, ureter (Ur), testes (T), vas deferens (Vd); epididymis (Ep), tunica vaginalis (Tv), Fallopian tube (Ft) urogenital sinus (Us) and bladder (B).



10a

♀

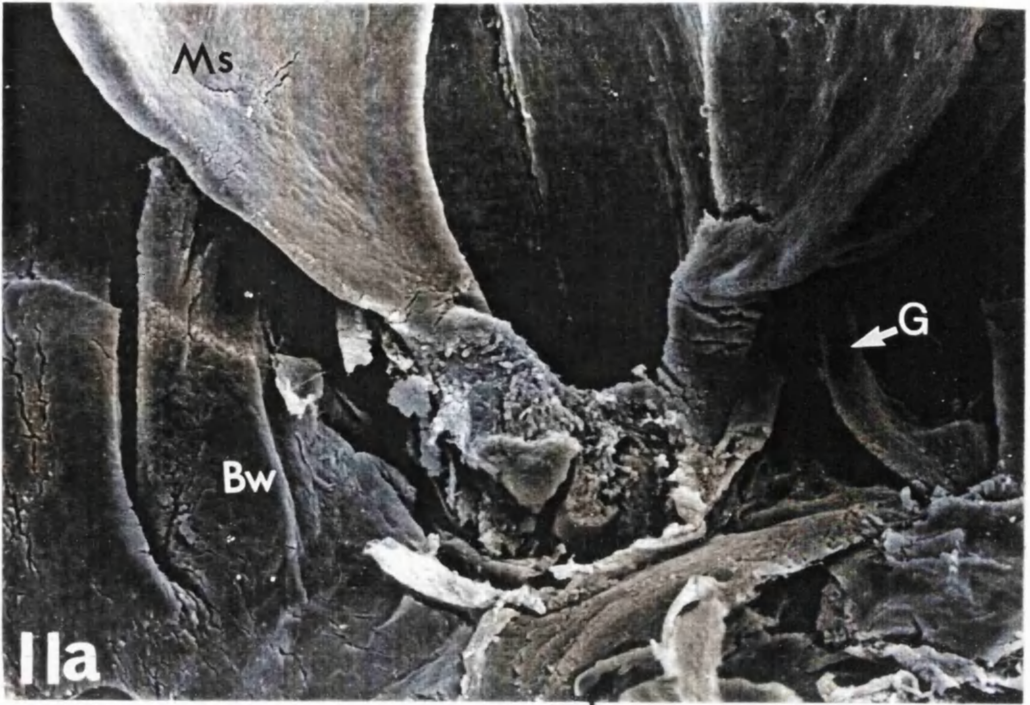


10b

Fig. 3.11a-b. Scanning electron micrographs of male (**a**) and female (**b**) gubernaculum on day 3.5 show the elongated male gubernaculum (G) extending further towards the body wall (Bw) when compared with the female. Note mesonephros (Ms).

a: Magnification: x 120

b: Magnification: x 200


Ms
G
Bw
11a

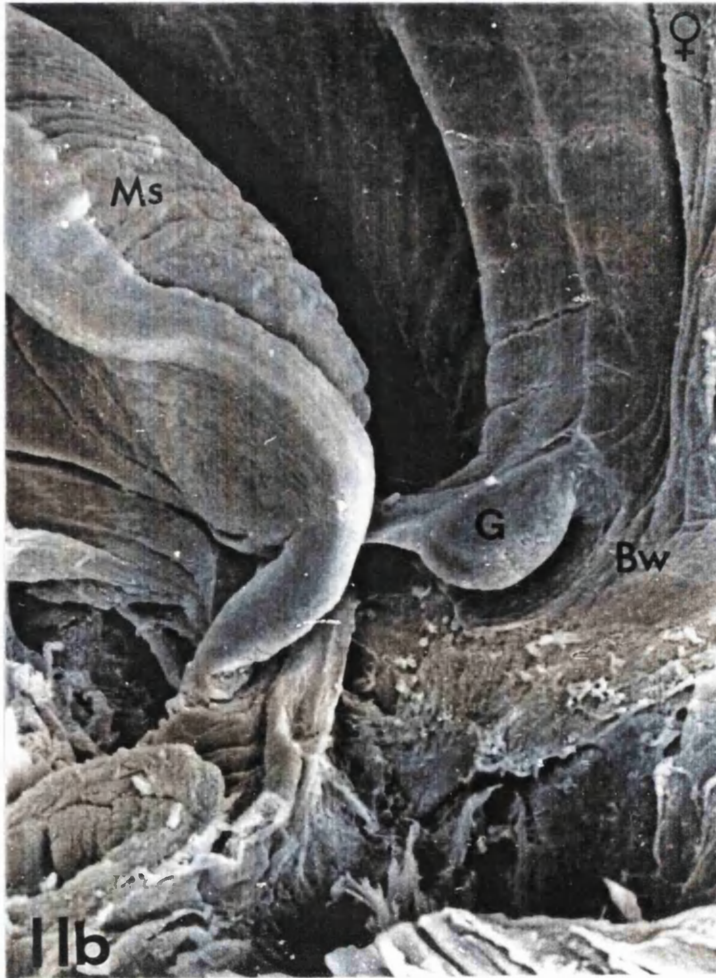
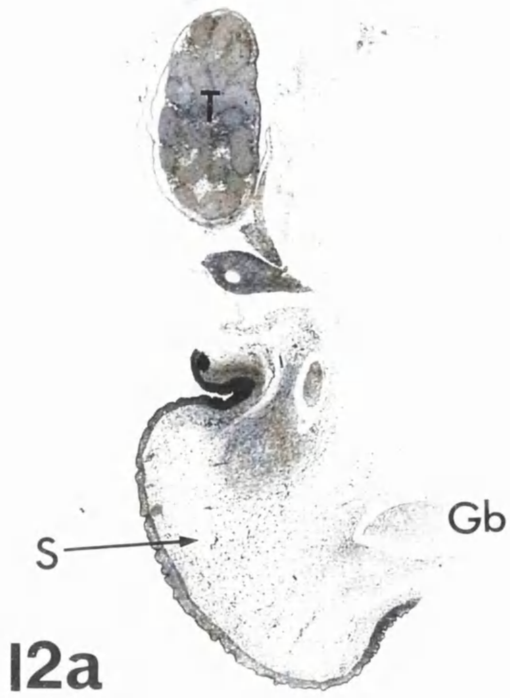

♀
Ms
G
Bw
11b

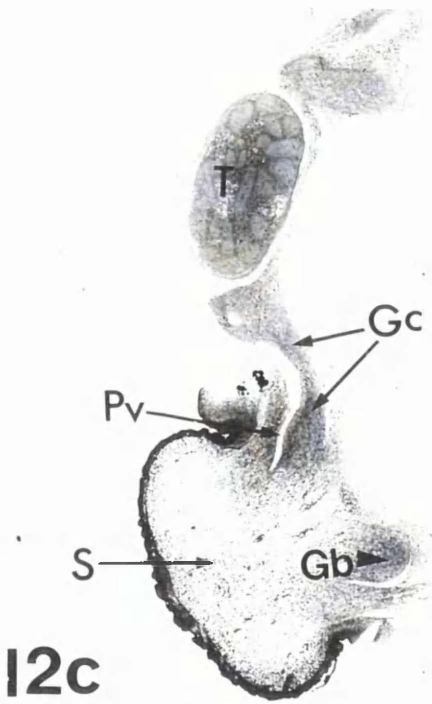
Fig. 3.12a-d. Longitudinal sections through the male opossum on day 15 shows the gubernaculum extending through the inguinal canal, developing myofibres (Mf) and mesenchyme cells (Mc).

Note gubernaculuar cord (Gc); gubernacular bulb (Gb); scrotum (S), and processus vaginalis (Pv).

Magnification: **a & c**: x 32; **b & d**: x 80.


T
Gb
S
12a

Mc
12b


T
Gc
Pv
S
Gb
12c

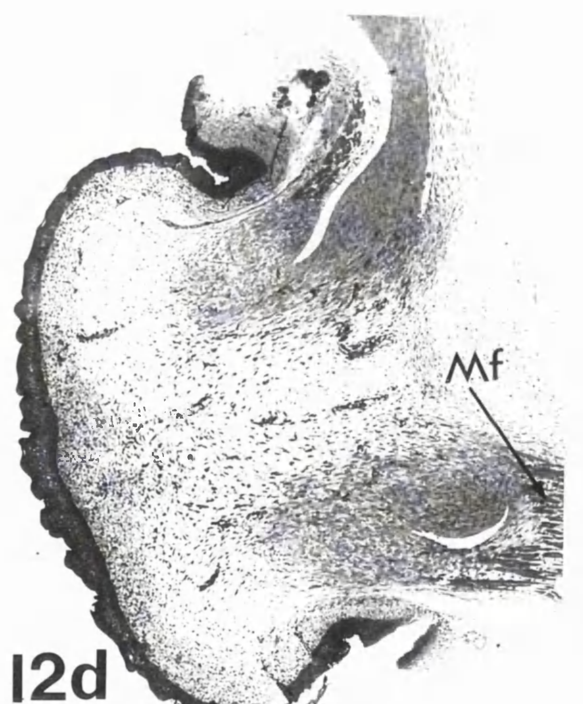
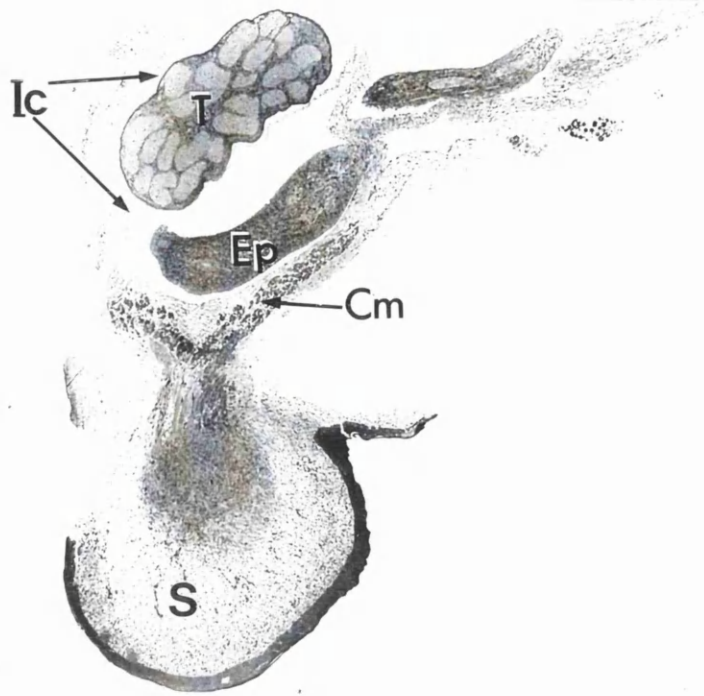

Mf
12d

Fig. 3.13a-b. Longitudinal sections through the male opossums on day 16:

a.) Low power micrograph of male opossum showing early differentiation of cremaster muscle (Cm) around the inguinal part of the gubernacular bulb.
Magnification: x 40.

b.) High power micrograph of gubernacular bulb showing developing myofibrs (Mf).
Magnification: x 80.

Note testis (T), inguinal canal (Ic), scrutum (S) and differentiation of epididymis (Ep).


Ic
T
Ep
Cm
S
13a

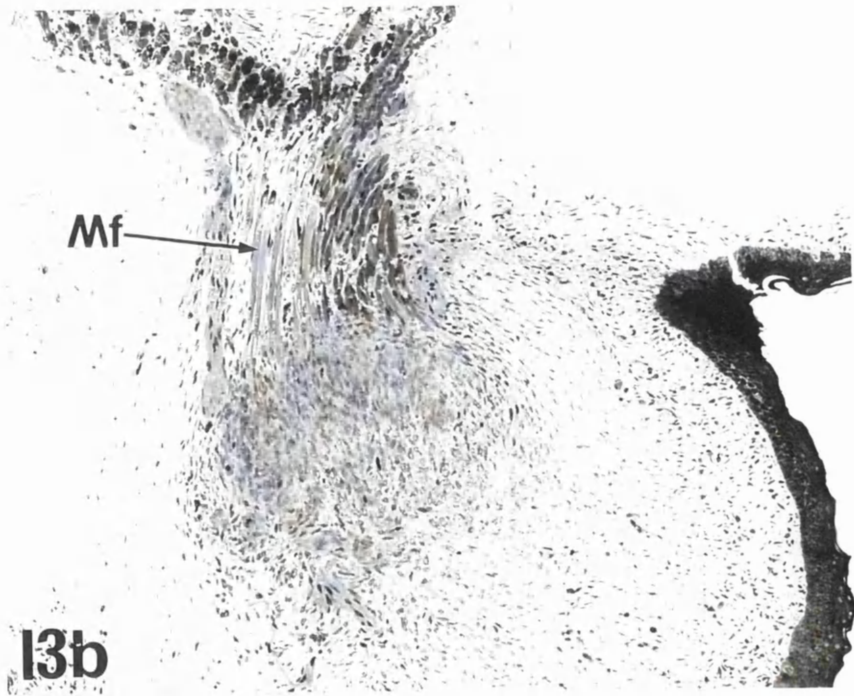
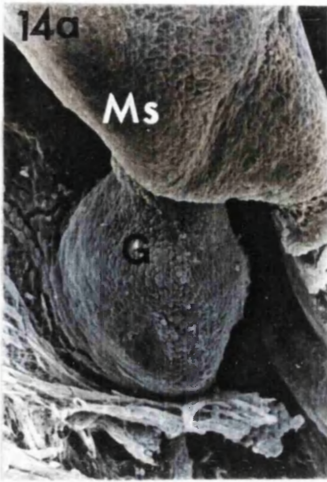
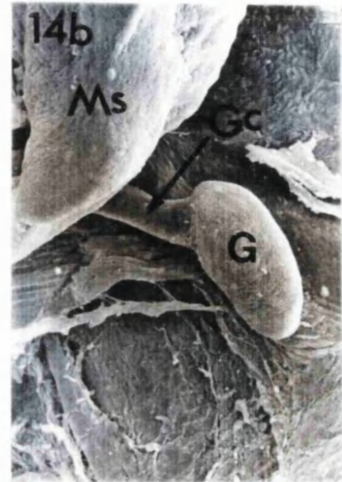

Mf
13b

Fig. 3.14a-f. Scanning electron micrographs of developing female gubernaculum:

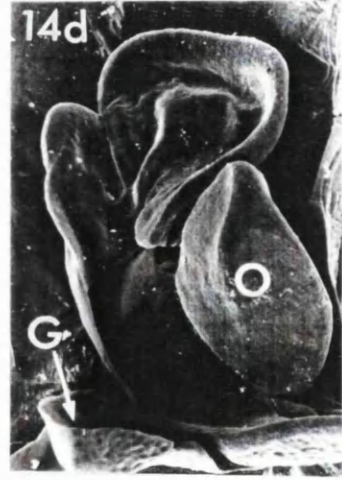
Day 5 (**a**) shows female gubernaculum bulb (Gb) connects with the ventral abdominal wall. Day 8 (**b**) showing female gubernaculum bulb (Gb) and cord (Gc). Further development of female gubernaculum on day16 (**c**), day 20 (**d**), day 28 (**e**) and day 35 (**f**).
Notes mesonaphros (Ms), ovary (O), ostium of the oviduct (arrows), Fallopian tube (Ft) and the uteri (Ut).

Magnification: **a & d**: x 220; **b & c**: x 110; **e:** x 170; **f:** x 200.


14a
Ms
G


14b
Ms
Gc
G

O
Ms
G

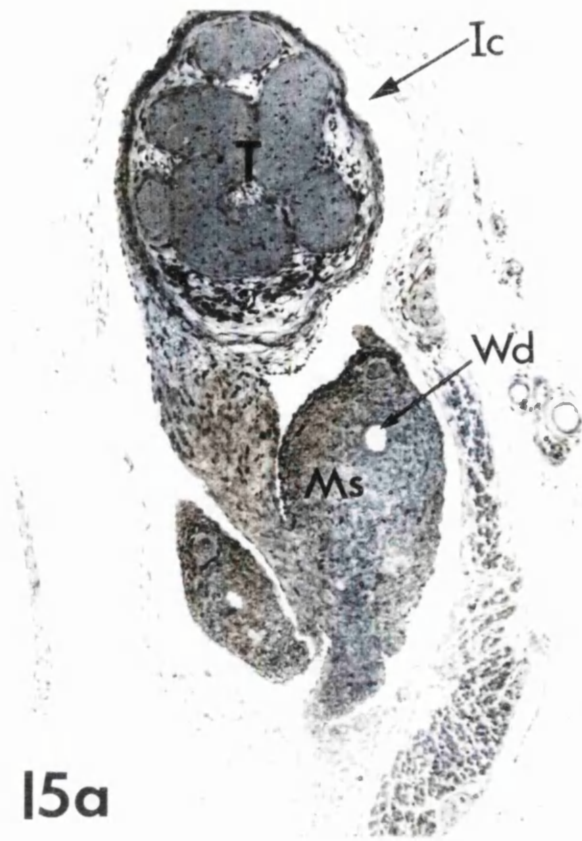

14d
O
G

14e
O
G

14f
Ft
Ut
G

Fig. 3.15a-b. Transverse sections through male opossums on day 16 (a) and day 17 (b) showing descent of both testis (T) and mesnephros (Ms) into the inguinal canal (Ic) and differentiation of the Wolffian duct (Wd) into epididymis.

Magnification: x 100.


Ic
T
Wd
Ms
15a

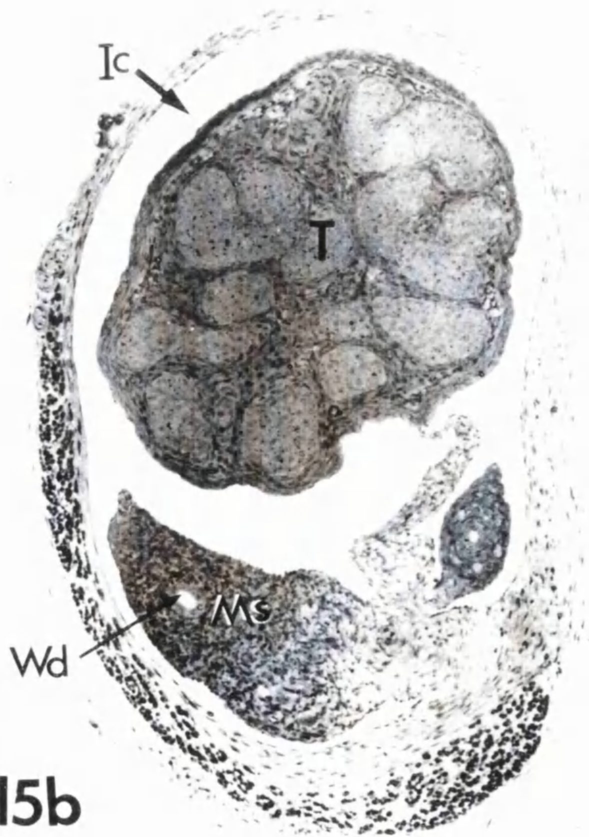

Ic
T
Ms
Wd
15b

Fig. 3.16a-d. Mouse urogenital complexes co-cultured with developing opossum testes:

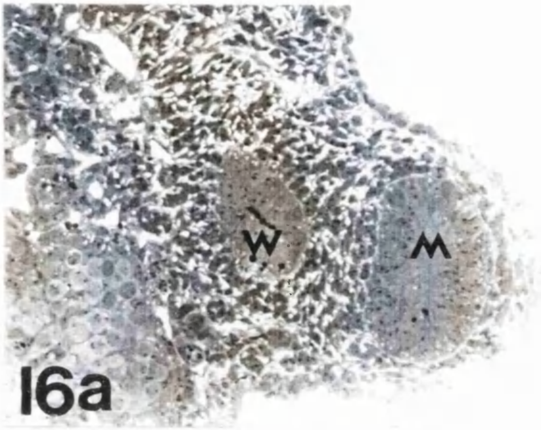
a.) A testis on day 7 causes no regression of the Müllerian duct (M).

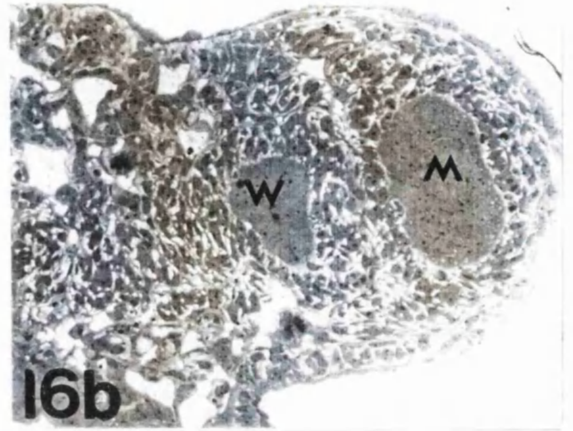
b.) Control experiment: An ovary induces no regression of the Müllerian duct (M) .

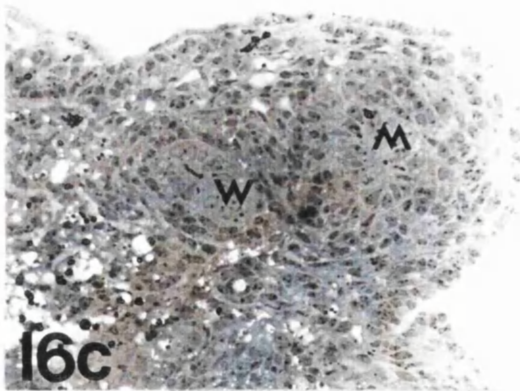
c.) A testis on day 15 causes Müllerian duct regression and also stimulates the Wolffian duct (W) when compared with control experiment.

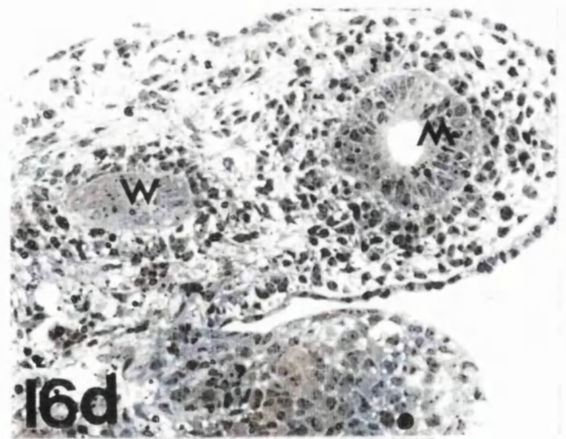
d.) Control experiment: An ovary induces no regression of the Müllerian duct (M). The Wolffian duct (W) has regressed in the absence of androgen.

Magnification: x 300.


W
M
16a


W
M
16b


W
M
16c


W
M
16d

Chapter 4: Immunocytochemical and Endocrinological Investigation of Postnatal Leydig Cell Development

4.1. Introduction

The course of sexual differentiation in male eutherians involves testis formation, followed by masculinisation of the reproductive tract, external genitalia and brain; much of this development occurs prenatally (Short, 1982; George & Wilson, 1994; Payne, 1996). The major androgen responsible for the masculinisation of the reproductive tract is testosterone, secreted by Leydig cells of the fetal testis (see chapter 1 p 10-11).

The enzymatic complex 3β-HSD, which is essential to the biosynthesis of forms of biologically active testosterone (Diagram. 4.1), can be readily detected by immunocytochemistry (Readhead *et al.*, 1983; Luu-The *et al.*, 1989; Dupont *et al.*, 1990a; 1990b; 1993). In eutherians, both enzyme histochemistry (Ziegler *et al.*, 1983; Haider *et al.*, 1986;) and immunocytochemistry (Dupont *et al.*, 1993) have shown 3β-HSD activity to be positively expressed in the mouse (13.5 dpc) and in the rat (15.5 dpc).

Testosterone is the principal gonadal androgen produced during marsupial sexual differentiation (Renfree *et al.*, 1992; Fadem & Harder, 1992). Gonadal testosterone in the Virginia opossum cannot be detected until day 10 (George *et al.*, 1985). In the tammar wallaby, gonadal testosterone levels are low in both sexes at birth, but in males they rise between days 2 to 10 with the formation of seminiferous tubules (Renfree *et al.*, 1992). Testosterone levels remain high in the testis until after day 40, by which time sexual differentiation of the internal genitalia is essentially complete (Renfree *et al.*, 1992; 1995).

Leydig cells can first be identified ultrastructurally in *Monodelphis* on postnatal day 3 (chapter 2). Surprisingly, Fadem and Harder (1992) reported that there were high levels of testosterone (comparable to adult male levels) in the peripheral plasma of newborn *Monodelphis*. However, there is no reported evidence of testicular hormone production at birth in either Australian (e.g. tammar wallaby: O *et al.,* 1988) or American marsupials (e.g. Virginia opossum: Burns, 1961a); moreover, Leydig cells cannot be distinguished prior to day 3 in *Monodelphis*, so the source of the testosterone measured by Fadem and Harder (1992) remains unclear.

The aim of the work described in this chapter is to shed further light on early sexual differentiation in *Monodelphis* by i) immunocytochemical identification of the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) involved in steroid hormone synthesis, and ii) measurement of androgen levels in the gonads, adrenal glands and peripheral plasma of both sexes.

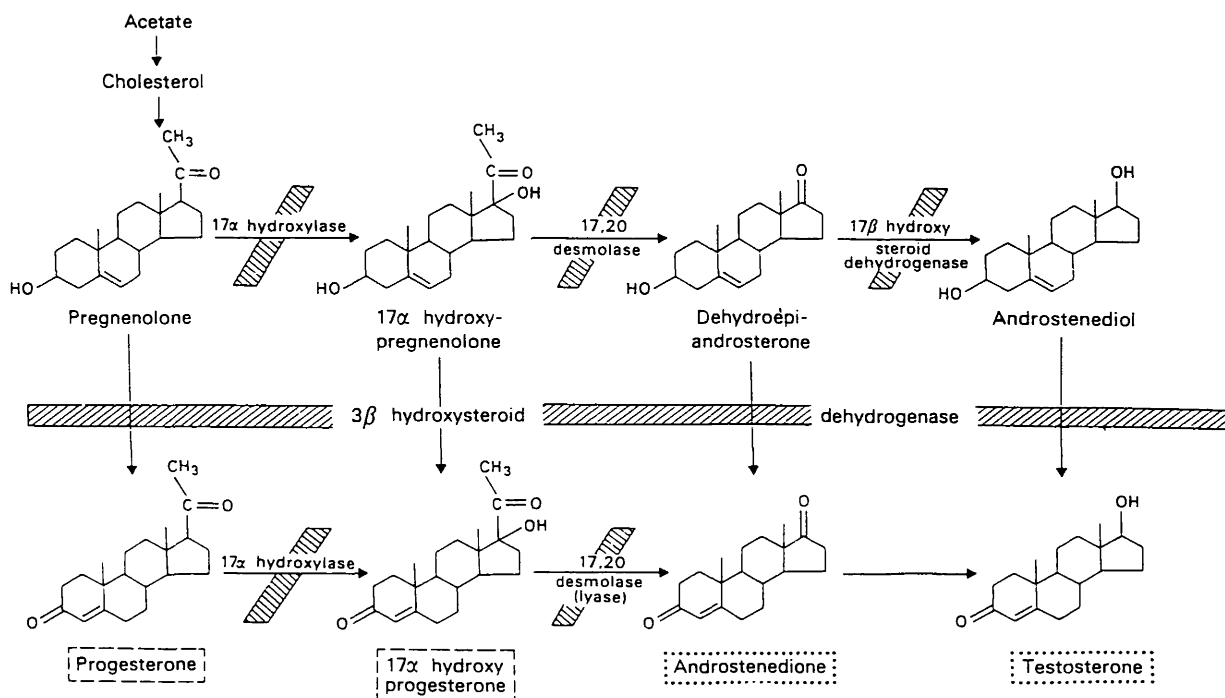


Diagram 4.1 Pathway of interconversion of steroid

4.2. Materials and Methods

4.2.1. Animals

The animals used in the present study were as described in chapter 2. For immunocytochemistry, male opossums of the following ages were used: (birth = day 0) days 3, 4, 5, 8, 16, 24 ; 4 weeks, 7-10 weeks; 3 - 3.5 months (pre-pubertal); 4 - 5 months (pubertal) and 1-3 years (adult). At least 3 animals were used at each age point (see Table 4.1). Animals were killed as chapter 2. For testosterone measurements, animals from day 5 to 4 weeks were decapitated and older animals from 7 weeks onwards were terminally anaesthetised.

4.2.2. Tissue Preparation

Testes were immersion- or perfusion-fixed with 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2-7.4) before being stored in fixative for over 24 hours at room temperature (20°C). Specimens were then rinsed in buffer for 10-30 minutes, dehydrated through an ascending ethanol series and embedded in paraffin wax at 57°C (Appendix 2).

4.2.3. Antibodies

The polyclonal rabbit anti-human placental 3β-HSD antibody was a gift from Prof. J.I. Mason (Department of Biochemistry, University of Edinburgh). Previous work has demonstrated cross-reactivity with *Monodelphis domestica* tissues (Ullmann *et al.*, 1995) (Appendix 5).

4.2.4. Immunocytochemistry

Immunostaining was carried out by the avidin-biotin technique on 5-7 μm paraffin sections (Appendix 5). Briefly, sections were deparaffinized and incubated in 0.1% hydrogen peroxide (H_2O_2) for 15-30 minutes to eliminate endogenous peroxidase activity. After rinsing in 0.01 M phosphate buffered saline (PBS), sections were treated with 10-20% normal goat serum (NGS) in PBS for 30-60 minutes to clear background staining, then incubated in a humidity chamber overnight at 4°C with the primary antibody (polyclonal rabbit anti-human placental 3β-HSD antibody). The primary antibody was diluted 1:1500 in PBS (containing 1% NGS + 0.3% X-100 triton). Sections were then washed in PBS and incubated at room temperature for 1-2 hours with secondary antibody (peroxidase-labelled goat anti-rabbit) at a dilution of 1:500 in PBS (containing 1% NGS and 0.3% X-100 triton). Peroxidase activity was revealed following a 5-10 minute incubation at room temperature in a medium containing 0.05% 3,3'-diaminobenzidine, 0.01% H_2O_2 and 0.02% $NiCl_3$ in 0.01 M phosphate buffer. The slides were counterstained with 0.5% methyl green, dehydrated and mounted in Histomount. Control experiments were performed on young and adult testis sections by substituting preimmunized 10-20% NGS in PBS instead of the primary antibody.

4.2.5. Blood, Gonadal and Adrenal Samples

Blood samples were obtained from the younger animals following decapitation and from animals seven weeks of age onwards by cardiac puncture (see Table 1). From day 5 to 4 weeks, the blood was collected in heparinized capillary tubes and pooled due to the very small volumes available (10-50 µl plasma/pools). From 7 weeks onwards blood was obtained from terminally anaesthetised individual animals by cardiac puncture with a heparinized syringe. Plasma was separated from chilled blood by centrifugation and stored in heparinized tubes at -20°C.

In animals up to 28 days of age, the gonads were dissected and pooled, as were the adrenals (see Table 4.2). Gonads from older animals were assayed individually. Upon removal, the organs were placed in 0.1 ml of 0.9% NaCl and homogenised. After centrifugation the supernatant was removed and stored at -20°C before being assayed.

The protein content of the tissue pellet remaining after centrifugation was determined from its absorbance at 595 nm after reaction with Coomassie Blue G250 (Pierce, Life Science Laboratories Ltd., Luton, UK). The weight of protein was calculated from a standard curve prepared using bovine serum albumin (Lowry *et al.*, 1951) (Appendix 6). The sample pellets were dispersed in 210 µl 0.1M NaOH and incubated overnight at 4°C. On the following day, duplicate 100 µl samples were transferred to two separate tubes and each treated with 0.9 ml of distilled water and 1ml of Coomassie Blue. The protein concentration in each tube was calculated by substitution of the absorbance into the regression equation of the standard curve (Table 4.3). The protein content of the duplicate tubes was summed to give the total protein content of the original sample. The testosterone concentrations were expressed as ng/mg protein.

4.2.6. Testosterone Measurements

Testosterone was measured in diethyl ether extracts of plasma and in the supernatant from the homogenised gonadal and adrenal tissue. Twenty five µl volumes of plasma were taken from the pooled samples; 50µl plasma volumes from the individual older opossums and from the supernatant and correction made for the different volumes taken. The assay was a double-antibody radioimmunoassay originally developed by Cook and Beastall (1987) for human studies and more

recently utilised by Gilmore *et al.* (1991) for studies in the sloth and by Kassim *et al.* (1997) for work on the rat (Appendix 7). The antiserum was raised against testosterone-3-(o-carboxymethyl)oxime-bovine serum albumin conjugate and ^{125}I-histamine-3-testosterone was used as tracer. The assay cross-reacts with 5α-dihydrotestosterone by 16%, 5α-androstane-3α,17β-diol by 5.8%, 5α-androstane-3β,17β-diol by 3.7%, androstenedione by 2.1%, dehydroepiandrosterone by 0.04% and cortisol by <0.01%. The intra- and inter-assay precision was calculated as coefficients of variation 8% and 12% respectively.

Testosterone was spiked into adult male opossum plasma samples at levels of 2 and 5 nanomoles/litre. Ninety percent recovery of added testosterone was achieved. Furthermore, adult male opossum plasma samples were analysed at doubling dilutions and diluted parallel to the standard curve used for the assay.

4.3. Results

4.3.1. 3β-HSD Immunocytochemistry

At birth, testicular cords in *Monodelphis* are composed of germ cells and Sertoli cells delimited by a basement membrane and surrounded by peritubular cells. Opossum Leydig cells are first distinguishable morphologically in the interstitial spaces by day 3 (chapter 1). This study of the immunolocalization of 3β-HSD during postnatal testis development shows that from days 3 - 8, no specific immunostaining for the enzyme is found in the interstitial tissue, but it is present in the testicular cords (Fig. 4.1a & b). By day 16, immunopositive staining is apparent in both the cytoplasm of the Leydig cells and in the testicular cords (Fig. 4.2). On day 24, immunostaining of the Leydig cells is more intense (Fig. 4.3). Staining intensity continues to increase between 7-12 weeks (Fig. 4.4); by contrast, the immunopositive staining within testicular cords declines during this period. By 4-5 months (puberty) immunostained Leydig cells reach peak numbers (Fig. 4.5) and some residual staining is still found peripherally in the testicular cords. In the adult opossum (1-3 years) positively-reacting cells are abundant in the interstitial tissue only (Fig 4.6a & b). In control incubations (where the primary antibody is omitted), no positive staining was found.

Table 4.1. Details of animals used for immunocytochemistry (ICC) and testosterone measurements*.

Age	No. of Animals for ICC	No. of plasma Samples for RIA
day 3-5	11 M	2 M + 2 F
day 8-10	4 M	2 M + 2 F
day 16	5 M	4 M + 3 F
day 24	4 M	1 M + 1 F
4 weeks	3 M	2 M
7-10.5 weeks	6 M	4 M
2-2.5 months	3 M	3 M
3-3.5 months	4 M	5 M
4 months	5 M	10 M
6 months		3 M
1-3 years	8 M	20 M + 10 F

*For testosterone measurement, blood samples were collected from a total of 78 animals from day 5 to 4 weeks of age. Plasma from animals up to 4 weeks was pooled. (male = M; female = F)

Table 4.2. Concentrations of gonadal and adrenal testosterone in the developing opossum*.

Age	Number of Gonads	Gonadal T in Supernatant (ng/mg protein)	Number of Adrenals	Adrenal T in Supernatant (ng/mg protein)
day 5 M	10 testes	0.1	8	<0.01
day 5 F	4 ovaries	<0.01	4	<0.01
day 8 M	6 testes	0.3	6	<0.01
day 8 F	6 ovaries	<0.01	6	<0.01
day 16 M	6 testes	0.45	14	<0.01
day 16 F	2 ovaries	<0.01	2	<0.01
day 28 M	5 testes	0.7	7	0.11
day 28 F	4 ovaries	< 0.01	4	0.11

*For the testosterone measurements samples from animals of the same sex and age were pooled. (M = Male, F = Female; T = Testosterone)

4.3.2. Testosterone Levels

Plasma testosterone concentrations during early postnatal development of the opossum (day 5 to 2.5 months) were uniformly below the level of detection i. e.

<0.3nmol/L. Testosterone levels in the male from week 4 onwards are illustrated in Fig.7. By four months testosterone levels had risen to 1.53 ± 0.35 nmol/L. After four months levels continued to increase steadily to 1.79 ± 0.4 nmol/L at six months and reached a peak of 2.71 ± 0.29 nmol/L in the adult (1 - 2 years).

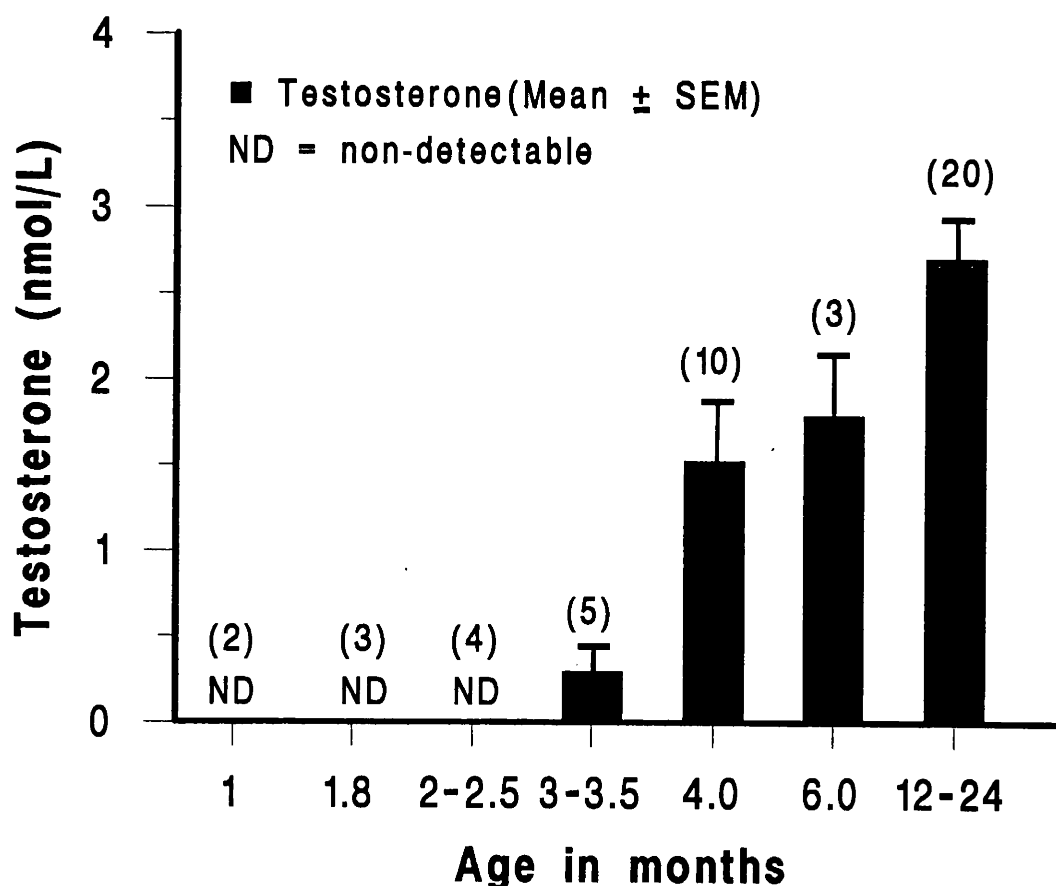


Fig. 4.7. Mean ± SEM testosterone concentrations in plasma of developing male opossums. Bars represent mean values of testosterone for animals aged from 1-24 months after birth. Number of pools or animals are shown in parentheses.

*ND = Non detectable (<0.3 nmol/L).

Gonadal and adrenal testosterone concentrations are shown in Table 4.2. Ovarian testosterone concentrations were uniformly low (<0.01 ng/mg protein). Testicular testosterone levels at day 5 were 0.1, at day 8 were 0.3, at day 16 were

0.45 and at day 28 were 0.7 ng/mg protein. The adrenal testosterone concentrations were uniformly low in both sexes until day 28.

4.4. Discussion

Although Leydig cells can be identified on the basis of ultrastructural features (numerous lipid droplets, abundant SER) on day 3 (chapter 2), 3β-HSD immunoreactivity is not detectable in the opossum testis before day 8. By day 16, positive staining is present in a few Leydig cells. This 3β-HSD reaction increases by day 24, reaches a peak at four months and remains maximal throughout adulthood. These data would therefore suggest that puberty is reached around four months, confirming the previous demonstration that sperm also first appear at this time (Xie *et al.*, 1995; 1996a). The achievement of puberty at this age is further confirmed by the rise in circulating testosterone levels.

In mammals, the main source of testicular androgen is the Leydig cells of the interstitial tissue (Christensen & Mason, 1965; Hall, 1994). However, some steroidogenic enzymes are also found within the seminiferous tubules (Hall *et al.*, 1969; Hall, 1994). Although both the seminiferous tubules and interstitial cells in rat testes are thus capable of converting progesterone to testosterone and androstenedione, the interstitial cells are considerably more efficient (Christensen & Mason, 1965; Hall *et al.*, 1969). Whole testis and interstitial cells are capable of converting cholesterol to androgens, whereas seminiferous tubules cannot synthesise androgens *de novo* from cholesterol; however, they are capable of converting more immediate precursors such as progesterone to testosterone (Hall *et al.*, 1969). In the Hokkaido brown bear, Tsubata *et al.*, (1993) reported that the cytochrome P450 enzymes i) cholesterol side-chain cleavage ($P450_{scc}$), ii) 17α-hydroxylase/C_{17-20} lyase ($P450_{17\alpha}$) and iii) aromatase ($P450_{arom}$) are localized in Leydig cells but that spermatids also stained very intensely in the testis. They suggested that steroidogenesis may occur not only in Leydig cells, but also in spermatids prior to the mating season and that Leydig cells and spermatids are the predominant sites of androgen and oestrogen synthesis respectively.

Surprisingly, during the first week after birth in *Monodelphis* positive immunostaining for 3β-HSD is present in the testicular cords, whereas the

interstitial tissue remains negative for this enzyme. This contrasts with studies in other species, such as the rat, in which 3β-HSD staining is restricted to Leydig cells (Dupont *et al.*, 1993). During subsequent development positive immunostaining in the opossum testicular cords declines gradually, being absent in the adult seminiferous tubules. This finding suggests that, in marsupials, there may be transient enzyme activity in the testicular cords, which disappears during development.

Sexual differentiation in eutherians is believed to be initially controlled genetically when the indifferent gonad is transformed into a testis or an ovary. Further differentiation of the Wolffian and Müllerian duct systems occurs under the influence of testicular hormones (Jost, 1947, 1953; Wilson *et al.*, 1981). The differentiation of Wolffian duct derivatives is caused by testosterone, secreted by the fetal Leydig cells (Jost *et al.*, 1963). In *Monodelphis*, this functional aspect of Leydig cells has been demonstrated by the activation of the 3β-HSD enzyme, which is first detected by day 16. Testicular androgen production in *Monodelphis* presumably starts around day 16 since Wolffian duct regression in the female begins at this time (see chapter 3). This result indicates the correlation between differentiation of the Wolffian duct and 3β-HSD enzyme synthesis by developing Leydig cells.

This study indicate that in *Monodelphis* testosterone synthesis at an early stage does not differ appreciably from that reported in other marsupials including the Virginia opossum (George *et al.*, 1985) and the tammar wallaby (Renfree *et al.*, 1992b). Although the testes are undoubtedly the major source of testosterone production throughout life, the adrenal glands have also been implicated as a source of this steroid in the eutherian fetus (Kime *et al.*, 1980). Fadem and Harder (1992) have suggested that the adrenals may also be a major source of androgen synthesis in the newborn opossum. However, this explanation is unlikely to hold since, as the present study shows, there is no evidence to indicate substantial testosterone secretion by the developing adrenal glands in either sex.

The presence of testosterone was undectable in peripheral plasma from *Monodelphis* until 3.5 months (pre-pubertal). However, by four months testosterone levels had risen significantly and reached a peak at adulthood Fadem & Harder (1992) reported that androgen levels in *Monodelphis* are measurable in the

circulation from the day of birth; curiously, they found levels to be significantly higher in mixed sex plasma pools from animals on day 4 than in adult males. In this study animals were readily sexed by external morphological features after day 3; prior to this, karyotyping is a useful confirmatory adjunct. Blood samples were obtained by pooling plasma from male and female animals separately and the testosterone levels were also measured separately in both sexes.

These results differ from those of Fadem and Harder (1992) in several respects. Firstly, no evidence was found to indicate testosterone levels at or above those of adult males in developing opossums of either sex. Secondly, Fadem and Harder (1992) found levels of testosterone from males on day 16 to be higher than those of adult males, whereas at this stage testosterone was undetectable in the peripheral plasma and even in the testes levels were low (0.45 ng/mg protein). However, testosterone levels from adults of both sexes were comparable in the two studies. Fadem and Harder's results are surprising because, if confirmed, extremely high testosterone levels in the female at the time of sexual differentiation would result in masculinisation unless the testosterone-sensitive tissues were in some way protected. The present findings are more consistent with those of previous studies in marsupials (George *et al.*, 1985; Renfree *et al.*, 1992b). Although the testosterone assay employed by Fadem and Harder exhibited more cross-reactivity with other steroids than did ours, this would be unlikely to fully explain the major discrepancy between the two studies. Moreover the authors themselves were unable to account for the very high levels of testosterone they measured in mixed sex plasma pools from animals 4 and 8 days of age.

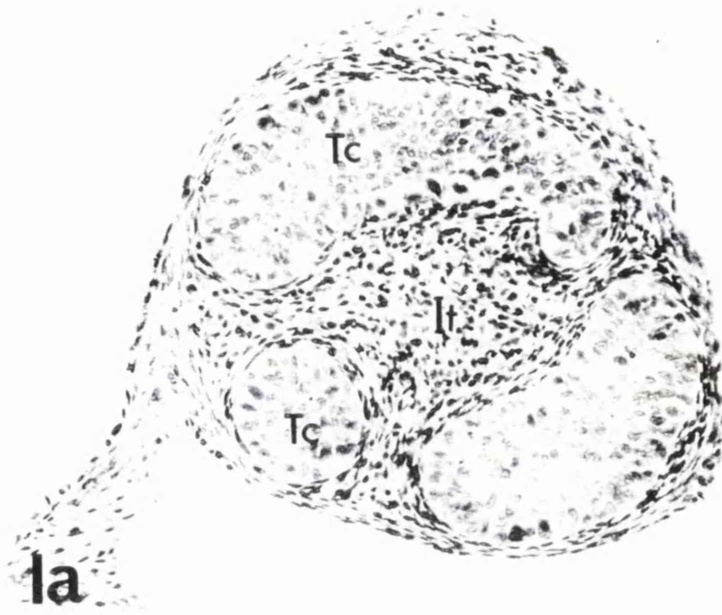
Previous work (chapter 2) described morphological differences between Leydig cells in early postnatal development and adulthood, the change occurring at about 3.5 months. However, it is unclear whether these differences betokened i) different populations of cells or ii) different stages of development of a single of population. The present work shows positive 3β-HSD staining from day 16, but does not resolve the continuing problem of Leydig cell differentiation. Inability to detect plasma testosterone until pre-puberty (3.5 months) can be explained by plasma testosterone concentrations being very low during early development.

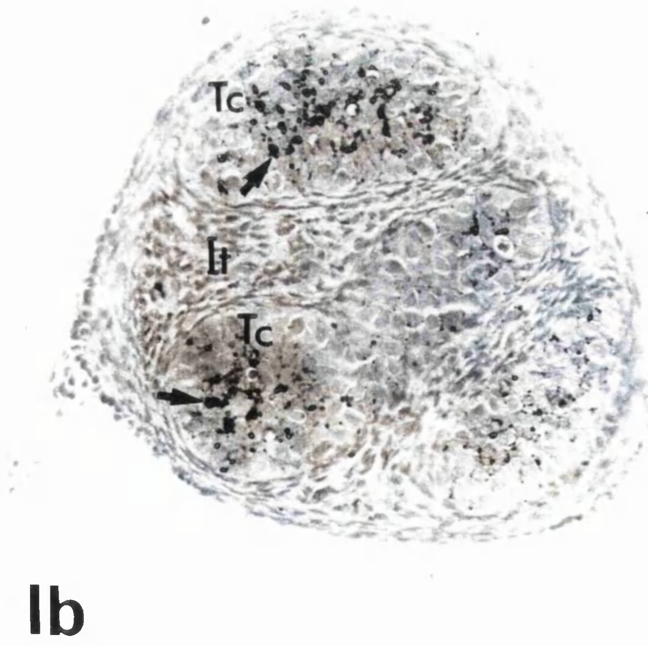
Fig. 4.1. Section through the testis on day 8:

a.) Stained with H & E showing testicular cords (Tc) and interstitial tissue cells (It)

b.) Immunostained for 3β-HSD showing the positive reaction (arrows) within the testicular cords (Tc) and the absence of 3β-HSD in the interstitial tissue (It).

Magnification: x 200.





Ib

Fig. 4.2. Section through the testis (day 16). Immunopositive staining has appeared in the interstitial tissue (arrowheads) and is still present in the testis cords (arrows).
Magnification: x 240.

Fig. 4.3. Section through the testis (day 24). Positive staining has increased in the interstitial tissue (arrows) and decreased in the testis cords (Tc).
Magnification: x 230.

2

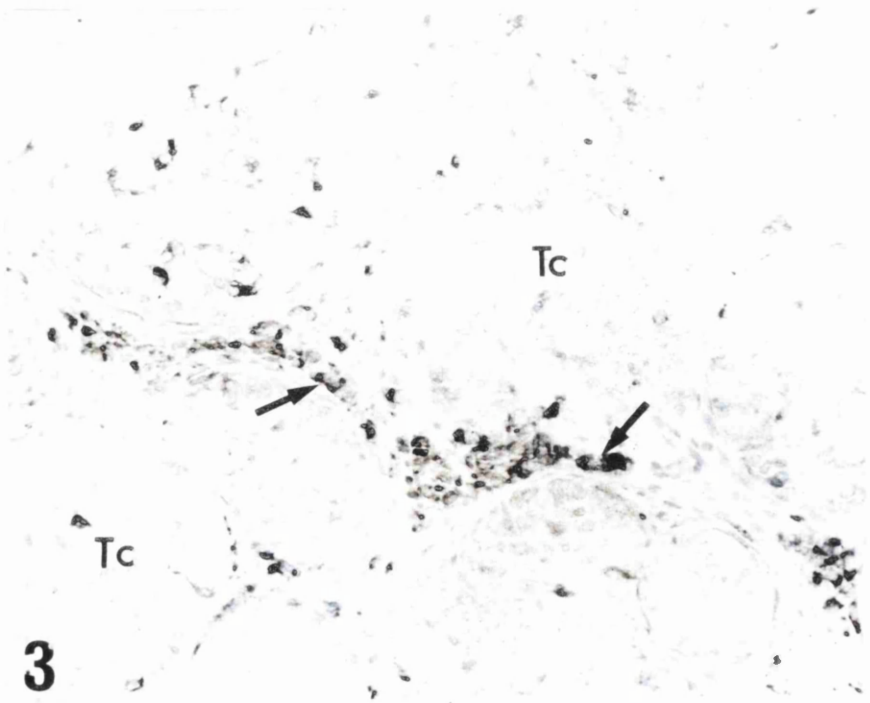
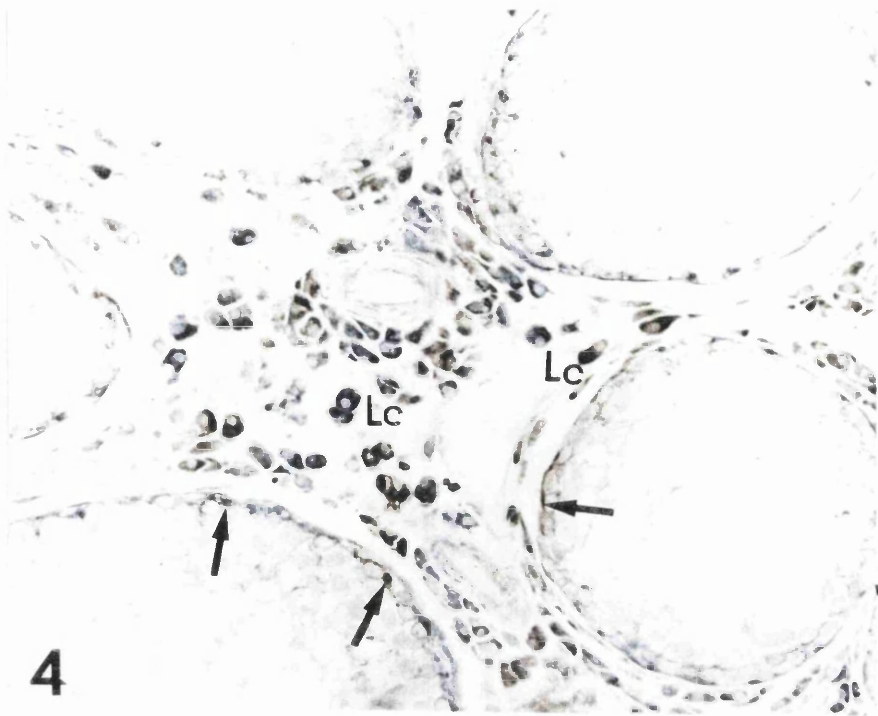

Tc
Tc
3

Fig. 4.4. Section through the pre-pubertal testis (12 weeks) showing immunostaining of Leydig cells (Lc) and at the periphery of the seminiferous tubules (arrow).

Magnification: x 200.

Fig. 4.5. Section through the pubertal testis (4 months). Note immunostaining in the numerous closely-packed Leydig cells and residual staining in the seminiferous tubules (arrow).

Magnification: x 200.


Lc
Lc
4

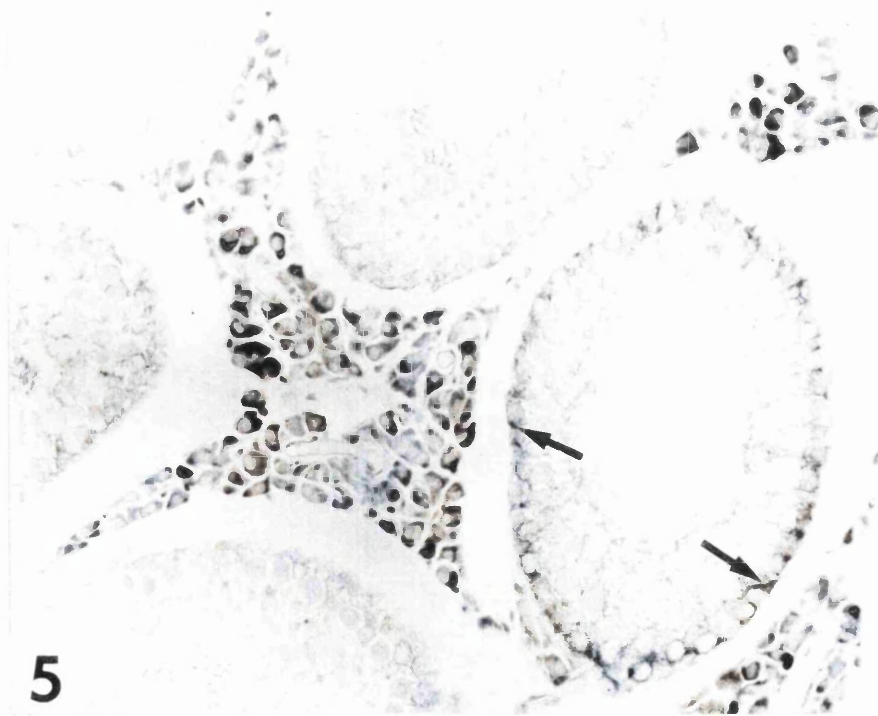
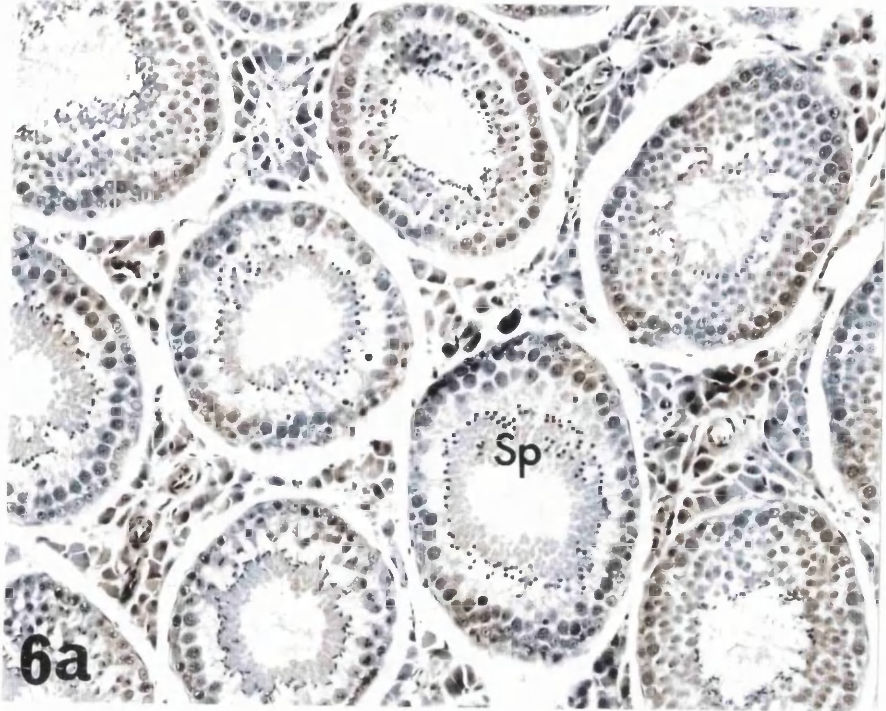
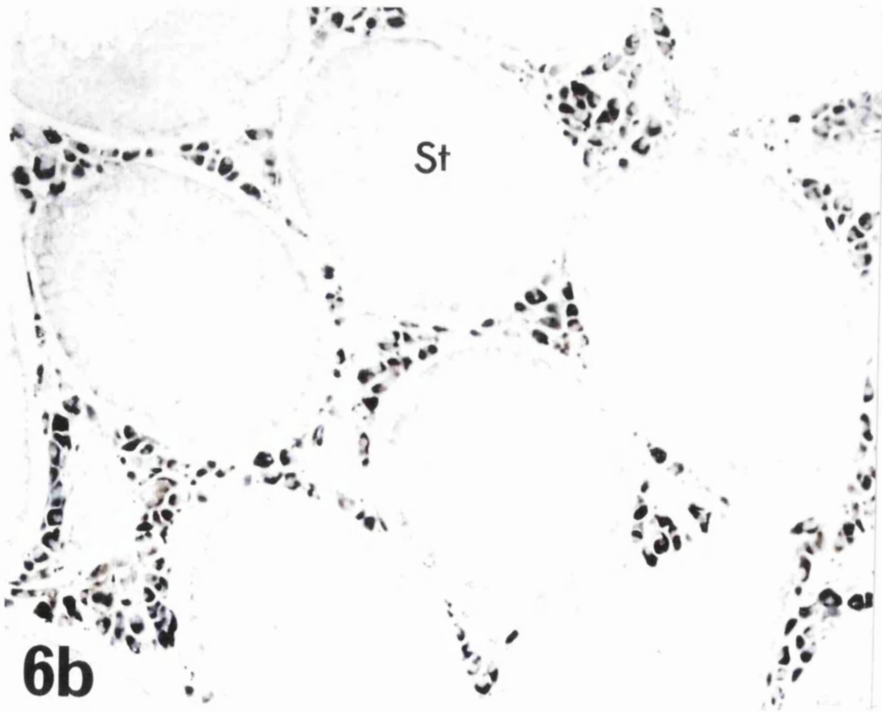

5

Fig. 4.6. Section through adult testis (1 year):

a.) stained with H & E showing abundant Leydig cells (Lc) and numerous spermatids (Sp).
Magnification: x 120

b.) staing for 3β-HSD, showing intense immunostaining in the Leydig cells and its absence in the seminiferous tubules (St).
Magnification: x 120


Sp
6a


St
6b

Chapter 5: General Discussion

The aim of the present study was to examine the sexual differentiation of the reproductive system in *Monodelphis domestica* since this marsupial has become an important model for the study of sexual differentiation. Previous individual chapters have included some discussion of the findings reported; the purpose of this chapter is to bring together major conclusions of this investigation and to make some recommendations for future research.

5.1. When do the Gonads Differentiate?

In marsupials, as in eutherians, gonadal differentiation is determined by the presence or absence of the testis-determining gene *SRY* on the Y-chromosome (Foster *et al.*, 1992) and the testis differentiates before the ovary (*Didelphis virginiana*: Baxter 1935; McCrady 1938, Burns 1939a, b; Moore 1939; the brushtail possum, *Trichosurus vulpecula*: Fraser, 1919; Ullmann 1993; the quoll, *Dasyurus viverrinus*: Ullmann, 1984; the bandicoot, *Isoodon macrourus*: Ullmann 1981; the tammar, *Macropus eugenii*: Renfree & Short 1988, the grey short-tailed opossum, *Monodelphis domestica*: Baker *et al.*, 1990; Moore & Thurstan, 1990; Fadem *et al.*, 1992; Maitland & Ullmann, 1993). The present study has resolved conflicting data as to whether gonadal differentiation in *Monodelphis* occurs prenatally (Baker *et al.*, 1990; Maitland & Ullmann, 1993) or postnatally (day 4: Fadem *et al.*, 1992; day 2: Moore & Thurstan, 1990). Testicular differentiation in *Monodelphis* is a truly perinatal phenomenon since only about 50% of XY gonads show differentiation at birth (Xie *et al.*, 1996a) and as defined by the emergence of testicular cord. This study represents the first careful examination by light, transmission and scanning electron microscopy of the developing gonad in karyotyped young pups in *Monodelphis*.

5.2. The Characteristics of Leydig Cells in *Monodelphis*

In eutherians, fetal and postnatal populations of Leydig cells have been well-documented (De Kretser & Kerr, 1988; Gondos *et al;*, 1974; Zirkin & Ewing, 1987). The course of sexual differentiation in phenotypic males involves masculinisation of the reproductive tract and this is controlled by testosterone, which is secreted by Leydig cells of the fetal testis (Jost, 1973). The postnatal population initiates puberty

and remains active during adult life (Zirkin & Ewing, 1987). However, the characterisation of Leydig cells in marsupials is poorly understood; indeed there is only one limited study, that in the brown marsupial mouse, (*Antechinus stuartii*) by Taggart *et al.*, 1993a) .

Leydig cells in *Monodelphis* can be first distinguished on postnatal day 3 by the expected ultrastructural features of steroidogenically active cells, i.e. abundant vesicles of SER, numerous lipid inclusions as well as by the formation of cell clusters surrounded by envelope cells. From 4 months onwards, Leydig cells lack lipid inclusions and show highly organised SER. These cells are closely packed, but no longer surrounded by envelope cells. In addition, unlike adult rats, the mesenchymal cells or fetal-type Leyig cells are not found in the adult *Monodephis*. The present extensive studies confirmed the earlier tentative identification of Leydig cells in *Monodelphis* on day 3 (Moore & Thurstan, 1990).

Although a morphological difference is evident in the Leydig cells of immature and mature animals, 2 separate and distinct populations (such as occur in eutherains) could not be distinguished. It is suggested that in *Monodelphis* fetal-type Leydig cells transform directly into adult-type cells by loss of lipid inclusions, loss of surrounding envelope cells and the development of highly organised arrays of SER.

5.3. The Developmental Time Course of Reproductive Tract Formation

The unusual reproductive anatomy of marsupials has been well-demonstrated in South American marsupials (virginia opossums, *Didelphis virginiana*: Cowper, 1740; Burns, 1945) and Australian species (Tyndale-Biscoe & Renfree, 1987). The marsupial urogenital system develops from an indifferent stage at birth, when both Wolffian and Müllerian ducts are present, to the phenotypically distinct male or female condition during early pouch life (for review see Renfree *et al.*, , 1996). During development, the caudal attachment of the ureter migrates ventrally to the neck of the urinary bladder and passes medially to the connection of the Wolffian and Müllerian ducts with the urogenital sinus (Buchanan & Fraser, 1918; Fraser, 1919; Tyndale-Biscoe & Renfree, 1987; Renfree *et al.*, 1994). Differentiation of the Wolffian and Müllerian ducts follows the normal eutherian pattern, and takes place after gonadal differentiation under the influence of gonadal hormones (Renfree *et al.*, 1996).

Monodelphis, like other marsupial species, is born with a fully functional mesonephros and at a sexually indifferent stage of urogenital system development, when the Wolffian duct is patent to the urogenital sinus. Furthermore, Müllerian ducts regression in the male occur earlier than the corresponding regression of Wolffian ducts in female.

Wolffian ducts first show evidence of regression in the female around day 15; presumably reflecting testosterone secretion in the male at this time and this is confirmed by the first detection of the enzyme 3β-HSD in Leydig cells at day 16 (Xie *et al.*, 1965; Xie *et al.*, 1997 *In Press*). In the male, regression of Müllerian duct occurs between days 12-16 after birth; although it has yet to be confirmed, the onset of MIS production presumably occurs prior to this period.

5.4. The Onset of Testosterone Production in *Monodelphis*

In well-documented marsupial species such as the tammar wallaby, the testes contain very little testosterone on the day of birth, but levels rise to 1 ng/mg protein by days 5-10 after birth. This is significantly different from ovarian testosterone which remains uniformly low (Renfree *et al.*, 1992a). The changing levels of fetal androgens may provide a greater understanding of the role of the different populations of Leydig cells in the development of the testis and differentiation of the male reproductive tract.

In *Monodelphis*, there is contradictory evidence on androgen availability during development. Fadem & Harder (1992) reported high levels of plasma testosterone in opossum pups of both sexes at birth, with testosterone levels by day 16 higher than in the adult male. The prsent immunocytochemical and endocrinolgical studies could not confirm these findings (Xie *et al.*, 1996b; Xie *et al.*, 1997 *In Press*). Only one population of Leydig cells is apparent in *Monodelphis* rather than the separate pre- and post-pubertal populations as in eutherians (Kerr & Knell, 1988). Patent seminiferous tubules with mature sperm appear by 4 months, the period when a) functional Leydig cells can be expressed immunocytochemically and ultrastructurally and b) peripheral testosterone begins to rise to adult levels. In conclusion, the pubertal period in *Monodelphis* is reached by 4 months.

5.5. Is Sexual Differentiation and Testicular Descent in *Monodelphis* Under Hormonal Control ?

Differentiation of phenotype poses particularly interesting questions in marsupials since it has been suggested that some aspects may not be hormonally controlled during development (O *et al.*, 1988, Renfree & Short, 1988; Renfree *et al.*, 1992). In eutherians, the formation of male sexual characteristics is dependent on the presence of hormones secreted by the embryonic testes. If gonads are removed from mammalian embryos, they will develop female characteristics, irrespective of their sex chromosome constitution (Jost, 1947).

In eutherian mammals the transabdominal phase of testis descent to the inguinal region is believed to be androgen-independent and regulated by MIS, while the subsequent inguinoscrotal phase is regulated by testosterone (Hutson, 1985). In marsupials, there is some evidence for a role for MIS in caudal migration in the tammar, since this coincides with high levels of testicular MIS production (Hutson *et al.*, 1988) and treatment with oestradiol, which blocks MIS action, prevents caudal migration (Shaw *et al.*, 1988, Moore & Thurstan, 1990). In *Monodelphis* these two phases of testicular descent broadly coincide with the timing of onset of MIS and testosterone production, thus reflecting the the regression of Müllerian ducts in males and Wolffian ducts in females.

The inguinoscrotal descent phrase in eutherians such as the rat appears to be dependent on androgen-induced calcitonin-gen-related peptide (CGRP) release from the genitofemoral nerves (Samarakkody and Hutson, 1992; Griffiths et al., 1993a, Goh *et al.*, 1993). However, the tammar gubernacula did not show any contractile response to CGRP added in vitro. Renfree *et al.* (1996) suggested that whether this reflects a fundamental difference in the control of testicular descent between marsupials and eutherians remains unclear. In *Monodephis*, the gubernaculum develops in both sexes; the function of the female gubernaculum is in suspensor support of the internal genitalia (van de Schoot *et al.*, 1996c). Development of the gubernaculum is not sexually dimorphic until 3.5 days after birth (this study).

The marsupial cremaster muscle presents an anomaly compared to eutherians in that it is well developed in both sexes possess (Tyndale-Biscoe & Renfree, 1987). In the male *Monodelphis*, this muscle is associated with the spermatic cord/scrotal sac as expected, but in the female it spans over the dorsal surface of part of the

mammary gland (van der Schoot *et al.*, 1996). The cremaster muscle is readily seen in this species on day 16 (this study), but it is not clear when the cremaster muscle differentiates in the two sexes or whether the period of differentiation coincides with a definable "androgenic" critical period.

Mammary primordia are present in both sexes at birth in American marsupials, although the male has fewer mammary glands than the female (e.g. *D. virginiana*: McCrady 1938; Burns, 1939; Renfree *et al.*, 1990 and *Monodelphis domestica*: Renfree *et al.*, 1990). In an extensive survey of Australian marsupials (such as the tammar wallaby, *Macropus eugenii*: Tyndale-Biscoe & Renfree, 1987), the mammary anlagen are found only in karyotypically female embryos (day 22 of gestation) (Renfree *et al.*, 1990). There is thus a fundamental distinction between American marsupials which have mammary primordia in both sexes and Australian marsupials in which no evidence of mammary anlagen could be found in the male (Bresslau, 1912, Renfree *et al.*, 1990). This could be explained by the long separation of marsupials into Australian and American groups. However, mammary glands and teats have never been described in any adult male marsupials (Tyndale-Biscoe & Renfree, 1987). Scrotal anlagen are only found in karyotypically male fetuses and cannot be induced in female tammars, even after prolonged treatment with androgen (Shaw *et al.*, 1988). In *Monodelphis* differentiation of the scrotal bulges occurs around postnatal day 2-3 (present observations).

The control of sexual differentiation in *Monodelphis* is influenced by genes, hormones and receptors, but the relative importance of these factors appears to be very different from that seen in eutherian mammals. Differentiation of the scrotum, mammary primordia, gubernaculum and cremaster muscle seem independent of androgens since testicular testosterone content is very low during early development. Although Leydig cells can be distinguished at day 3, 3β-HSD immunoreactivity is not detectable until day 16. Sexual differentiation in marsupials therefore differs from eutherians perhaps reflecting their long evolutionary separation for over 100 million years.

5.6. Future Work

5.6.1. Differentiation of Sertoli Cells in *Monodelphis*

In eutherians, the origin of the somatic components of the gonada remain controversial: potential contributors include the mesothelium, the mesonephros and the general mesenchyme. Current orthodoxy holds that the mesonephros is a major source of gonadal blastema cells (Byskov, 1986; Byskov & Hoyer, 1994). The steps involved in testis differentiation in marsupials and eutherians are broadly similar except for timing differences. The indifferent gonad forms medial to the mesonephros. Since the mesonephros is functional postnatally in marsupials (Tyndale-Biscoe & Renfree, 1987; Hughes & Hall, 1988) and believed not to be involved in the formation of gonadal blastema cells in some marsupials (Bandicoots: Ullmann, 1981; 1989; brushtail possum: Ullmann; 1996), it is still not clear whether the sex cords in *Monodelphis* derive from a mesothelial proliferation (Yoshinaga *et al.*, 1988), or from the mesenchymal cells of the mesonephros as in the eutherian (Byskov, 1986). An investigation of prenatal gonadal development in *Monodelphis* would be necessary to resolve this problems. The genesis of Sertoli cells is regarded as the first sign of male gonadal differentiation. This study has shown 50% of genetically male opossums to have sexually differentiated gonads present at birth with readily distinguished pre-Sertoli cells (Xie *et al.*, 1996a). Further work should investigate the initial apppearance of these cellls.

5.6.2 Leydig Cells and Androgen Production

Critical periods of sexual differentiation can be investigated by altering the hormonal environment and subsequently examining appropriate morphological, physiological or behavioural parameters. Where the timing of the critical period is not known it may often be deduced by evidence such as the appearance of fetal populations of active Leydig cells, detectable levels of androgens in the testis or plasma, steroid receptor densities in target tissues etc. In eutherian mammals, exposure of fetuses or neonates to ethylene-1,2-dimethane sulphonate (EDS) has a specifically cytotoxic action on Leydig cells with androgen production suppressed and Leydig cells killed within a few hours of administration (Bartlett *et al.*, 1986; Molenarr *et al.*, 1985; Sharpe *et al.*, 1986). EDS does not prevent Leydig cells reappearing in the adult testis and this has been used to examine development of a

second wave of Leydig cell genesis - perhaps from fibroblast-like precursors (Thomson & Lendon, 1988). Since it is hypothesised that only one population of Leydig cells are present in *Monodelphis*, the employment of EDS would resolve these problems and contribute to our understanding of Leydig cell characteristics in marsupial species.

5.6.3. MIS Detection

The functional status of pre-Sertoli cells, producing Müllerian inhibiting substance, has been assessed by the use of co-culture bioassay *in vitro*, MIS causes regression of the Müllerian duct in the male (Jost *et al.*, 1973; Wilson *et al.*, 1981; Short, 1982, Josso & Picard, 1986), actions on early gonad formation (Jost, 1972b; Jost & Magre, 1988) and may control testicular descent (Hutson *et al.*, 1988; Munsterberg & Lovell-Badge, 1991). The onset of MIS production in *Monodelphis* presumably occurs prior to day 12 since the male Müllerian duct starts regression at this time. However, MIS cannot be detected in *Monodelphis* until day 15, this may have been due to the small size of the gonad causing the dilution of MIS in the co-culture medium and is a limitation of the bioassay. The sequence of events involved in testicular cord formation could be investigated by an *in vitro* approach, radioimmunoassay or immunostaining for cell adhesion molecules. Unfortunately the anti-Müllerian hormone antibody (a polyclonal antibody raised in a rabbit against human recombinant AMH) used in the current work did not crossreact with *Monodelphis*.

Adam E, Bunce CE, Saunders NR and Snart C (1988) *Monodelphis domestica* (grey short-tailed opossum): a model for studies of very early stages of development. *Journal of Physiology* **400**: 13

Alcorn GT (1975) Development of the ovary and urino-genital ducts in the tammar wallaby *Maropus eugenii* (Desmarest, 1817). Thesis, Macquarie University, Sydney

Backhouse KM (1964) The gubernaculum testis Hunteri, testicular descent and maldescent. *Annals of the Royal College of Surgeons* **35**: 27

Baggott LM, Davis-Butler S and Moore HDM (1987) Characterisation of oestrus and timed collection of oocytes in the grey short-tailed opossum, (*Monodelphis domestica*). *Journal of Reproduction and Fertility* **79**: 105-114

Baker PJ, Moore HDM, Burgess AMC and Mittwoch U (1993) Gonadal sex differentiation in embryos and neonates of the marsupial, *Monodelphis domestica*: arrest of testis development in postterm embryos. *Journal of Anatomy* **182**: 267-273

Baker PJ, Moore HDM, Penfold LM, Burgess AMC and Mittwoch U (1990) Gonadal sex differentiation in the neonatal marsupial *Monodelphis domestica*. *Development* **109**: 699-704

Baker TG (1972) Primordial germ cells. In: *Reproduction in Mammals* (1st edn) (eds: CR Austin & RV Short) pp. 1-13 Cambridge: Cambridge University Press

Baker TG (1982) Oogenesis and ovulation. In: *Reproduction in Mammals,* (2nd edn), vol. 1 (eds: CR Austin & RV Short) pp. 17-45 Cambridge: Cambridge University Press

Baker TG, Metcalfe SA and Hutson JM (1990) Serum levels of Müllerian inhibiting substance in boys from birth to 18 years, as determined by enzyme immunoassay. *Journal of Clinical Endocrinology and Metabolism* **70**: 11-15

Barnes RD (1968a) *Marmosa mitis*, a small marsupial for experimental biology. In: *Symposium on Animal Models for Biomedical Research, Animal Models for Biomedical Research.* Publication No, 1594. pp. 88-97. (Natl Acad. Sci., Washington, DC)

Barnes RD (1968b) Small marsupials as experimental animals. *Laboratory Animal Care* **18**: 251-257

Barnes RD and Barthold SW (1969) Reproduction and breeding behaviour in an experimental colony of *Marmosa mitis* Bangs (*Didelphidae*). *Journal of Reproduction and Fertility Supplement* **6**: 477-482

Bartlett JMS, Kerr JB and Sharpe RM (1986) The effect of selective destruction and regeneration of rat Leydig cells on the intratesticular distribution of testosterone and morphology of the seminiferous epithelium. *Journal of Andrology* **7**: 240-253

Baxter JS (1935) Development of the female genital tract in the American opossum. *Carnegie Institute Contributions to Embryology* **25**: 15-35

Beddard JM (1891) On the pouch and brain of the male *Thylacinus. Proceedings of the Zoological Society of London* **1891**: 138-145

Benarous R Guerrier D, Josso N and Kahn A (1986) Cloning and expression of cDNA for anti-Müllerian hormone. *Proceedings of the National Academy of Sciences* USA **83**: 5464-5468

Bentley PJ and Shield JW (1962) Progesterone-induced development of dormant blastocysts in the tammar wallaby, *Macropus eugenii* Desmarest: Marsupialia. *Journal of Reproduction and Fertility* **20**: 201-210

Bezard J, Vigier B, Tran D, Mauleon P and Josso N (1987) Immunocytochemical study of anti-Müllerian hormone in sheep ovarian follicles during fetal and post-natal development. *Journal of Reproduction and Fertility* **80**: 509-516

Billingham RE and Silvers WK (1960) Studies on tolerance of the Y chromosome antigen in mice. *Journal of Immunology*, **85**: 14-26

Black VH and Christensen AK (1969) Differentiation of interstitial cells and Sertoli cells in fetal guinea pig testes. *American Journal of Anatomy* **124**: 211-238

Blanchard MG and Josso N (1974) Source of the anti-Müllerian hormone synthesised by the fetal testis: Müllerian-inhibiting activity of fetal bovine Sertoli cells in tissue culture. *Pediatric Research* **8**: 968-971

Blecher ST and Wilkinson LJ (1989) Non hormone-mediated sex chromosomal effects in development: another look at the Y chromosome-testicular hormone paradigm. In: *Evolutionary Mechanisms in Sex Determination* (ed: SS. Wachtel) chapter 21, pp. 219-229 Baca Raton, Florida: CRC Press

Bouin and Ancel (1903) Recherches sur les cellules interstitielles du testicule des mammiferes. *Arch. Zool. Exp. Gen.* **1**: 437-523

Bresslau E (1912) Die Entwickelung des Mammarapparates der Monotremen, Marsupialier und einiger Placentalier. In: *Zoologische Forschungsreisen in Australien,* vol. 4, (ed: R Semon) pp. 653-874 Jena: Gustav Fischer

Buchanan G and Fraser EA (1918) The development of the urogenital system in the Marsupialia, with special reference to *Trichosurus vulpecula*. *Journal of Anatomy* **53**: 35-95

Buehr M, Gu S and McLaren A (1993) Mesonephric contribution to testis differentiation in the fetal mouse. *Development* **117**: 273-281

Burnett (1830) In: Walker's Mammals of the World. vol. 1. 5th edition (1991). (ed: RM Nowak) pp.16 Baltimore & London: The John Hopkins University Press

Burns RK (1939a) The differentiation of sex in the opossum (*Didelphys virginiana*) and its modification by the male hormone testosterone propionate. *Journal Morphology* **65**: 79-119

Burns RK (1939b) Sex differentiation during the early pouch stages of the opossum (Didelphy virginiana) and a comparison of the anatomical changes induced by male and female sex hormones. *Journal of Morphology* **65**: 497-545

Burns RK (1939c) Effect of testosterone propionate on sex differentiation in pouch young of opossum. *Proceedings of the Society for Experimental Biology* **41**: 60-62

Burns RK (1939d) Effect of female sex hormones in young opossums. *Proceedings of the Society for Experimental Biology* **41**: 270-272

Burns RK (1945) The differentiation of the phallus in the opossum and its reaction to sex hormones. *Contrib Embryol Canegie Inst Wash* **31**: 147-162

Burns RK (1961a) Role of hormones in the differentiation of sex. In: *Sex and Internal Secretions.* (3rd Edn), (ed: WC. Young) pp. 76-158 Baltimore: Williams & Wilkins

Burns RK (1961b) Hormones in the differentiation of sex. In: *Sex and Internal Secretions.* (ed: WC Young), London: Tindall & Cox Ltd.

Butcher L (1995) Ovarian follicle growth and yolk formation in the New World marsupial *Monodelphis domestica* Thesis University of Glasgow U. K.

Byskov AG (1979) Regulation of meiosis in mammals. *Ann Biol Anim Biochim Biopys* **19**: 1251-1261

Byskov AG (1986) Differentiation of mammalian embryonic gonads. *Physiology Review.* **66**: 71-117

Byskov AG and Hϕyer PE (1994) Embryology of mammalian gonads and ducts. In: *The Physiology of Reproduction* (eds: E. Knobil, J. Neill et al.) chapter 9, pp. 487-540. New York: Raven Pres

Cate RL and Wilson CA (1993) Müllerian-Inhibiting Substance. *Genes in Mammalian Reproduction* **185:** 185-205

Cate, RL, Donahoe, Pk and Maclaughlin, DT (1990) Müllerian inhibiting substance. In: *Peptide growth factors and their receptors, vol. II* (eds: MB Sporn & AV Roberts) pp. 179-210. Berlin: Springer-Verlag

Cavicchia JC and Sacerdote FL (1991) Correlation between blood-testis barrier development and onset of the first spermatogenic wave in normal and in busulfan-treated rats: a lanthanum and freeze-fracture study. *The Anatomical Record* **230**: 361-368

Chase EB (1939) The reproductive system of the male opossum, *Didelphis virginiana* Kerr and its experimental modification. *Journal of Morphology* **65**: 215-240

Christensen AK and Fawcett DW (1961) The normal fine structure of opossum testicular interstitial cells. *The Journal of Biophysical and Biochemical Cytology* **9**: 653-670

Christensen AK and Mason NR (1965) Comparative ability of seminiferous tubules and interstitial tissue of rat testes to synthesise androgens from progesterone-4-^{14}C *in vitro. Endocrinology* **76**: 646-656

Clemens WA (1968) Origin and early evolution of marsupials. *Evolution* **22**: 1-18

Clermont Y and Huckins C (1961) Microscopic anatomy of the sex cords and seminiferous tubules in growing and adult male Albino rats. *American Journal Anatomy* **108**: 79-97

Cook B and Beastall GH (1987) Measurement of steroid hormone concentrations in blood, urine and tissues. In: *Steroid Hormones a Practical Approach.* (eds: B Green & RE Leeke) pp.1-65 Oxford:IRL Press

Cooper DW (1993) The evolution of sex determination, sex chromosome dimorphism, and X-inactivation in eutherian mammals: a comparison of metatherians (marsupials) and eutherians ('placentals'). In: *Sex chromosomes & sex determining genes* (eds: KC Reed & JA Marshall-Graves), pp. 183-200 Harwood Academic Publishers, GmbH, Chur, Switzerland

Cothran EG, Aivaliotis MJ and VandeBerg, JL (1985) The effects of diet on growth and reproduction in gray short-tailed opossums. (*Monodelphis domestica*). *Journal of Experimental* Zoology. **236**: 103-114

Cowper W (1740) II Carigueya, seu Marsupiale Americanum Masculum or, The Anatomy of a male opossum: in a letter to Dr Edward Tyson from Mr William Cowper. *Philosophical Transactions of the Royal Society* **24**: 1565-1590

Crisp EA, Messer, M and Vandberg JL (1989) Changes in milk carbohydrates during lactation in a Didelphid marsupial, *Monodelphis domestica*. *Physiological Zoology* **62**: 1117-1125

Davis RM (1981) Localisation of male determining factors in man: a thorough review of structural anomalies of the Y chromosome. *Journal of Medical Genetics* **18**: 161-195

De Kretser DM and Kerr JB (1988) The cytology of the testis. In: *The Physiology Of Reproduction*, (ed. E. Knobil. & J. Neill), pp. 837-932. London & New York: Raven Press

Duesberg J (1919) On the interstitial cells of the testicle in *Didelphis*. *Biological Bulletin* **15**: 175-194

Dupont E, Labrie F, Luu-The V and Pelletier G (1993) Ontogeny of 3β-hydroxysteroid dehydrogenase / Δ^5 - Δ^4 - isomerase (3β- HSD) in rat as studied by immunocytochemistry. *Anatomy and Embryology* **187**: 583-589

Dupont **E, Luu-The V, Labrie F and Pelletier G** (1990a) Light microscopic immunocytochemical localization of 3β-hydroxy-5-ene-steroid dehydrogenase / Δ^5 - Δ^4 - isomerase (3β-HSD) in the gonads and adrenal glands of the guinea pig. *Endocrinology* **126**: 2906-2909

Dupont E, Zhao H-F, Rheaume E, Simard J, Luuthe V, Labrie F and Pelletier G (1990b) Localization of 3β-hydroxysteroid dehydrogenase / Δ^5 - Δ^4 - isomerase in the rat gonads and adrenal glands by immunocytochemistry and *in situ* hybridization. *Endocrinology* **127**: 1394-1403

Dyche WJ (1979) A comparative study of the differentiation and involution of the Müllerian duct and Wolffian duct in the male and female fetal mouse. *Journal of Morphology* **162:** 175-210

Dym M and Fawcett DW (1970) The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. *Biology of Reproduction* **3**: 308-326

Eichwald EJ and Silmser CR (1955) Untitled communication. *Transplantation Bulletin* **2**: 148-149

Enders RK (1966) Attachment, nursing and survival of young in some didelphids. *Symposia of the Zoological Society of London* **15**: 195-203

Etgen AM and Fadem BH (1987) Estrogen binding macromolecules in hypothalamus-preoptic area of male and female gray short-tailed opossums (*Monodelphis domestica*). *Gen Comp Endocrinol* **66**: 441-446

Evans EP (1987) Karyotyping and sexing of gametes, embryos and fetuses and in situ hytndisation to chromosomes In: *Mammalian Development A Practical Approach*, (ed. M. Mont), pp. 93-114. Oxford: IRL Press

Fadem BH (1985) Evidence for the activation of female reproduction by males in a marsupial, the grey short-tailed opossum (*Monodelphis domestica*). *Biology of Reproduction* **33:** 112-116

Fadem BH and Erskine MS (1987) Estradiol and testosterone in the blood of gray short-tailed opossums *Monodelphis domestica. Biology of Reproduction* **36(Suppl. 1):** 128

Fadem BH and Harder JD (1992) Evidence for high levels of androgen in peripheral plasma during postnatal development in a marsupial: the gray short-tailed opossum (*Monodelphis domestica*). *Biology of Reproduction* **46**: 105-108

Fadem BH and Rayve RS (1985) Characteristics of the oestrous cycle and influence of social factors in grey short-tailed opossums (*Monodelphis domestica*). *Journal of Reproduction and Fertility* **73**: 337-342

Fadem BH Hill HZ, Huselton CA and Hill GJ (1988) Transplantation, growth, and regression of mouse melanoma xenografts in neonatal marsupials. *Cancer Investigation* **6**: 403-408

Fadem BH, Tesoriero JV and Whang M (1992) Early differentiation of the gonads in the grey short-tailed opossums (*Monodelphis domestica*). *Biology of the Neonate* **61**: 131-136

Fadem BH, Trupin GL, Maliniak E, VandeBerg JL and Hayssen V (1982) Care and breeding of the gray short-tailed opossum (*Monodelphis domestica*). *Laboratory Animal Science* **32**: 405-409

Fadem, BH & Tesoriero, JV (1986) Inhibition of testicular development and feminization of the male genitalia by neonatal estrogen treatment in a marsupial. *Biology of Reproduction* **34**: 771-776

Finkel MP (1945) The relation of sex hormones to pigmentation and to testis descent in the opossum and ground squirrel. *American Journal of Anatomy* 76: 93-151

Foster JW, Brennan FE, Hampikian GK, Goodfellow PN, Sinclair AG, Lovell-Badge R, Selwood L, Renfree MB, Cooper DW and Graves JAM (1992) Evolution of sex determination and the Y chromosome: *SRY*-related sequences in marsupials. *Nature* **359**:531-533

Fox RC (1971) Marsupial mammals from the early Campanian, Milk River Formation, Alberta, Canada. In: *Early Mammals* (eds: DM & KA Kermack) *Journal Linn. Sco. Suppl.* **50**: 145-164

Fraser EA (1919) The development of the urogenital system in the Marsupialia, with special reference to *Trichosurus vulpecula.* Part II. *Journal of Anatomy* **53**: 97-129

Frederick WG and Wilson JD (1994) Sex determination and differentiation. In: *The Physiology of Reproduction,* (2nd ed) (eds: E. Knobil, J. Neill *et al.*) chapter 1, pp. 3-28. New York: Raven Press

Fujimoto T, Miyayama Y and Fuyuta M (1977) The origin, migration and fine morphology of human primordial germ cells. *Anatomical Record* **188**: 315-330

Gardner RL, Lyon MF, Evans EP and Burtenshaw MD (1985) Clonal analysis of X-chromosome inactivation and the origin of the germ line in the mouse embryo. *Journal of Embryology and Experimental Morphology* **88**: 349-363

George FW and Peterson KF (1988) Partial characterisation of the androgen receptor of the newborn rat guberncaulum. *Biology of Reproduction* **39**: 536-539

George FW and Wilson JD (1994) Sex determination and differentiation. In: *The Physiology of Reproduction*, (2nd ed.) (ed. E. Knobil. & J. D. Neill), pp. 1-28. London & New York: Raven Press

George FW, Hodgins MB and Wilson JD (1985) The synthesis and metabolism of gonadal steroids in pouch young of the opossum, *Didelphis virginiana.* *Endocrinology* **116**: 1145-1150

Gilmore DP, Peres da Costa C, Valenca M, Duarte DPF, Wilson CA and Gray CE (1991) Effects of exogenous LHRH on plasma LH and sex steroid levels in the three-toed sloth *Bradypus tridactylus.* *Medical Science Research* **19**: 333-335

Ginsburg M, Snow MHL and McLaren A (1990) Primordial germ cells in the mouse embryo during gastrulation. *Development* **110**: 521-528

Godfrey GK (1975) A study of oestrus and fecundity in a laboratory colony of mouse opossums (*Marmosa robinsoni*). *Journal of Zoology (London)* **175:** 93-96

Goh DW, Momose Y, Middlesworth W and Hutson JM (1993) The relationship among calcitonin gene-related peptide, androgens and gubernacular

development in 3 animal models of cytptorchidism. *Journal of Urology* **150**: 574-576

Goldman AS, Yakovac WC and Bongiovanni AM (1966) Development of activity of 3β-hydroxysteroid dehydrogenase in human fetal tissue and in two anencephalic new borns. *Journal of Clinical Endocrinology* **26**: 14-22

Gondos B (1980) Development and differentiation of the testis and male reproductive tract. In: *Testicular Development, Structure and Function* (eds: A Steinberger & E Steinberger) pp. 3-20 New York: Raven Press

Gondos B, Paup DC, Ross J and Gorski RA (1974) Ultrastructural differentiation of Leydig cells in the fetal and postnatal hamster testis. *The Anatomical Record* **178**: 551-566

Gould E (1984) Ultrasonic communication in terrestrial mammals with some new data about *Monodelphis domestica* (Marsupialia). *Acta Zool. Fennica.* **171**: 93-96

Greene RR, Burrill MW and Ivy AC (1939) Experimental intersexuality The effect of antenatal androgens on sexual development of female rats. *American Journal of Anatomy* **65**: 415-469

Griffiths A, Renfree MB, Shaw G, Watts LM and Hutson JM (1993a) The tammar wallaby *Macropus eugenii* and the Sprague-Dawley rat: comparative anatomy and physiology of inguinoscrotal testicular descent. *Journal of Anatomy* **183**: 441-450

Griffiths AL, Middlesworth W, Goh DW, Hutson JM (1993b) Exogenous calcitonin gene-related peptide causes gubernacular development in neonatal (*Tfm*) mice with complete androgen resistance. *Journal of Pediatric Surgery* **28**:1028-1030

Grino FB, Griffin JE and Wilson JD (1990) Testosterone at high concentrations interacts with the human androgen receptor similarly to dihydrotestosterone. *Endocrinology* **126**: 1165-1172

Gubbay J, Collignon J, Koopman P, Capel B, Economou A, Münsterberg A, Vivian N, Goodfellow P and Lovell-Badge R (1990) A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature* (*London*) **346**: 245-250

Hϕyer PE (1980) Histoenzymology of the human ovary: dehydrogenases directly involved in steroidogenesis. In: *Biology of Ovary* (eds: PM Motta & ES Hafez) pp. 52-67 The Hague: Nijhoff

Hϕyer PE and Byskov AG (1981) A quantitative cytochemical study of Δ5, 3β-hydroxysteroid dehydrogenase activity in the rete system of the immature mouse ovary. In: *Development and function of Reproduction Organs* (eds: Byskov AF & Peters H) pp. 216-224 Amsterdam Excerpta Med

Haider SG, Passia D and Overmeyer G (1986) Studies on the fetal and postnatal development of rat Leydig cells employing 3β-hydroxysteroid dehydrogenase activity. *Acta Histochemica Supplement Band* XXXII. S. pp 197-202

Hall PF (1994) Testicular steroid synthesis: organization and regulation. In: Knobil N, JD Neill JD. (eds), *Physiology of Reproduction* (2nd ed.) vol. 1 London & New York: Raven Press, pp. 1335-1357

Hall PF, Irby DC and de Kretser DM (1969) Conversion of cholesterol to androgens by rat testes: comparison of interstitial cells and seminiferous tubules. *Endocrinology* **84**: 488-496

Harder JD and Fleming, MW (1982) Husbandry of a small breeding colony of opossums (*Didelphis virginiana*). *Laboratory Animal Science* **32**: 547-549

Harding HR, Carrick FN and Shorey CD (1982). Crystalloid inclusions in the Sertoli cell of the koala, *Phascolarctos cinereus* (Marsupialia). *Cell and Tissue Research* **221**: 633-642

Harry JL, Koopman P, Brennan FE, Graves JAM and Renfree MB (1995) Widespread expression of the testis-determining gene SRY in a marsupial *Nature Genetics*

Hayman DL (1990) Marsupial cytogenetics. *Australian Journal of Zoology* **37**: 331-349

Heyns CF (1987) The gubernaculum during testicular descent in the human fetus. *Journal of Anatomy* **153**: 93-112

Hughes RL and Hall LS (1988) Structural adaptations of the newborn marsupial. In: *The Developing Marsupial*, pp. 8-27 (Eds: C.H Tyndale-Biscoe & P.A. Janssens) Springer Verlag

Hunsaker D II (1977) Ecology of New World marsupials. In: *The biology of Marsupials* (ed: B Hunsaker II) pp. 95-156 New York: Academic Press

Hunter J (1762) Observations on the state of the testis in the foetus, and on the hernia congenita. In: *Medical Commentaries.* part 1 pp. 75-90 by Willian Hunter

Hunter RHF (1995) Mechanisms of sex determination. In: *Sex Determination, Differentiation and Intersexuality in Placental Mammals,* (1st ed.), pp. 22-62. Cambridge: Cambridge University Press

Hutson J, Ikawa H, Donahoe PK (1981) The ontogeny of Müllerian inhibiting substance in the gonads of the chicken. *Journal of Pediatric Surgery* **16**: 822-827

Hutson JH, Shaw G, O W-S, Short R and Renfree MB (1988) Müllerian inhibiting substance production and testicular migration and descent in the pouch young of a marsupial. *Development* **140**: 549-556

Hutson JM (1985) Abiphasic model for the hormonal control of testicular descent. *Lancet* **II:** 419-421

Hutson JM (1986) Testicular feminization: a model for testicular descent in mice and men. *Journal of Pediatric Surgery* **21:** 195-198

Hutson JM and Donahoe PK (1986) The hormonal control of testicular descent. *Endocrine Reviews* **7**: 270-283

Hutson JM, Shaw G, O WS, Short R & Renfree MB (1988) Müllerian inhibiting substance production and testicular migration and descent in the pouch young of a marsupial. *Development* **104**: 549-556

Hutson JM, Williams MPL, Fallat ME and Attah A (1990) Testicular descent: new insights into its hormonal control. *Oxford Reviews of Reproductive Biology* **12**: 1-56

Johnson MH and Everitt BJ (1980) Sex. In: *Essential Reproduction* (1st ed) chapter 1 pp.1-17 Blackwell Scientific Publications

Josso N (1972) Evolution of the Müllerian-inhibiting activity of the human testis. Effect of fetal, peri-natal and post-natal human testicular tissue on the Müllerian duct of the fetal rat in organ culture. *Biology of the Neonate* **20**: 368

Josso N (1973) *In vitro* synthesis of Müllerian-inhibiting hormone by seminiferous tubules isolated from the calf fetal testis. *Endocrinology* **93**: 829

Josso N (1994) Anti-Müllerian hormone: a masculinizing relative of TGF-β. *Oxford Reviews of Reproductive Biology* (ed HM Charlton) pp.139-163 Oxford University Press

Josso N and Picard JY (1986) Anti-Müllerian hormone. *Physiological Reviews* **66**: 1038-1090

Josso N Picard JY and Tran D (1977) The anti-Müllerian hormone. *Recent Progress in Hormone Research* **33**: 117-167

Josso N, Lamarre I, Picard JY, Berta P, Davies N, Morichon N, Peschanski M and Jeny R (1993) Anti-Müllerian hormone in early human development. *Early Human Development* **33:** 91-99

Josso N, Legeai L, Forest MG, Chaussain JL and Brauner R (1990) An enzyme-linked immunoassay for anti-Müllerian hormone in early human development. *Early Human Development* **33**: 91-99

Josso N, Vigier B, Cate RL, Behringer R, diClemente N and Lyet L (1992) Hormonal control of gonadal differentiation. In: *Gonadal Development and Function* pp. 31-39 New York Raven Press

Jost A (1953) Problems of fetal endocrinology: the gonadal and hypophyseal hormones. *Recent Progress in Hormone Research* **8**: 379-413

Jost A (1947) Recherches sur la differenciation sexuelle de l'embryon de lapin. III. Role des gonades foetals dans la defferenciation sexuelle somatique. *Archives d'Anatomie Microscopique et de Morphlogie Experimentale* **36**: 271-315

Jost A (1957) Embryonic sexual differentiation (morphology, physiology, abnormalities). In: *Hermaphroditism, Genital Anomalies and Related Endocrine Disorders.* (eds: HW Jones, Jr. & WW Scott) pp. 14-45 Williams & Wilkins Co., Baltimore

Jost A (1961) The role of fetal hormones in prenatal development. *Harvey Lecture Series* **55**: 201-226

Jost A (1970a) General outline about reproductive phyiology and its developmental background. In: *Mammalian Reproduction* (eds: H. Gibian & E. J. Platz) pp. 4-32. Berlin: Springer Verlag

Jost A (1970b) Hormonal factors in the sex differentiation of the mammalian foetus. *Philosophical Transactions of the Royal Society of London* **Series B 259**: 119-130

Jost A (1972a) A new look at the mechanisms controlling sex differentiation in mammals. *John Hopkins Medical Journal* **130**: 39-53

Jost A (1972b) Donnees preliminaires sur les stades initiaux de la differenciation du testicule chez le rat. *Archives d'Anatomie Microscopique et de Morphologie Experimentale* **61**: 415-438

Jost A and Magre S (1984) Testicular development phases and dual hormonal control of sexual organogenesis. In: *Sexual Differentiation* Basic and Clinical Aspacts (eds: M. Serio *et al.*) New York: Raven Press

Jost A and Magre S (1988) Control mechanisms of testicular differentiation. *Philosophical Transactions of the Royal Society of London*, Series B, **322**: 55-61

Jost A and Magre S (1993) Sexual differentiation. In: *Reproduction in mammals and man* (eds: C Thibault & MC Levasseur MC) pp.197-212 Paris

Jost A, Chodkiewicz M and Mauleon P (1963) Intersexualite du foetus de Veau produite par les androgenes. Comparaison entre l'hormone foetale responsable du free-martinisme et l'hormone testicularire adulte. C. R. Acad. Sci. (Paris). 256:274

Jost A, Magre S and Agelopoulou J (1981) Early stages of testicular differentiation in the rat. *Human Genetics* **58**: 59-63

Jost A, Vigier B Prepin J and Perchellet JP (1973) Studies on sex differentiation in Mammals. *Recent Progress in Hormone Research* **29**: 1-35

Jurgelski WJr (1974) The opossum (*Didelphis virginiana* Kerr) as a biomedical model. I. Research perspective, husbandry, and laboratory techniques. *Laboratory Animal Sciences* **24**: 376-403

Jurgelski Wjr, Forsythe W, Dahl D, Thomas LD, Moore JA, Kotin P, Falk HL and Vogel FS (1974) The opossum (*Didelphis virginiana* Kerr) as a biomedical model. II. Breeding the opossum in captivity: facility design. *Laboratory Animal Sciences* **24:** 404-411

Karnovsky M.J (1965) A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *Journal of Cell Biology* **27**: 137A-138A

Kassim NM, McDonald SW, Reid O, Bennett NK, Gilmore DP, Payne AP (1997) The effects of pre- and postnatal exposure to the non steroidal antiandrogen flutamide on testis descent and morphology in the albino swiss rat. *Journal of Anatomy* **190**: 577-588

Keast A (1977) Historical biogeography of the marsupials In: *The Biology of Marsupials* (eds: B Stonehouse & D. Gilmore) pp. 69-95 London: The Macmillan Press Ltd

Kerr JB and Knell CM (1988) The fate of Leydig cells during the development of the fetal and postnatal rat testis. *Development* **103**: 535-544

Kime D, Vinson G, Major P and Kilpatrick R (1980) Adrenal-gonad relationships. In: *General Comparative and Clinical Endocrinology of the Adrenal Cortex* (eds: I Jones & I Henderson), vol. 3. New York: Academic Press, pp.183-264

Kirsch JAW (1977) The six-percent solution: second thoughts on the adaptedness of the marsupials. (eds: B Stonehouse & DP Gilmore) Macmillan Press Led

Kitahara Y (1923) Uber die Entstehung der Zwischenzellen der Keimdrusen des Menschen und der Saugetiere und uber deren physiologische Bedeutung. *Arch. Entwicklungsmech. Org.* **52**: 571-604

Kraus DB and Fadem BH (1987) Reproduction, development and physiology of the gray short-tailed opossum (*Monodelphis domestica*). *Laboratory Animal Sciences* **37**: 478-482

Krause WJ, Cutts H and Leeson CR (1979a) Morphological observations on the mesonephros in the postnatal opossum, *Didelphis virginiana. Journal of Anatomy* **129:** 377-397

Krause WJ, Cutts H and Leeson CR (1979b) Morphological observations on the metanephros in the postnatal opossum, *Didelphis virginiana. Journal of Anatomy* **129:** 459-477

Kuopio T, Paranko J and Pelliniemi LJ (1989) Basement membrane and epithelial features of fetal-type Leydig cells in rat and human testis. *Differentiation* **40**: 198-206

Kuopio T, Tapanainen J, Pelliniemi LJ and Huhtaniemi I (1989) Developmental stages of fetal-type Leydig cells in prepubertal rats. *Development* **107**: 213-220

Ley RD (1987b) Photoreactivation in mammalian skin: mouse, marsupial and man. *Photodermatology* **4**: 173-175

Ley RD (1987a) *Monodelphis domestica*: a new animal model for studies on photodermatology. *Photochemical Photobiology* **46**: 223-227

Ley RD, Applegate LA, Stuart TD and Fry RJM (1987) UV radiation induced skin tumors in *Monodelphis domestica. Photodermatology* **4**: 144-147

Lipsett MB and Tullner (1965) Testosterone synthesis by the fetal rabbit gonad. *Endocrinology* **77**: 273-277

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* **193**: 265-275

Luu-The V, Lachance Y, Labrie C, Leblanc G, Thomas JL, Strickler RC and Labrie F (1989) Full length cDNA structure and deduced amino acid sequence of human 3β-hydroxy - Δ^5- ene steroid dehydrogenase. *Molecular Endocrinology* **3:** 1310-1312

Mackay S and Smith RA (1989) Mouse gonadal differentiation in vitro in the presence of fetal calf serum. *Cell Differentiation and Development* **27**: 19-28

Mackay S, Bashir AA and Birnie DH (1989) Primordial germ cells and gonadal development in the golder hamster. *Journal of Anatomy* **164**: 155-163

Mackay S, Strachan L and McDonald SW (1993) Increased vascularity is the first sign of testicular differentiation in the mouse. *Journal of Anatomy* **183**: 171

Magre S and Jost A (1983) Early stages of the differentiation of the rat testis: relations between Sertoli and germ cells. In: *Current Problems in Germ Cell Differentiation* (eds: A McLaren & CC Wylie) Cambridge University Press

Maitland P and Ullmann SL (1993) Gonadal development in the opossum, *Monodelphis domestica*: the rete ovarii does not contribute to the steroidogenic tissues. *Journal of Anatomy* **183**: 43-56

McCrady E (1938) The embryology of the opossum. In: *American Anatomical Memoirs*, No. 16. pp. 1-233 (eds: C. R. Stockard & H. M. Evans). Philadelphia: Wistar Institute

McKay DG, Hertig AT, Adams EC and Danzinger S (1953) Histochemical observations on the germ cells of human embryos. *Anatomical Record* **177**: 201-219

Mckeever S (1970) Male reprodictive organs. In: *Reproduction and Breeding Techniques for Laboraloty Animals* (Ed: E. S. E. Hafez) Cpapter 2 Lea & Febiger: Philadelphia pp.28-55

McLaren A (1983) Studies on mouse germ cells inside and outside the gonad. *Journal of Experimental Zoology* **288**: 167-171

McLaren A (1991a) Development of the mammalian gonad: The fate of the supporting cell lineage. *Biological Essays* **13**: 151-156

McLaren A (1991b) Sex determination in mammals. In: *Oxford Reviews of Reproductive Biology*, Vol. 13 ed. SR Milligan pp. 1-33 Oxford: Oxford University Press

Merry DE, Pathak S and VandeBery JL (1983) Differential NOR activities in somatic and germ cells of *Monodelphis domestica* (Marsupialia, Mammalia). *Cytogenet. Cell Genet.* **35**: 244-251

Mittwoch U (1970) How does the Y chromosome affect gonadal differentiation? *Philosophical Transactions of the Royal Society of London* **Series B 259**: 113-177

Mittwoch U (1985) Males, females and hermaphrodite. *Annals of Human Genetics* **50**: 103-1121

Molenarr D, De Rooij DG, Rommerts FFG, Reuvers PJ and Van Der Molen JH (1985) Specific destruction of Leydig cells in mature rats after "in vivo" administration of ethylene dimethanesulfonate (EDS). *Biology of Reproduction* **33**: 1213-1222

Moore CR (1939) Modification of sexual development in the opossum by sex hormones. *Proceedings of the Society for Experimental Biology* **40**: 544-546

Moore HDM and Thurstan SM (1990) Sexual differentiation in the grey short-tailed opossum, *Monodelphis domestica*, and the effect of oestradiol benzoate on development in the male. *Journal of Zoology* **221**: 639-658

Morgan CF (1943) The normal development of the ovary of the opossum from birth to maturity and its reactions to sex hormones. *Journal of Morphology* **72**: 27-85

Munsterberg AD & Lovell-Badge R (1991) Expression of the mouse anti-Müllerian hormone gene suggests a role in both male and female sexual differentiation. *Development* **113**: 613-624

Neumann F, Von Berswordt-Wallrabe R, Elger W, Steinbeck H, Haha JD and Kramer M (1970) Aspects of androgen-dependent events as studied by anti-androgens. *Recent Progress in Hormone Research* **26**: 337-410

O'Rahilly R (1977) The development of the vagina in the human. *Birth Defects* **13**: 123-136

O W-S and Baker TG (1978) Germinal and somatic cell interrelationships in gonadal sex differentiation. *Ann. Biol. Anim. Bioch. Biophys.* **18 (2B)**: 351-357

O W-S, Short RV, Renfree MB and Shaw G (1988) Primary genetic control of somatic sexual differentiation in a mammal. *Nature* **331**: 716-717

Ohno S (1976) Major regulatory genes for mammalian sexual development. *Cell* **7**: 315-321

Olson GE and Winfrey VP (1991) Changes in actin distribution during sperm development in the opossum, *Monodelphis domestica. The Anatomical Record* **230**: 209-217

Payne AP (1996) Gonadal hormones and the sexual differentiation of the nervous system: mechanisms and interactions. In: *CNS Neurotransmitters and Neuromodulators Neuroactive Steroids* (ed: Stone TW), New York: CRC Press, pp. 153-175

Pehleman FW and Lombard MN (1978) Differentiation of ovarian and testicular interstitial cells during embryonic and post-embryonic development in mice. *Cell Tissue Research* **188**: 465-480

Pelliniemi LJ (1975) Ultractructure of gonadal ridge in male and female pig embryos. *Anatomic Embryology* **147**: 19-34

Peters H (1969) The development of the mouse ovary from birth to maturity. *Acta Endocrinologica* **62**: 98-116

Picard J-Y, Benarous R, Guerrier D, Josso N and Kahn A (1986) Cloning and expression of cDNA for anti-Müllerian hormone. *Proceedings of the National Academy of Sciences* USA **83**: 5464-5468

Picard J-Y, Guerrier D, Kahn A and Josso N (1989) Molecular biology of anti-Müllerian hormone. In: *Evolutionary Mechanisms in Sex Determination* (ed: SS Wachtel) pp. 209-217 Boca Raton, Florida: CRC Press

Picon R (1969) Action du testicule foetal sur le developpement in vitro des canaux de Müller chez le rat *Archives d'Anatomie Microscopique et de Morphlogie Experimentale* **58**: 1-19

Pocock RJ (1926) Description des organes de la generation chez le Macropus (Halmaturus) bennettii femalle. *l'Academie Royale des Sciences de Belgique* **18**: 595-599

Rattner JB (1972) Nuclear shaping in marsupial spermatids. *Journal of Ultrastructural Research* **40**: 498-512

Raynaud A (1958) Inhibition, sous I'effec d'une hormone oestrogene, du developpement du gubernaculum du foetus male de souris. *C.r. hebd. Seanc. Acad. Sci., Paris.* 246: 176-179

Readhead C, Lobo RA and Kletzky DA (1983) The activity of 3β-hydroxysteroid dehydrogenase and Δ^4 - Δ^5 isomerase in human follicular tissue. *Am J Obstet Gynecol* **145**: 491-495

Renfree MB (1992a) Brief Reviews: The role of genes and hormones in marsupial sexual differentiation. *Journal of Zoology* London **226**: 165-173

Renfree MB (1992b) The role of genes and hormones in marsupial sexual differentiation. *Journal of Zoology* **266**: 165-173

Renfree MB (1994) Sexual dimorphisms in the gonads and reproductive tracts of marsupial mammals. In: *The Differences Between the Sexes* (eds: Balabane & Short RV) pp. 213-230 Cambridge University Press

Renfree MB and Short RV (1988) Sex determination in marsupials: evidence for a marsupial-eutherian dichotomy. *Philosophical Transactions of the Royal Society* (B) **322**: 41-53

Renfree MB, Shaw G and Harry JL (1997) Differentiation of sexual dimorphisms during marsupial development **43**(Suppl): 25-26

Renfree MB, Harry JL and Shaw G (1995) The marsupial male: a role model for sexual development. *Philosophical Transactions of the Royal Society* **B 350** 243-251

Renfree MB, O W-S and Short RV (1996) Sexual differentiation of the urogenital system of the fetal and neonatal tammar wallaby, *Macuopus eugenii. Anatomical Embryololgy* **194**: 111-134

Renfree MB, Robinson ES, Short RV and Vandeberg JL (1990) Mammary glands in male marsupials: Primordia in neonatal opossums *Didelphis virginiana* and *Monodelphis domestica. Development* **110**: 385-390

Renfree MB, Shaw G, Clarke J, Short RV and Kerr JB (1992) Morphology of the developing urogenital system of the tammar wallaby, *Macropus eugenii. Journal of Reproduction and Fertility*, Abstract Series, **9**: 134

Renfree MB, Wilson JD, Short RV, Shaw G and George FW (1992) Steroid hormone content of the gonads of the tammar wallaby during sexual differentiation. *Biology of Reproduction* **47**: 644-647

Reynolds ES (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *Journal of Cell Biology* **17**: 208

Robinson ES, Renfree MB, Short RV and VandeBerg (1991) Mammary Glands in male marsupials. 2. Development of teat primordia in *Didelphis virginiana* and *Monodelphis domestica. Reprod. Fertil. Dev.* **3**: 295-301

Rodger JC (1982) The testis and its excurrent ducts in American Caenolestid and Didelphid marsupials. *The American Journal of Anatomy* **163**, 269-282

Rubin D (1944) Embryonic differentiation of Cowper's and Bartholin's glands of the opossum following castration and ovariectomy. *Journal of Experimental Zoology* **94**: 463-473

Russell LD, Bartke A and Goh JC (1989) Postnatal development of the Sertoli cell barrier, tubular lumen, and cytoskeleton of Sertoli and myoid cells in the rat, and their relationship to tubular fluid secretion and flow. *The American Journal of Anatomy* **184**: 179-189

Russo J and Rosas JC-De (1971) Differentiation of the Leydig cell of the mouse testis during the fetal period: an ultrastructural study. *American Journal of Anatomy* **130**: 461-480

Samarakkody UK and Hutson JM (1992) Intrascrotal CGRP 8-37 causes a delay in testicular descent in mice. *Journal of Pediatric Surgery* **27**: 874-875

Schwanzel-Fukuda M, Fadem BH, Garcia MS and Pfaff DW (1988) The immunocytochemical localization of luteinizing hormone-releasing hormone (LHRH) in the brain and nervus terminalis of adult and early neontal gray short-tailed opossums (*Monodelphis domestica*). *Journal of Comp. Neurol.* **276**: 44-60

Sharpe RM, Kerr JB, Cooper I and Bartlett JMS (1986) Intratesticular factors and testicular secretion: the effect of ethane dimethane sulphonate (EDS) and the induction of seminiferous tubule damage. *International Journal of Andrology* **9**: 285-298

Shaw G, Harry JL, Whitworth DJ and Renfree MB (1995) Sexual determination and differentiation in the marsupials. In: *Recent Advances in Marsupial Biology* (eds: Saunders NA & Hinds LA) University of New South Wales Press

Shaw G, Renfree MB and Short RV (1990) Primary genetic control of sexual differentiation in marsupials. *Journal of Zoology* **37**: 443-450

Shaw G, Renfree MB, Short RV and O W-S (1988) Experimental manipulation of sexual differentiation in wallaby pouch young treated with exogenous steroids. *Development* **104**: 689-701

Short RV (1982) Sex determination and differentiation. In: *Reproduction in Mammals* 2: 70-113

Short RV (1972) Species differences. In: *Reproduction in Mammals* (eds: CR Austin & RV Short) Cambridge University Press

Short RV, Renfree MB and Shaw G (1988) Sexual development in marsupial pouch young. In: *The Developing Marsupial Models for Biomedical Research* pp. 200-238 New York Press

Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, Foster JW, Frischauf A-M, Lovell-Badge R and Goodfellow PN (1990) A gene from the human sex-determining region encodes a protein with homology to a conserved DNA binding motif. *Nature* (*London*). **346**: 240-244

Sinha AK and Kakati S (1976) C- and G-bands of the opossum chromosomes: Terminal sequences of DNA replication. *Can J. Genet. Cytol.* **18**: 195-205

Sinha AK, Kakati S and Pathak S (1972) Exclusive localisation of C-bands within opossum sex chromosomes. *Experimental Cell Research* **75**: 265-268

Smith C and Mackay S (1990) The development of mesonephric tubules and their possible contribution to gonadal and adrenal cortical anlagen in the CBA mouse Abstract in *Journal of Anatomy* **170**: 240

Taggart DA, Johnson J and Temple-Smith PD (1993a) Testicular and epididymal development in the brown marsupial mouse, *Antechinus stuartii* (Dasyuridae, Marsupialia). *Anatomy and Embryology* **188**: 87-100

Taggart DA, Johnson JL, O'Brien HP and Moore HDM (1993b) Why do spermatozoa of American marsupials form pairs? A clue from the analysis of sperm-pairing in the epididymis of the grey short-tailed opossum, *Monodelphis domestica. The Anatomical Record* **236**: 465-478

Takahashi M, Hayashi M, Manganaro TF and Donahoe PK (1986a) The ontogeny of Müllerian duct inhibiting substance in granulosa cells of the bovine ovarian follicle. *Biology of Reproduction* **35**: 447-453

Takahashi M, Koide SS and Donahoe PK (1986b) Müllerian inhibiting substance as oocyte meiosis inhibitor. *Molecular Cell Endocrinology* **47**: 255-234

Taketo T, Saeed J, Manganaro T, Takahashi M and Donahoe PK (1993) Müllerian inhibiting substance production associated with loss of oocytes and testicular differentiation in the transplanted mouse XX gonadal primordium. *Biology of Reproduction* **49**: 13-23

Tam PPL and Snow MHL (1981) Proliferation and migration of primordial germ cells during compensatory growth in mouse embryos. *Journal of Embryology and Experimental Morphology* **64**: 133-147

Theiler K (1972) The house mouse. In: *Development and normal stages from fertilisation to four weeks of age.* Berlin, Heidelberg, New York: Springer Verlag

Thomson SD and Lendon RG (1988) The effects of ethane-1, 2-dimethanesulphonate (EDS) on the neonatal rat testis. *Journal of Anatomy* **158**: 203

Tsubota T, Nitta H, Osawa Y, Mason JI, Kita I, Tiba T and Bahr JM (1993) Immunolocalization of steroidogenic enzymes, P450, 3β-HSD, P450c17, and P450arom in the Hokkaido brown bear (*Ursus arctos yesoensis*) testis. *General and Comparative Endocrinology* **92**: 439-444

Tran D and Josso N (1982) Localisation of anti-Müllerian hormone in the rough endoplasmic reticulum of the developing bovine Sertoli cell using immunocytochemistry with a monoclonal antibody. *Endocrinology* **111**: 1562-1567

Tran D, Meusy-Dessolle N and Josso N (1977) Anti-Müllerian hormone is a functional marker of foetal Sertoli cells. *Nature* (*London*) **269**: 411-412

Tran D, Picard JY, Campargue J & Josso N (1987) Immunocytochemical detection of anti-Müllerian hormone in Sertoli cells of various mammalian species. *Journal of Histochemistry & Cytochemistry* vol. 35 **7**: 733-743

Trupin GL and Fadem BH (1982) Sexual behaviour of the gray short-tailed opossum (*Monodelphis domestica*). *Journal of Mammalology* **63**. 409-414

Tung PS, Skinner MK and Fritz IB (1984) Cooperativity between Sertoli cells and peritubular myoid cells in the formation of the basal lamina in the seminiferous tubule. *Annals New York Academy of Sciences.* **438**: 435-446

Tyndale-Biscoe CH (1973) *Life of Marsupials.* London: Arnold

Tyndale-Biscoe CH and Renfree MB (1987) *Reproductive Physiology of Marsupials* Cambridge: Cambridge University Press

Tyson E (1698) Carigueya, Seu Marsupiale Americanum or The Anatomy of an Ppossum Dissected at Gresham college. *Philoophical Transactions of the Royal Society* **(B) 20**: 105-164

Ullmann SL (1981) Sexual differentiation of the gonads in bandicoots (Peramelidae, Marsupialia). In *Development And Function of Reproductive Organs* (ed. A. G. Byskov & H. Peters). Amsterdam: Elsevier North-Holland

Ullmann SL (1984) Early differentiation of the testis in the native cat, *Dasyurus viverrinus* (Marsupialia). *Journal of Anatomy* **138** 675-688

Ullmann SL (1989) Ovary development in bandicoots: sexual differentiation to follicle formation. *Journal of Anatomy* **165**: 45-60

Ullmann SL (1993) Differentiation of the gonads and initiation of mammary gland and scrotum development in the brush tail possum *Trichosurus vulpecula* (Marsupialia). *Anatomy and Embryology* **187**: 475-484

Ullmann SL (1996) Development of the ovary in the brushtail possum *Trichosurus vulpecula* (Marsupialis). *Journal of Reproduction and Fertility* Abstract Series **18**: 81

Ullmann SL Maitland P and Mason JI (1995) Distribution of 3β-Steroid dehydrogenase in the ovaries of the opossum *Monodelphis domestica. Journal of Reproduction and Fertility* Abstract Series 15: 131

Van der Schoot P (1992) Androgens in relation to prenatal development and postnatal inversion of the gubernacula in rats. *Journal of Reproduction and Fertility* **95**: 145-158

Van der Schoot P (1993a) Doubts about the 'first phase of testis descent' in the rat as a valid concept. *Anatomy and Embryology* **187**: 203-208

Van der Schoot P (1993b) Foetal testes control the prenatal growth and differentiation of the gubernacular cones in rabbits - a tribute to the late Professor Alfred Jost. *Development* **118:** 1327-1334

Van der Schoot P (1996a) Development, structure and function of the cranial suspensory ligaments of the mammalian gonads in a cross-species perspective; their possible role in effecting disturbed testicular descent. *Human Reproduction Update* **2**: 399-418

Van der Schoot P (1996b) Towards a rational terminology in the study of the Gubernaculum testis: arguments in support of the notion that the cremasteric sac should be considered the Gubernaculum in postnatal rats and other mammals. *Journal of Anatomy* **189**: 97-108

Van der Schoot, Xie Q, Payne A, Mackay S, Ullmann SL and Gilmore D (1996) Development of the gubernaculum in female marsupial mammals. *Journal of Reproduction and Fertility* Abstract Series **18**: 43

VandeBerg JL (1983) The gray short-tailed opossum: a new laboratory animal. *ILAR News* **26**: 9-12

VandeBerg JL (1990) The gray short-tailed opossum (*Monodelphis domestica*) as a model Didelphid species for genetic research. In: *Mammals from Pouches and Eggs*. (ed. J. A. Marshall Graves, R. M. Hope & D. W. Cooper). Australia: C.S. I. R. O

VandeBerg JL Cothran DG and Kelly CA (1986) Dietary effects on hematologic and serum chemical values in gray short-tailed opossums. (*Monodelphis domestica*). *Laboratory Animal Science* **36**: 32-36

VandeBerg JL, Robinson ES, Samollow PB and Johnston PG (1987) X linked gene expression and X chromosome inactivation: marsupials, mouse, and man compared. *Isozymes Curr. Top. Biol. Med. Res.* **15**: 255-253

Vigier B, Picard JY, Tran D, Legeai L and Josso N (1984) Production of anti-Mullerian hormone: another homology between Sertoli and granulosa cells. *Endocrinology* **144**: 1315-1320

Vigier B, Tran D, du Mesnil du Buisson F, Heyman Y and Josso N (1983) Use of monoclonal antibody techniques to study the ontogeny of bovine anti-Müllerian hormone. *Journal of Reproduction and Fertility* **69**: 207-214

Vigier B, Watrin R, Magre S, Tran D and Josso N (1987) Purified bovine AMH induces a characteristic freemartin effect in fetal rat prospective ovaries exposed to it *in vitro*. *Development* **11**: 43-55

Wachtel SS, Ohno S, Koo GC and Boyse EA (1975) Possible role for H-Y antigen in the primary determination of sex. *Nature (London)* **257**: 235-236

Wartenberg H (1981) The influence of the mesonephric blastema on gonadal development and sexual differentiation. In: *Development and Function of Reproductive Organs* (eds: AF Byskov & J Peters) pp. 3-12 Amsterdam: Exverpta Medica

Wartenberg H (1982) Structural aspects of gonadal differentiation in mammals and birds. In: *Differentiation* (eds:U Müller & WW Franke) Brelin: Springer-Verlag pp. 64-71

Wartenberg H (1983) Development of the early human ovary and role of the mesonephros in the differentiation of the cortex. *Anatomical Embryology* **165**: 253-280

Wartenberg H (1989a) Differentiation and development of the testis. In: *The Testis* (2nd edn) (eds: H Burger & D de Kretser) pp. 67-118 New York: Raven Press

Wartenberg H (1989b) Ultrastructure of fetal ovary including oogenesis. In: *Ultrastructure of Human Gametogenesis and Early Embryogenesis* (eds: von blerkom J, Motta PM) Boston: Kluwer Academic Publishers, **2989**: 61-85

Wensing CJG and Colenbrander B (1986) Normal and abnormal testicular descent. *Oxford Reviews of Reproductive Biology* **8**: 130-164

Wensing CJG (1973) Testicular descent in some domestic mammals. II. The nature of the gubernacular changes during the process of descent in the pig. *Proceedings of the Koninklijke Nederlandse Akademin van Wetenschappen Series C*, 76:190-195

Wensing CJG (1986) Testicular descent in the rat and a comparison of this process in the rat with that in the pig. *Anatomical Record* **214**: 154-160

Wensing CJG (1988) The embryology of testicular descent. *Hormone Research* **30:** 144-152

Wilson JD (1978) Sexual differentiation. *Annual Review of Physiology* **40**: 279-306

Wilson JD (1992) Syndromes of androgen resistance. *Biology of Reproduction* **46**: 168-173

Wilson JD (1994) Translating gonadal sex into phenotypic sex. In: *The Differences Between the Sexes* (eds: Balabane & Short RV) pp. 203-212 Cambridge university Press

Wilson JD Frederick W, George FW and Renfree MB (1995) The endocrine role in mammalian sexual differentiation. *Recent Progress in Hormone Research* **50**: 349-364

Wilson JD George FW, Griffin JE (1981) The hormonal control of sexual development. *Science* **211**: 1278-1284

Wilson JD and Siiteri PK (1973) Developmental pattern of testosterone synthesis in the fetal gonad of the rabbit. *Endocrinology* **92**: 1182-1191

Witschi E (1948) Migration of the germ cells of human embryos from the yolk sac to the primitive gonadal folds. *Contributions to Embryology of the Carnegie Institution* **32**: 67-80

Witschi E (1951) Embryogenesis of the adrenal and the reproductive glands. *Recent Progress in Hormone Research* **6**: 1-23

Xie Q, Mackay S, Ullmann SL, Gilmore DP and Payne AP (1996a) Testis development in the opossum *Monodelphis domestica. Journal of Anatomy* **189**: 393-406

Xie Q, Mackay S, Ullmann SL, Gilmore DP and Payne AP (1996b) A study of the early postnatal development of the reproductive tract of *Monodelphis* domestica. *Journal of Reproduction and Fertility* Abstract Series 18: 101

Xie Q, Mackay S, Ullmann SL, Gilmore DP and Payne AP (1995) An ultrastructural and immunocytochemical study of postnatal development of Leydig cells in the poossum, *Monodelphis domestica. Journal of Reproduction and Fertility* Abstract Series **16**: 83

Xie Q, Mackay S, Ullmann SL, Gilmore DP, Payne AP and Gray CE (1997) Postnatal development of Leydig cells in the opossum *Monodelphis domestica*: an immunocytochemical and endocrinological study. *Biology of Reproduction* **(In Press)**

Yoshinaga K, Hess DL, Hendrickx AG and Zamboni L (1988) The development of the sexually indifferent gonad in the prosimian, *Galago crassicaudatus crassicaudatus. American Journal of Anatomy* **181**: 89-105

Ziegler HG, Haider SG, Passia D and Hilscher W (1983) Enzyme histochemical and morphometrical studies on Δ^5 - 3β-hydroxysteroid dehydrogenase during the fetal and neonatal development of rat Leydig cells. *Andrologia* **15** **(4)**: 392-397

Zirkin BR and Ewing LL (1987) Leydig cell differentiation during maturation of the rat testis: A stereological study of cell number and ultrastructure. *The Anatomical Record* **219**: 157-163

Zuckerman S and Baker TG (1977) The development of the ovary and the process of oogenesis. In *Ovary* (2nd edn) (eds: S Zuckerman & BJ Weir) chapter 2, pp. 41-67. New York & London: Academic Press

Appendix 1: Chromosome Karyotyping Analysis

Solution:

0.56% KCl :	0.56 gms KCl in 100 ml H_2O
3:1 methanol : glacial acetic acid:	must be freshly made up
60% glacial acetic acid:	must be freshly made up

Hank's Buffer:

Double distilled H_2O	9 mls
Hank's buffer (10X HBSS on bottle **Gibco**)	1 ml
Sodium bicarbonate (**Gibco**)	0.1 mls

Phosphate Buffered Saline (PBS):

Phosphate buffer	10 mls
NaCl	0.9 mls
Double distilled H_2O	90 mls

Williams E-Medium:

Williams medium	8.8 ml
Fetal calf serum (nutrients)	1.0 ml
Gentamycin (antibiotic)	0.1 ml
L-glutamine (**Gibco**)	0.2 ml

CMRL-100 Medium:

CMRL-100	8.9 ml
Fetal calf serum	1.0 ml
Gentamycin	0.1 ml

Colcemid Stock (mitotic arrest agent 1μg/ml):

Supplied as 1mg in 30 ml vial, add 20 ml of sterile phosphate buffered saline (0.1 M) and passing through the millpore (......) to sterile. Aliquoted 1 ml for each (0.05mg/ml) and keep in the freezer.

Took 1 for "using" stock add 49 ml sterile PBS and leave in 5°C fridge in sterle bottle. The concentration of Solcemid Stock is 1 μm/ml.

Colcemid (0.4 μg/ml):

Colcemid stock (1μg/ml)	2 ml
Williams medium	3 ml

0.2 M phosphate buffer at pH = 7.2-7.4 solution:

$NaH_2PO_4.2H_2O$	15.6 gm in 500 ml of distilled water
NaH_2PO_4	56.8 mg in 2000 ml of distilled water

*Combine the two solutions to give 2.5 litres of 0.2M phosphate buffer at pH 7.4

0.1M phosphate buffer solution:

Stock A: 0.1 M sodium dihydrogen orthophosphate (mw 156) 1.56g in 100 ml H_2O

Stock B: 0.1 M disodium hydrogen orthophosphate (mw 142) 1.42g in 100 ml H_2O

Compisition of buffer:

x ml of A + y ml of B made up to 100 ml with distilled water

pH	x ml of A	y ml of B
6.8	25.5	24.5
7.2	14.0	36.0
7.4	9.5	40.5

Methods:

Chromosome karyotyping were carried out on young aged opossum from day 0-3 using a modification of a technique described for mice by Evans *et al.* (1972).

1. The pups were killed by decapitation.
2. Dissect out liver in Willians' E-medium (Gibco) at 37°C.
3. Transfer to Williams' E- medium containing 0.4 mg/ml of colchicine.
4. Break up liver into fragments with needles and pipette up and down in a syringe to disperse cells prior to incubation. Incubate for 1-2 hours at 37°C.
5. Transfer the cell suspension to a centrifuge tube and spin at 1000 rpm for 10 minutes
6. Discard supernatant and resuspend pellet in 0.56% KCl at room temperature for 10 minutes.
7. Centrifuge cell suspension at 1000 rpm for 10 minutes.
8. Discard supernatant and fix pellet slowly by carefully pipetting in 3:1 methanol : glacial acetic acid.
9. Remove fixative. Resuspend cells by flicking centrifuge tube, simultaneously adding further fixative. Complete fixation should take 2-5 minutes.
10. Centrifuge at 1000rpm for 10 minutes. Discard supernatant and resuspend in 60% glacial acetic acid. Place 3 drops in a row onto a cleaned slide.

Giemsa (For chromosome karyotyping) Stain:

1. 0.5M HCl - 15 minutes
2. Wash in running tap water - 5 minutes
3. Stain with 2.5% Giemsa's in 0.1 M phosphate buffer (pH 6.8) - 15 minutes
4. Wash in phosphate buffer
5. Dry in air.
6. Histoclear.

Mount in DPX.

Results: Chromosome stain blue to purple.

Appendix 2: Processing Tissue for Light and Transmission Electron Microscopy

Solution:

Fixative for Light Microscopy (Paraffin Sections) and Immunocytochemistry:

To prepare 4% 1000 ml paraformaldehyde in 0.1M phosphate buffer (pH 7.2-7.4)

1) Heat 400 ml ml distilled water in a conical flask until water temperature reaches 68-70°C.
2) Measure 40 gms of paraformaldehyde and empty into the above flask and stir. Add 1 N sodium hydroxide solution drop wise (a few drops) until solution becomes clear.
3) Add 500 ml of 0.2 M phosphate buffer.
4) Make the volume to 1000 ml with distilled water and filter.

Primary Fixative for Light (semi-thin sections) and Electron Microscopy:

To prepare 1000 ml 3%glutaraldehyde + 1%formaldehyde in 0.1M PB (pH 7.2-7.4)

1) Heat 350 ml distilled water in a conical flask until water temperature reaches 68-70°C.
2) Measure 10 gms of paraformaldehyde and empty into the above flask and stir. Add 1 N sodium hydroxide solution drop wise (about 10 - 12 drops) until solution becomes clear.
3) Add 120 ml of 25% glutaraldehyde and 4 ml of 0.5 calcium chloride.
4) Add 500 ml of 0.2M phosphate buffer.
5) Make the volume to 1000 ml with distilled water and filter.

Post-Fixative (1% OsO_4 in 0.1 M PB): 1 gm OsO_4 in 100 mls PB

Araldite Resin Mixture:

Araldite Resin	12.5 ml
DDSA (Dodecenyl Succinic Anhydride)	12.5 ml
DBP (Dihutyl Phthalate)	0.63 ml
DMP-30 (2,4,6,-tri(dimethylaminomethyl) phenol)	0.31 ml

Histokinette Automatic Tissue Processor for Paraffin sections:

Fixed specimens were rinsed to remove as much fixative as possible with three changes of 0.1 M phosphate buffer. Any bony components were decalcified with buffered 10% EDTA to facilitate cutting. Specimens were placed in separate histokinette baskets and labelled.

1) 70% ethanol	2 hours
2) 90% ethanol	2 hours
3) Absolute alcohol	2 hours
4) Amyl acetate	2 hours
5) Wax bath	2 hours

Multi-tissue specimens were subjected to vacuum extraction to ensure they were completely degassed. After embedding in wax they were then sectioned in 5-7μm thickness.

Processing Tissue for Light (semi-thin sections) Electron Microscopy:

1. Fix in (3%glutaraldehyde + 1%formaldehyde)	1-24 hours
2. Rinse in 0.1 M PB	10-30 minutes
3. Post fix in 1% OsO_4	30-60 minutes
4. Rinse in 0.1M PB	10-15 minutes
5. 30%, 50%, 70%, 90% ethanol	10-30 min each
6. Absolute ethanol	3x10-30 minutes
7. Propylene oxide	2 X 5 minutes
8. Propylene oxide/ resin (75:25)	1 hour
9. Propylene oxide/ resin (50:50)	1-2 hours
10. Propylene oxide/ resin (25:75)	1-4 hours
11. Pure Araldite resin mixture	≥ 24 hours(2 changes)
12. Embedding in resin	≥ 24 hours at 60°C

*Note: All stages from first rinse in PB to last infiltration in Araldite resin mixture - use rotator.

Appendix 3: Morphological Staining

A. Haematoxylin and Eosin (For paraffin sections):

Solutions:

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

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

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

Method:

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

Results:

Nuclei stain blue/purple. Cytoplasm stains pink.

B. Haematoxylin and Eosin (For semi-thin sections)

Solution:

Celestine blue:

Ferric ammonium solphate	5 mgs
Distilled water	100mls
Celestine blue	0.5 gms
Glycerine	14 mls

Method:

1. Hotplate	30 - 60 minutes
2. Saturated sodium hydroxide in ethanol	30 minutes
3. Ethanol series (100%, 90%, 70%, 50%, 30%)	3 minutes in each
4. Distilled water	
5. Celestine blue	30 minutes
6. Wash in tap wate	
7. Haematoxylin (Meyer's)	5 mintues
8. Wash in running tap water	5 miuntes
9. Scott's tap water substitute until blue.	
10. Wash in running tap water	
11. Differentiate, if necessary, in acid alcohol.	
12. Putt's eosin	2 minutes
13. Rinse in tap water.	
14. Ethanol series (30%, 50%, 70%, 90%, 100%)	30 seconds each
15. Histoclear	5 minutes
16. Mount in DPX.	

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

C. Toluidine Blue (For semi-thin sections)

Solution:

A: Sodium tetraborate (borax) 1 g in 100 mls H_2O

B: Toluidine blue 1 g in 100 mls H_2O

*60 mls solution A + 40 mls solution B stir and filter before use.

Method:

1. Stain 1-2 m resin sections on 60°C hotplate for 10 to 30 seconds.
2. Rinse with water and dry in air.
3. Xylene
4. Mount in DPX.

Results: Various shades of blue.

D. Ultra-thin sections staining

Solutions:

A: Lead Citrate Solutions:

Pb nitrate	1.33 g
Na citrate	1.76 g
distilled water	30 ml

*Shake until solution is well mixed (20-30 minutes) and add 8 mls 1 N NaOH, then add distilled H_2O to top up to 50 mls.

B: Saturated Uranyl Acetate Solution in 60% ethanol.

Method:

1. Uranyl acetate solution 5-10 minutes
2. Wash in distilled water
3. Lead citrate solution 5-10 minutes

(*NaOH pellets placed in petri dish to absorb carbon dioxide)

4. Wash in distilled water

Result: Electrical density

Appendix 4: Processing Tissue for Scanning Electron Microscopy

The neonates were killed by rapid decapitation. They were then immediately fixed in 3% glutaraldehyde + 1% formaldehyde in 0.1 m phosphate buffer (pH 7.2-7.4). The neonates were then dissected to expose the abdomen i.e. the urogenital ducts, bladder, kidneys, gonads adrenals etc. The colon was removed at the base of the bladder so that the reproductive tract can be easily observed.

Method:

1. Opossums are fixed with 3% glutaraldehyde + 1% formaldehyde in 0.1 m phosphate buffer (pH 7.2-7.4).
2. Transfer to same buffer — 1 hour
3. Post fix with 1% OsO_4 — 30 minutes
4. Transfer to buffer — 30-60 minutes
5. Dehydrate through the following series:

50% Acetone	1 hour
70% Acetone	1 hour
90% Acetone	1 hour
1st Absolute Acetone	1 hour
2nd Absolute Acetone	1 hour
3rd Absolute Acetone	1 hour

6. Critical Point Dry using liquid CO_2
7. Mount on stub and coated with gold

Appendix 5: Immunocytochemical (ICC) Staining

Solution:

Blocking serum diluent:

1.5% goat serum in PBS containing Triton X-100 (0.3%) = 1ml PBS containing Triton X-100 (0.3%) + 15µl NGS.

Primary antibody:

The primary antibody used is a polyclonal rabbit anti-human placental 3β-HSD antibody was a gift from Prof. J.I. Mason (Department of Biochemistry, University of Edinburgh). This antibody has been wildly used on rat, mouse and human tissues and previous work has demonstrated cross-reactivity with *Monodelphis domestica* tissues (Ullmann *et al.*, 1995).

Biotinylated secondary antibody (anti-rabbit IgG - 1:200 in diluent):

1 ml PBS containing Triton X-100 (0.3%) + 15µl NGS + 5µl biotinylated anti-rabbit IgG.

ABC reagent:

1 ml PBS + 20µl solution A + 20µl solution B

(mix well and stand for ≥ 30 minutes; A and B must be from same kit)

DAB solution (make up just before use):

50 ml PBS + 1 vials DAB + 1ml 3.5% $NiCl_3$- filter and add 15 µl (30%) H_2O_2

Antibody dilutions from 1:100 Stock solution:

10 µl of stock in 90 µl of diluent = 1:1000

5 µl of stock in 95 µl of diluent = 1:2000

4 µl of stock in 96 µl of diluent = 1:2,500

3.33 µl of stock in 96.66 µl of diluent = 1:3000

2.5 µl of stock in 97.5 µl of diluent = 1:4000

Methods:

Step	Time
1. Xylene	20 minutes
2. Ethanol series (100%, 90%, 70%)	10 minutes each
3. Water	5 minutes
4. Per-incubation in 1-5% BSA	30-60 minutes
5. 0.3% H_2O_2 in methanol	30 minutes
6. PBA	3 X 5 minutes
7. 1-10% NGS containing Triton X-100	30-60 minutes
8. Incubation in primary antibody (1:1000)	overnight
9. Rinse in PBS	3 X 5 minutes
10. Incubation in secondary antibody (1:200)	60 minutes
11. Rinse in PBS	3 X 5 minutes
12. Incubation in ABC reagent	30-60 minutes
13. Rinse in PBS	3 X 5 minutes
14. DAB containing 0.01% H_2O_2 and 0.02% $NiCl_3$	5-10 minutes
15. Wash in PB	5-10 minutes
16. Counterstain in 0.5% methyl green	30 seconds
17. Ethanol series (70%, 90%, 100%)	5 minutes each
18. Xylene	
19. Mount in DPX	

*Note: Incubation most be carried out in a humidity chamber.

Results:

3β-HSD positive cells - dark Leydig cell cytoplasm

All nuclei - green (methyl green)

Appendix 6: Protein Estimation

Solution:

Weigh out 10mg BSA and dissolve this in 100ml of 0.1 NaHO (100µg/ml)

The total volume of the BSA dilutions is 10ml and the small plastic capped bottles are used, these must be carefully labelled.

1. Blank 10ml 0.1 NaOH
2. A dilution of 10µg BSA is formed - 1ml BSA stock +9ml 0.1M NaOH
3 A dilution of 20µg BSA is formed - 2ml BSA stock + 8ml 0.1M NaOH
4. A dilution of 30µg BSA is formed - 3ml BSA stock + 7ml 0.1M NaOH
5. A dilution of 40µg BSA is formed - 4ml BSA stock + 6ml 0.1 M NaOH
6. A dilution of 50µg BSA is formed - 5ml BSA stock + 5ml 0.1 M NaOH
7. A dilution of 60µg BSA is formed - 6ml BSA stock + 4ml 0.1 M NaOH
8. A dilution of 70µg BSA is formed - 8ml BSA stock + 3ml 0.1 M NaOH
9. A dilution of 80µg BSA is formed - 9ml BSA stock + 2ml 0.1 M NaOH
10. A dilution of 90µg BSA is formed - 10ml BSA stock + 1ml 0.1 M NaOH
11. Adilution of 100µg BSA is formed - 1ml BSA stock

Weigh out 20mg BSA, dilute it in 100me of 0.1M NaOH (200µg/ml)

12. A dilution of 120µg BSA is formed - 6ml BSA stock + 4ml 0.1 M NaOH
13. A dilution of 140µg BSA is formed - 7ml BSA stock + 3ml 0.1 M NaOH
14. A dilution of 160µg BSA is formed - 8ml BSA stock + 2ml 0.1 M NaOH
15. A dilution of 180µg BSA is formed - 9ml BSA stock + 1ml 0.1 M NaOH
16. A dilution of 200µg BSA is formed - 10ml BSA stock

Weigh out 30mg BSA, dilute it in 100ml of 0.1M NaOH (300µg/ml)

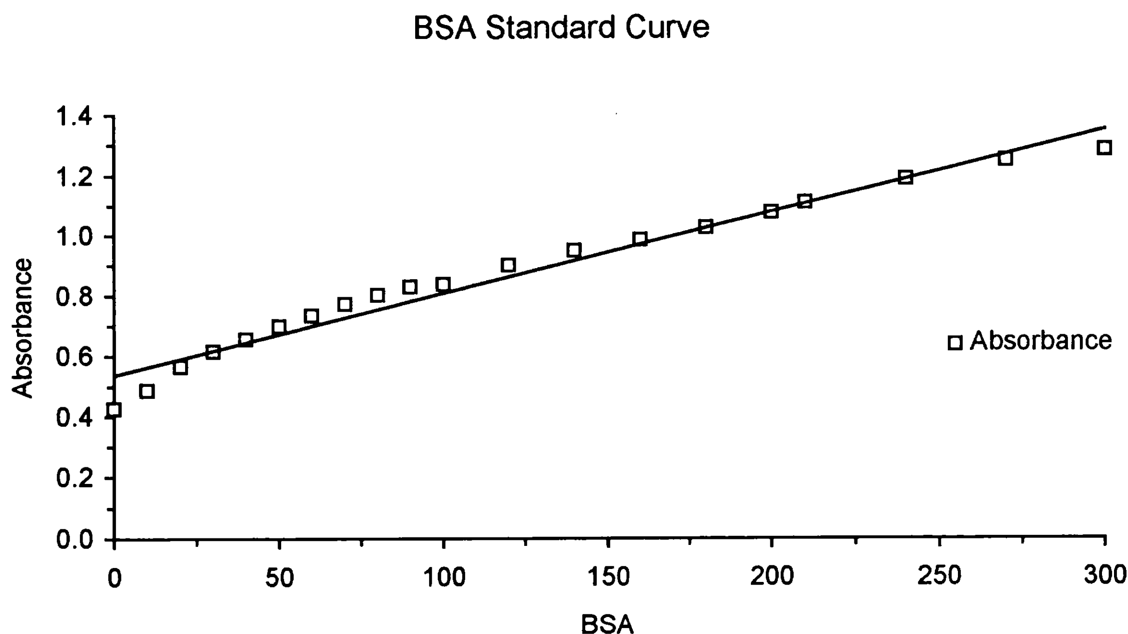
17. A dilution of 210µg BSA is formed - 7ml BSA stock + 3ml 0.1 M NaOH
18. A dilution of 240µg BSA is formed - 8ml BSA stock + 2ml 0.1 M NaOH
19. A dilution of 270µg BSA is formed - 9ml BSA stock + 1ml 0.1 M NaOH
20. A dilution of 300µg BSA is formed - 10ml BSA stock

The samples cell membranes must be broken down thus pellets are left overnight, 210μg of 0.1 M NaOH are added to each of these, they are placed in the vortex mixer for 1 minute and incubated overnight at 4°C.

The protein content of the tissue pellet is determined from its absorbance at 595nm after reaction with Coomassie Blue G250 (Pierce, Life Science Laboratories Let., Luton, UK). The weight of protein is calculated from a standard curve prepared using bovine serum albumin by modified method of Lowry *et al.* (1951).

To each cuvette add 1ml Coomassie Blue + 900μl Distilled water + 100μl of each of the 10, 20 & 30 μg BSA solutions in 0.1M NaOH (i.e. the series of solutions prepared as in the above). The first reading is a blank 200μl of 0.1M NaOH in 1800μl of distilled water. The first cell contains the blank, between each reading the spectrophotometer must be reset to zero. The protein concentration in each tube was calculated by substitution of the absorbance into the regression equation of the standard curve (shown below).

X(mg/mg protein) = $Y - 0.51551 \div 3.413 \times 3^{-3}$ (Y is your reading from a sample)



Appendix 7: Radioimmunoassay (RIA)

Solution:

Diluent Buffer: RIA buffer pH 7.4 containing 0.25% BSA (Sigma)

Diethyl Ether: B.D.H. AR grade.

Antiserum:

Supplied by Biochemical Services Ltd., Cardiff. The reagent was obtained by immunisation of rabbits with testosterone-3-0 (carboxymethyloxime)-BSA.

a. Working Stock as - Stock antiserum is diluted 1:100 with RIA buffer and stored -20°C in aliquots of 300 μl.
b. Working stock (1:100) is diluted sixty-fold e.g. 300 μl to 18 ml to give an initial titre of 1:6000. This dilution is prepared for each assay and 100μl added to each tube (except total count and NBS tubes). The incubation volume is 500 μl and therefore the final titre of the antiserum is 1:30,000.

Tracer:

a. Stock tracer - ^{125}I-histamine-testosterone is prepared every 6-8 weeks according to instructions in RIA method book. The stock solution of tracer in ethanol is stored at 4°C. The appropriate dilution for use of each batch of tracer to yield 10,000 counts/100μl/60s should be marked on the container. This dilution varies from batch to batch of tracer.
b. Working solution - To prepare a working solution of the tracer, take the volume of stock tracer indicated and evaporate to dryness under an air stream in a conical flask, then add the diluent buffer and allow to stand for several minutes with occasional swirling. This solution should be prepared fresh for each assay.

Extraction of testosterone:

All tubes are set up in duplicate. For the standard curve, the tubes consisted of total counts, normal bovine serum and standards T0 to T9, and the quality control (QC) pools (low, medium, high, normal male and high female).

For the samples, testosterone was extracted from plasma and testicular and adrenal homogenates: 2 measure of 25, 50 or 100μl of plasma (or 50μl of testicular homogenates) were placed in two separate tubes, (for testicular homogenate - dilute with an equal amount (50μl) of distilled water to make the volume to 100μl). Then, 3 ml of diethyl ether was added before agitating the mixture using a multi-tube vortexer for 3 minutes with a speed set to ensure that the lower aqueous phase is spun off the bottom of the tube.

The tubes were allowed to stand for 5 minutes at room temperature to separate the different phases (i.e. the lower aqueous phase and the upper ether phase); the lower phase was then allowed to freeze in a bath of methanol containing carbon dioxide pellets for about 30 seconds before decanting the solvent phase into another assay tube. The solvent was then allowed to evaporate in the 'Buchler Vortex Evaporator', thus leaving the testosterone as sediment at the bottom of the tube.

Staining the Testosterone:

300μl of 0.25% bovine serum albumen (NSB) in buffer pH 7.4 (prepared fresh for each assay) was added to each tube containing the dried testosterone sediment or control (without testosterone) followed by 100μl of the primary antibody (1° antibody against testosterone raised in rabbit with titre 1:6000) and 100μl of the radioactive tracer (^{125}I-histamine-testosterone). The mixture was then briefly agitated and incubated for 1-2 hours at room temperature. Afterwards, 500μl of doubled antibody reagent (donkey anti-rabbit in rabbit serum) was added and then incubated at 4 °C overnight.

Separation:

Finally, the assay tubes were centrifuged at 2,500 rpm for 25 minutes at 4°C. The supernatant fraction was then removed using a finely drawn out glass Pasteur pipette. Assay tubes were counted for sufficient time on the NEN 1600 gamma

counter (linked to the Commodore 4032 computer and printer 4022) to accumulate 10,000 counts in the total count tubes. (NB: where a 25 or 50µl sample has been extracted, the result obtained should be multiplied by 4 or 2).

Methods:

1. Measured samples of plasma, adrenal and testis (25-50µl) were pipetted in duplicate into large numbered test-tubes.
2. Ether was added to the tubes which were vortexed, taking care not to foam the samples.
3. Test-tube were also vortexed while evaporating the ether off. The residue was transferred to smaller test-tubes.
4. Dilutent buffer was added to the test-tubes.
5. Primary antibody (100µl) raised in rabbit, was added to both standards and samples, which were again vortexed.
6. Iodine tracer was added (100µl) to all tubes including standards, samples, non-specific buffer and tracer tubes. These were then incubated for 1-2 hours at room temperature.
7. After vortexing a secondary antibody (raised in donkeys), 100µl was finally added to all the tubes. The samples were again vortexed and incubated overnight at 4°C.
8. The samples were centrifuged, the supernatant of each was discarded and radioactive counts made.

*Note: All samples should be assayed together to avoid inter-assay anomalies