

**Y CHROMOSOME EFFECTS ON THE KINETICS OF
SPERMATOGENESIS IN THE DEVELOPING MOUSE**

by

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**This thesis is dedicated to my parents
William J and Doris L Atkins
with love and gratitude for everything**

ABSTRACT

The mammalian Y chromosome has a fundamental role in the control of primary sex determination, diverting the undifferentiated bi-potential gonad to form a testis. In addition, the Y chromosome has been implicated in a number of other male-specific functions. This study aims to provide additional details of the function of the Y chromosome in spermatogenesis during development.

A quantitative analysis of germ cells in XOSxr^b mice compared to their XY \pm Sxr^b sibs, during the first two post-natal weeks, investigated the function of the spermatogenesis gene *Spy*. The spermatogenesis gene was shown to act on the survival and proliferation of early differentiating A spermatogonia by five days after birth.

By a quantitative analysis of germ cells in XOSxr^a and XYSxr^a mice - the latter identified by DNA analysis - throughout puberty, it was shown that there was no pre-meiotic cell loss. Cell degeneration was first observed at metaphase I in XOSxr^a testes, but earlier, at pachytene, in XYSxr^a mice. XYSxr^a testes are mosaic for normal and defective germ cell patches. The 'pairing site' hypothesis of Miklos (1974) states that there is a correlation between the number of 'unsaturated' pairing sites and the extent of spermatogenic impairment. It is suggested that the earlier spermatogenic breakdown in XYSxr^a testes, results from the presence of two unpaired univalents (as opposed to one in XOSxr^a) with the consequent increased number of 'unsaturated' sites.

The XSxr^a chromosome was provided with a pairing partner - the chromosomal product, Y^{del}, derived from XY^{*} mice - which could satisfy at least some of the pairing sites, without adding Y long arm material. The germ cells in XSxr^aY^{del} testes overcame the block at MI seen in XOSxr^a mice, and proceeded to sperm. All sperm, however, were abnormal, confirming previous findings that a sperm morphology gene (*Smy*), present on the long arm, is essential for fertility.

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CHAPTER 1

INTRODUCTION

1. THE SEX CHROMOSOMES - A HISTORICAL PERSPECTIVE

Genetical sex determination is characterised by the establishment of male or female sex at conception and involves some hereditary material (Deeming and Ferguson, 1988). The concept that this hereditary material might be certain chromosomes dates back to Mendel, 1865 (published 1866 - reviewed by Sturtevant, 1965) who wrote to Nageli suggesting that sex determination might follow the same segregational pathway as other inherited characteristics.

Whilst many plants, a few lower animals, and several fish species, such as teleosts, remain synchronously or functionally hermaphroditic because no particular chromosome has accumulated enough sex-determining factors to qualify as a sex chromosome, most species are gonochorists and do possess a chromosomal sex-determining mechanism (Ohno, 1967).

1.1 Discovery of the X Chromosome

The description 'X' was assigned to a chromatin element observed by Henking (1891 - reviewed by Mittwoch, 1967) which passed to only half of the spermatozoa in the plant bug *Pyrrhocoris apterus*. Montgomery (1898 - reviewed by McClung, 1902) recognised the element as a chromosome, but it was McClung's (1902) studies of the sex-determining accessory X chromosome and spermatogenesis in Orthoptera and Hemiptera that heralded the birth of the chromosome theory of sex determination. Stevens (1905 - reviewed by Mittwoch, 1973) observed dimorphic sperm in the mealworm *Tenebrio molitor*, whilst Wilson (1905) investigated members of the Hemiptera group and found that males had

one less chromosome than females. He concluded, therefore, that males determined the sex of offspring by donating X or O gametes. From this simple XX-XO sex-determining system arose the concept of a heterogametic sex, two types of gametes, and a homogametic sex with one type of gamete. Simultaneously, Correns (1907 - reviewed by Mittwoch, 1967) working with *Bryonia*, also provided definite evidence of the heterogametic male role in the inheritance of sex in plants.

1.2 Discovery of the Y Chromosome

The terms 'Y' and 'sex' chromosome were introduced by Wilson (1909) to describe the observation in various insects that one of a pair of chromosomal bodies showed a size difference in males as opposed to that seen in females. He argued that there must be a causal connection between these chromosomes and sex, although he believed the number of X chromosomes, rather than the Y chromosome, determined sex; a theory that proved correct for the insect species he observed. Experiments on XXY *Drosophila melanogaster* were carried out by Morgan (1910, 1911) and Bridges (1914, 1916 - reviewed by Whitehouse, 1969) who confirmed Wilson's theory of sex determination as a function of the X chromosome to autosome ratio, with males XY or XO and females XX or XXY. Although the Y chromosome in *Drosophila melanogaster* does not have a role in sex determination, it is nonetheless essential to male fertility. Stern (1929 - reviewed by Hess and Meyer, 1968) recognised that factors on the Y were involved in the formation of normal functioning sperm and by the late 1930's, five fertility genes were identified on the Y chromosome (Neuhaus, 1939). More recently, it has been shown that there are at least five regions on the long arm and two on the short arm of

the *Drosophila melanogaster* Y chromosome, which are involved in spermatogenesis (Brosseau, 1960; Hess and Meyer, 1968; Hess, 1970; Baker and Lindsley, 1982).

1.3 Mammalian Sex Chromosomes

The latter part of the 19th and early 20th century also saw the birth of mammalian cytogenetics. Tafani (1889 - reviewed by Evans, 1981), was the first to score the haploid chromosome number as 20 in the mouse, and although some numerical discrepancy followed, this chromosome number was later confirmed by Long (1908), Painter (1925) and Cox (1926) . The 40, XY karyotype was first described by Crew and Koller (1932) with the Y described as the shortest chromosome in the complement.

Attempts to study human chromosomes were first made in 1882, with the human X chromosome described in the early 1900's (Flemming, 1882; Guyer, 1910 and Winiwater, 1912 - all reviewed by Mittwoch, 1967). Wieman (1917 - reviewed by Painter, 1923) and Painter (1924 - reviewed by Mittwoch, 1967) provided evidence for a Y, as well as an X chromosome, but the correct autosome number was to remain in dispute for a further thirty years. Although in 1949, Matthey reviewed sex chromosomes in a variety of vertebrates, the Y chromosome was still considered unnecessary for male determination (Matthey, 1949 - reviewed by Mittwoch, 1967).

It was not until 1956, that Tjio and Levan demonstrated that there were 46 chromosomes in man comprising 22 pairs of autosomes and one pair of sex chromosomes, XY in males and XX in females. The human

chromosome number was confirmed shortly after by Ford and Hamerton (1956). Three years later, Ford *et al.* (1959) and Welshons and Russell (1959) established that in humans and mouse, an XO constitution was female, and concluded that to be male required a Y chromosome. The original belief that sex determination in humans was controlled by the X to autosome ratio, therefore, proved incorrect, and the importance of the Y chromosome was established.

This XX-XY sex-determining mechanism is now known to prevail in virtually all eutherian mammals, with the male the heterogametic sex; the few exceptions involve multiple or aberrant sex chromosomes (Fredga, 1988). Another system known as the ZW-WW sex chromosomal mechanism, in which the female is the heterogametic sex, is the form of sex determination for birds, moths and some reptiles and amphibians (Ohno, 1967).

1.4 The Y Chromosome, Testes and Hormones

In 1953, Jost's experiments involving castration and hypophysectomy of foetal rabbits conclusively demonstrated that testicular factors (androgens and the 'Mullerian regression factor') were responsible for masculinisation of foetal structures. Female organogenesis was shown to result from the mere absence of testes, hence the concept that male determination equated to testis determination (Jost, 1953; Jost, 1970; Jost *et al.*, 1973). The role of the Y chromosome was thus established to be that of testis determination.

1.5 Conclusion

From findings based on insect experiments, it was originally assumed that in mammals also, the Y chromosome had no role in sex determination. Half a century later, it was acknowledged that a testis-determining factor on the mammalian Y chromosome diverted the indifferent foetal gonad to form a testis, but no other function was ascribed to it. During the last few decades, however, following research mainly in mouse and man, the Y chromosome has been implicated in a number of other male-specific morphogenetic and fertility functions.

2. THE MAMMALIAN Y CHROMOSOME

There are two functionally distinct parts to the mammalian Y chromosome. The Y chromosome pairing and exchange or pseudoautosomal region (see Section 2.2.5.1) with an equivalent region on the X chromosome, and the Y-differential region which is normally protected from any recombination with the X chromosome. This differential region carries Y-specific genes, including the gene for testis determination, and is unique in the mammalian genome because it is perpetually monosomic.

2.1 Y Chromosome Structure

Three types of genetic mapping - deletion, meiotic and physical - have been used to construct a structural and functional plan of the Y chromosome.

2.1.1 Human

In man, the Y chromosome comprises approximately 2% of the haploid male genome (Laird, 1971) and has a long arm, Yq, and a short arm, Yp. The pairing and exchange region lies at the distal tip of the Y short arm (Burgoyne, 1982; Pritchard *et al.*, 1987) (see Section 2.2.5.1) and the boundary between the pseudoautosomal and differential sectors is defined by *Alu* repeat sequences which provide an abrupt change between the two regions (Ellis *et al.*, 1989). The testis-determining factor lies proximal to this boundary on Yp (see Section 2.2.1). The Y centromeric region, like centromeres throughout the genome, comprises alphoid satellite repeat sequences (Wolfe *et al.*, 1985; Tyler-Smith, 1987).

The human Y chromosome can also be divided into two equal sized portions on the basis of staining characteristics. The euchromatic region spans Ypter - q11 and the heterochromatic region Yq11 - qter (Goodfellow *et al.*, 1985). More than 50% of the Y chromosome consists of tandemly repeated sequences - 'junk' DNA - with no apparent function (Goodfellow *et al.*, 1985). These sequences are concentrated in the Yq heterochromatin, such that individuals lacking this heterochromatic region can be normal fertile males (Smith *et al.*, 1987; Muller, 1987a).

Whilst there is complete sequence homology between distal Xp and distal Yp consistent with a pairing and exchange region (Bishop *et al.*, 1984; Muller, 1987a; Petit *et al.*, 1988), a larger region of 99% sequence homology has been identified between Yp and the X long arm, Xq13.2 - 21.2 (Page *et al.*, 1984; Vergnaud *et al.*, 1986). This Xq and Yp homology is evidence for a 'recent' (in evolutionary terms) large

transposition between the sex chromosomes. The Y chromosome also shares sequence homology with a number of autosomal regions (Bishop *et al.*, 1984; Affara *et al.*, 1986; Smith *et al.*, 1987).

2.1.2 Mouse

All chromosomes in the mouse genome have been described as telocentric including the Y chromosome (Nesbitt and Francke, 1973; Eicher, 1981), despite Ford's (1966) observations of the Y 'short arm' morphology in mitotic preparations. However, Ford's (1966) view has recently been vindicated (Roberts *et al.*, 1988; McLaren *et al.*, 1988). Unlike the situation in humans, the mouse pairing and exchange region is located distally on the long arm (Burgoyne, 1982). The information for testis determination is located in the short arm (Roberts *et al.*, 1988; McLaren *et al.*, 1988). This means that in the mouse, the testis determinant (*Tdy*) is at the opposite end of the Y to the pairing and exchange region, so that unlike the situation in humans, there is no danger of accidental transfer of *Tdy* to the X during male meiosis.

Much of the sex-determining region of the mouse Y has sequence homology to a Bkm satellite sequence isolated from the heterogametic female banded krait snake (Singh *et al.*, 1981; Jones and Singh, 1981) (see Section 3.2.1). Such sequence homology has been shown to be highly conserved in most vertebrates, especially mammals, in a sex specific manner (Jones and Singh, 1981).

The mouse Y long arm has a large heterochromatic region, as in man, and much of this region has been presumed to be 'junk' DNA. A viral probe,

M720, recognises two repeat retroviral sequences comprising approximately 3% and 10% of the mouse Y chromosome, the latter, MuRVY, present as 300 or more copies of tandem repeats (Eicher *et al.*, 1983; Eicher and Washburn, 1986). Blatt *et al.* (1983) have also identified Y chromosome murine virus-related sequences. A genomic sequence, the pY353B insert, cloned by Bishop and Hatat (1987), occurs in multiple copies along the length of the mouse Y long arm. As in man, large deletions of long arm heterochromatin are compatible with fertility. However, mice with such deletions are prone to higher than normal levels of X-Y non-disjunction (Burgoyne - personal communication) and have a higher incidence of abnormal sperm (Moriwaki *et al.*, 1988) (see Section 2.2.4). A schematic labelled Fig. 1a, outlines the structural information known to date in man and mouse.

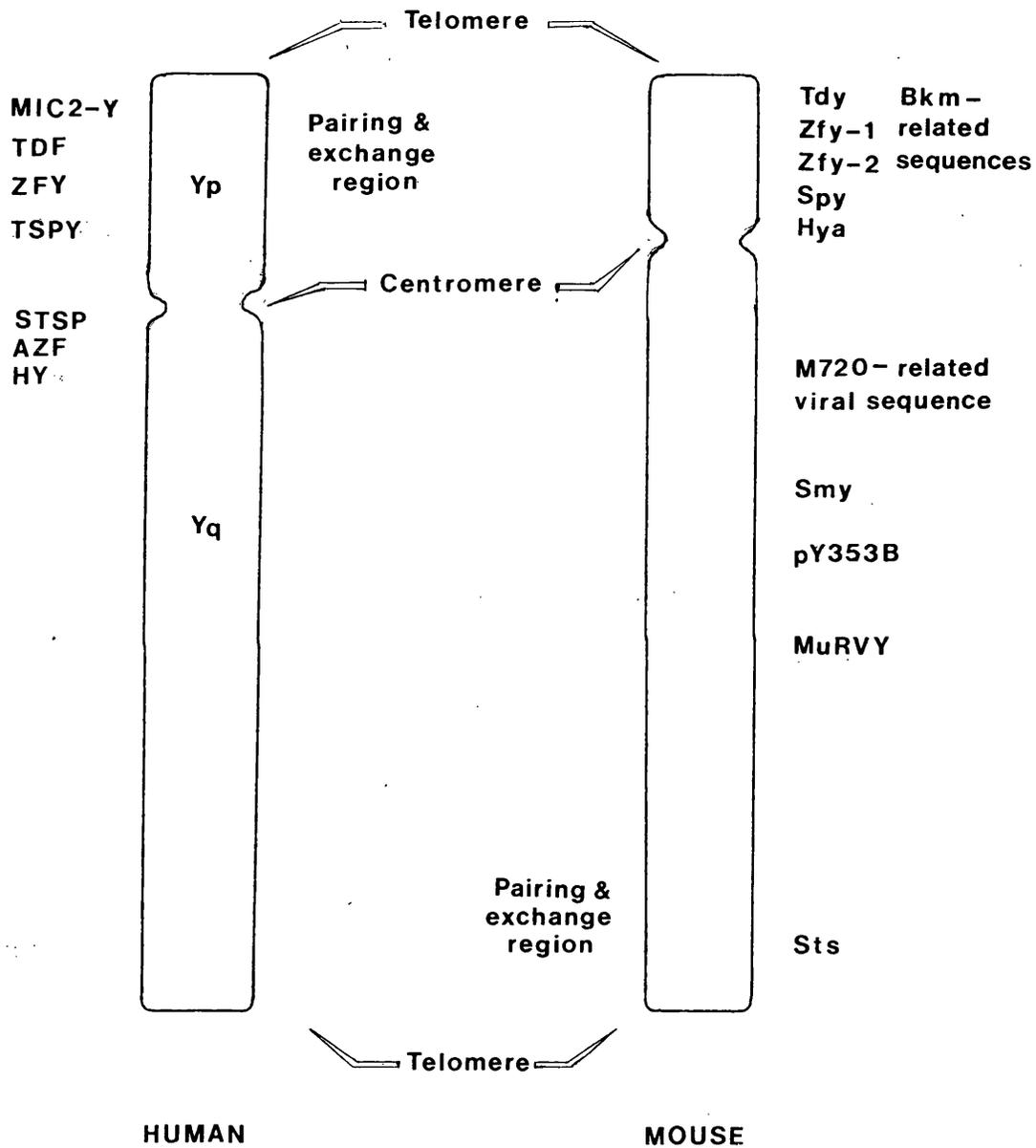


Fig. 1a. Schematic showing the Y chromosome in mouse and man. (after Craig *et al.*, 1987; Weissenbach *et al.*, 1989) All sequences indicated on the mouse long arm are thought to be multiple copy sequences. MIC2-Y in humans and Sts in mouse are both pseudoautosomal genes. The STS pseudogene on the Y chromosome in humans has been variously assigned to the distal region of the long arm (Craig and Tolley, 1986) and Yq11 (Fraser *et al.*, 1987)

2.2 Y Chromosome Function

In spite of its small size, absence of extensive recombination and minimal genetic markers, the Y chromosome has been the subject of extensive classical (from phenotypic level to the gene and DNA level) and molecular (from the DNA sequence to the phenotypic and functional level) genetic investigations to determine its functional role (Goodfellow *et al.*, 1985; Weissenbach, 1988).

2.2.1 Testis determination

Deletion mapping and pedigree analysis in humans has been possible because sex chromosome mutations are generally not lethal. Using a cytogenetic approach, Jacobs and Strong (1959) constructed deletion maps of XX males, who usually retain a fragment of Y chromosome within their genome, and XY females who usually have deletions of Y chromosome material. They postulated that male determination in humans resulted from a gene, or genes, located on the short arm, Yp. Both cytogenetic and molecular techniques have since confirmed the assignment of the testis determination factor *TDF* to the distal Yp region (Rosenfeld *et al.*, 1979; Davies, 1981; Magenis *et al.*, 1982; Fryns *et al.*, 1985; Buhler, 1985; Vergnaud *et al.*, 1986; Disteché *et al.*, 1986; Pritchard *et al.*, 1987; Muller, 1987b). (Note: It is accepted practice that genes assigned to humans are coded by capitals, e.g. *TDF*, whereas those for the mouse have only an initial capital, e.g. *Tdy*).

In 1987, Page *et al.* (1987a) identified a gene, close to the human pseudoautosomal boundary, which was generally accepted to be the testis-determining factor *TDF*. This gene has been given the label *ZFY*

because it encodes a protein with thirteen zinc fingers. One of the prerequisites for a candidate testis-determining gene, was high sequence conservation on the Y chromosome in other mammals and *ZFY* appeared to amply satisfy this criterion (Page *et al.*, 1987a). In addition to *ZFY*, a highly homologous region was found on the X chromosome and designated *ZFX* (Page *et al.*, 1987a; Page, 1988).

Concurrent with human studies, the testis-determining gene *Tdy* in mouse has also been the subject of investigation via Y chromosome rearrangements, partial deletions and molecular studies (Bishop *et al.*, 1987a; Burgoyne, 1988). Recent molecular evidence has demonstrated that *Zfy*, which has 80% homology to human *ZFY*, is present in two copies *Zfy-1* and *Zfy-2* in mouse, both of which map to the proximal short arm and are present in the sex-reversed factor *Sxr^a* (see Section 3.2.1) (Roberts *et al.*, 1988; McLaren *et al.*, 1988; Mardon *et al.*, 1989). However, male determination can occur in the absence of *Zfy-2* (Mardon and Page, 1989; Nagamine *et al.*, 1989). A homologous sequence on the X chromosome, *Zfx*, and an autosomal sequence, *Zfa*, have also been described (Page, 1988).

Despite its Y-specific and highly conserved nature, questions remained as to whether *ZFY* was equivalent to *TDY*. A small number of rare XX(Y-) human males have been recorded (Vergnaud *et al.*, 1986; Ferguson-Smith and Affara, 1988) and it has been suggested that male determination may result from a cascade of genetic events or alternatively, be the result of a complex interaction between the Y and one or more autosomes (Page, 1986; Bishop *et al.*, 1987a; McLaren, 1987b; de la Chapelle, 1987; Craig *et al.*, 1987; Eicher, 1988; Page,

1988). It has even been hypothesised that testis determination may occur in the absence of a Y chromosome in XX hermaphrodites, although these individuals have not been probed for the Y-specific pseudoautosomal boundary sequences (Waibel *et al.*, 1987; Ferguson-Smith and Affara, 1988; Mittwoch, 1988). Findings in marsupials (who have an XX-XY sex-determining mechanism) that *ZFY* homologues are autosomal, rather than on the Y chromosome, did nothing to inspire confidence in the testis-determining role of *ZFY* (Renfree and Short, 1988; Sinclair *et al.*, 1988; Hodgkin, 1988).

The most recent molecular and transcriptional evidence strongly opposes *ZFY* and *Zfy-1* as the primary testis-determining gene in man and mice (Palmer *et al.*, 1989a; Koopman *et al.*, 1989). However, Burgoyne (1989) has suggested that in man, *ZFY* together with *ZFX* may nevertheless be necessary to ensure that the primary testis-determining gene can pre-empt the ovarian pathway.

2.2.2 Control of H-Y antigen expression

The H-Y antigen was originally defined by female rejection of male skin grafts from within the same inbred strain in mice. A gene required for H-Y antigen expression has been assigned to the Y chromosome but not yet characterised (Eichwald and Silmser, 1955; Billingham and Silvers, 1960). Male specific 'H-Y' antigen has subsequently been detected serologically (Goldberg *et al.*, 1971) and by cytotoxic T-cell assay (Goldberg *et al.*, 1973; Gordon *et al.*, 1975).

Conflicting results and controversy centred on whether the transplantation and serological methods recognised the same or different antigens (Melvold *et al.*, 1977; Ohno, 1985; Wiberg, 1985; Stewart, 1986) and whether H-Y was the primary male determinant (Wachtel *et al.*, 1975; Wolf *et al.*, 1980; McLaren 1987a; McLaren, 1987b). The consensus of opinion to date is that the transplantation and cytotoxic T-cell assay detect the same antigen. This antigen retains the label H-Y. The Y-linked gene which is required for expression of H-Y maps within deletion interval 4B-7 on the human Y (*TDY* maps to interval 1) (Wiberg, 1987; Simpson *et al.*, 1987a) and along with *Tdy*, to the mouse Y short arm (Simpson *et al.*, 1986; Simpson *et al.*, 1987b). The serologically-detected antigen may be distinct from H-Y and has been variously referred to as: SDM/SDMA = serologically-determined male antigen, Sxs/Ssxs = serological sex-specific antigen, and possibly also Hye = histocompatibility Y expression antigen (reviewed by Goldberg, 1988) and MEA = male enhanced antigen (Lau, 1987). Although it remains unclear whether H-Y and SDM are separate antigens, the finding that XXSxr^b male mice (see Section 3.2.2) lack male specific antigen(s) by transplantation (Simpson *et al.*, 1986), cytotoxic T-cell (Simpson *et al.*, 1986) and serological tests (Goldberg, 1988), excludes both antigens from being required for primary male sex determination.

2.2.3 Spermatogenesis gene

From a conservational viewpoint and from findings of fertility factors on the *Drosophila melanogaster* Y chromosome, a multi-functional role for the mammalian Y chromosome might well be expected (Hess, 1970; Ohno, 1967; Vogt, 1989). Recent molecular investigations suggest that many genes are associated with HTF islands (Hpa II tiny fragment sequences)

and approximately 40 of these have been estimated for the human Y chromosome (Wolfe, 1987). So far, less than 10 Y genes have been described.

Yq deletions in humans have often been correlated with azoospermia and led to suggestions that a spermatogenesis gene may be located on the Y chromosome long arm (Neu *et al.*, 1973; Tiepolo and Zuffardi, 1976; Yunis *et al.*, 1977; Alvesalo and de la Chapelle, 1981; Affara *et al.*, 1986). The Y chromosome was first implicated in spermatogenesis in mice by Evans *et al.* (1969). Evidence for a spermatogenesis gene, *Spy*, acting germ cell autonomously, was provided by Levy and Burgoyne (1986b) in their study of XO germ cells in XO/XY/XYY mosaics. From the germ cell block apparent in histological sections of XOSxr^b compared to XOSxr^a testes, it was concluded that *Spy* was present in the Sxr^a region (but missing from Sxr^b) and was therefore located in the mouse Y short arm (Burgoyne *et al.*, 1986; McLaren *et al.*, 1988) (see Section 3.2.2). It is not yet clear whether the spermatogenic defect in men with Yq deletions is equivalent to that seen in XOSxr^b mice.

2.2.4 Other genes on the Y chromosome

Additional genes implicated on the human Y chromosome relate to body growth, skeletal maturation and dental development (Tanner *et al.*, 1959; Alvesalo and de la Chapelle, 1981; Lau *et al.*, 1989), suppression of Turner's stigmata (Jacobs and Ross, 1966; Rosenfeld *et al.*, 1979; Fryns *et al.*, 1985; Buhler, 1985, Levilliers *et al.*, 1989) and gonadoblastoma predisposition in dysgenetic gonads (Page, 1987). Recently, Y-encoded RNA transcripts have been found in human and

mouse testes which are thought to be part of a family of testis-specific transcripts whose function is, as yet, unknown (Arneemann *et al.*, 1987; Bishop *et al.*, 1987a; Bishop *et al.*, 1987b).

In mice, comparing different Y chromosomes on the same genetic background, demonstrated Y-linkage for genes involved in testis size (Hayward and Shire, 1974; Herrick and Wolfe, 1977), predisposition to non-disjunction (Beamer *et al.*, 1978), sexual/aggressive behaviour and androgen level/sensitivity (reviewed by Stewart, 1983; Jutley and Stewart, 1985) and sperm head development (Krzanowska, 1971; Krzanowska, 1976; Stewart, 1983). Recent evidence from Moriwaki *et al.* (1988) investigating mice with a mid-region long arm deletion, revealed more than 70% of spermatozoa with abnormal head shape, confirming that a sperm head morphology gene, probably present in multiple copies, is located on the Y long arm.

An XY to XX developmental difference has been recorded in CD1 mouse embryos at the blastocyst stage (Tsunoda *et al.*, 1985), with males ahead of females. This difference is controlled by the Y chromosome (Burgoyne - personal communication). XY embryos are also more advanced at early somite stages (Seller and Perkins-Cole, 1987) but in this case the Y does not appear to be responsible (Burgoyne - personal communication).

2.2.5 The Y chromosome as a meiotic pairing partner

As well as a direct genetic function in the male germ line, the Y chromosome provides a pairing partner for the X chromosome during meiotic prophase (Hulten, 1974; Burgoyne, 1987a).

2.2.5.1 The pseudoautosomal region

Autosomal chromosome pairing is an essential meiotic mechanism ensuring successful recombination, segregation and fertility (Federley, 1931 - quoted by Miklos, 1974; Darlington, 1937 - quoted by Burgoyne, 1982; Hulten, 1974). The hypothesis that pairing and exchange is a necessary feature of sex chromosomes also, was proposed by Koller and Darlington (1934) and was reiterated by Burgoyne (1982). With the advent of silver staining techniques and visualisation of meiotic pachytene cells both at the light and electron microscope levels, X and Y synaptonemal complexes were identified, and sequence homology was inferred (Moses *et al.*, 1975; Polani, 1982). Unfortunately, it is now known that the formation of a synaptonemal complex *per se*, whilst being an essential prerequisite for recombination, neither necessarily implies homology nor ensures that a chiasma has occurred (Chandley *et al.*, 1984). Despite late challenges to the concept of regular X-Y exchange (Ashley, 1984; Ashley, 1985), proof that X-Y crossing over is a regular event in man and mouse has now been obtained.

By linkage analysis and molecular techniques, the short arms of the human X and Y chromosomes have been shown to have both non-homologous and homologous regions (Cooke *et al.*, 1985; Simmler *et al.*, 1985). Both homology and recombination have now been demonstrated by sex linkage analysis of three pseudoautosomal loci (Rouyer *et al.*, 1986), by mapping of the pseudoautosomal region (Page *et al.*, 1987b) and by cloning of the pseudoautosomal gene, *MIC2*, in man (Goodfellow *et al.*, 1988). *MIC2* codes for the cell surface antigen 12E7 (Buckle *et al.*, 1985; Goodfellow *et al.*, 1986; Goodfellow and Goodfellow, 1987; Goodfellow *et al.*, 1987). The X and Y copies of *MIC2*

are homologous (Darling *et al.*, 1986) and *in situ* hybridisation has assigned *MIC2* to Xp22.3 and Yp11-pter respectively (Pritchard *et al.*, 1987; Goodfellow *et al.*, 1988).

In mice, the steroid sulfatase gene, *Sts*, has been shown to be pseudoautosomally located with functional X and Y copies (Keitges *et al.*, 1985). Breeding experiments using high and low activity *Sts* alleles have proved unequivocally that there is at least one obligatory cross-over event between the X and Y chromosomes at meiosis (Keitges *et al.*, 1985; Nagamine *et al.*, 1987). A *Mov15* viral insert in a strain of transgenic mice (Harbers *et al.*, 1986), and recent linkage analysis, has indicated more than one cross-over may be possible in this region (Soriano *et al.*, 1987).

Although in humans *STS* is X-linked rather than pseudoautosomal, there is a non-functioning Y-chromosomal pseudogene at Yq11 (Craig and Tolley, 1986; Fraser *et al.*, 1987). It is thought that an inversion event may have removed the Y copy of *STS* from the pseudoautosomal region, and that at the same time, brought *TDF* close to the pseudoautosomal boundary (Fraser *et al.*, 1987; Craig *et al.*, 1987).

The size of the pseudoautosomal region is estimated at only 5×10^6 base pairs in length, so a recombination rate at meiosis in this region is likely to be ten times higher in males than the rate for the genome in general. This factor is a persuasive indicator that, in spite of the limited size of the region, not just pairing, but the recombinational event may be of paramount importance in males (Rouyer *et al.*, 1986; Goodfellow and Goodfellow, 1987).

2.2.5.2 Consequence of pairing failure

Disruption of sex chromosome pairing, like that observed for autosomes, is associated with varying degrees of spermatogenic breakdown. Findings such as those of Beechey (1973) of spermatogenic failure in an XY mouse with 100% non-association of sex chromosomes, and of Chandley *et al.* (1976) who found a positive correlation between X-Y non-association and infertility, have lent support to the hypothesis that X-Y chromosome pairing is a prerequisite for normal spermatogenesis (Searle, 1982).

By what mechanism could failure of sex chromosome bivalent formation at meiosis lead to spermatogenic faults? Two major models were proposed in the early 70's relating to spermatogenic failure. Lifschytz and Lindsley (1972), reviewing X-autosome translocations, concluded that since the X chromosome in all organisms with heteromorphic X and Y chromosomes was positively heteropycnotic, (which is correlated with late replication and inactivation - Monesi, 1965; Kofman-Alfaro and Chandley, 1970), X-inactivation was a basic meiotic control mechanism at the chromosome level. They proposed that X-inactivation was a necessary requirement for spermatogenesis and anything that interfered with this, e.g. X-chromosome translocations, would result in a disturbance to spermatogenesis. Further studies of autosomal translocations, led Forejt and Gregorova (1977) and Forejt (1982) to suggest that delays at the pachytene stage could result in non-homologous sex chromosome to autosome associations, which would in turn interfere with X-inactivation, and therefore lead to spermatogenic impairment. This model cannot readily be extended to explain spermatogenic failure associated with X-Y

dissociation and X univalence. The second model was proposed by Miklos (1974), who extrapolated data and rationale from his extensive studies of chromosome pairing in *Drosophila melanogaster*. He postulated that chromosomes have pairing sites which must interact and become 'saturated' for normal post-meiotic development. Any situation, such as sex chromosome univalence, leading to 'unsaturated' sites, would result in spermatogenic disruption and sterility. The Miklos model can be put to the test when observing a number of anomalous sex chromosome situations.

XYY individuals frequently have unpaired sex chromosomes at meiosis, so this potentially seems to be a good test of the pairing hypothesis. XYY sex chromosome aneuploidy was first recorded in humans by Sandberg *et al.* (1961) and has subsequently been identified in mouse and rat (Cattanach and Pollard, 1969) as well as various other mammals (reviewed by Searle and Wilkinson, 1986; Hale and Greenbaum, 1986). The fertility in humans ranges from near normal to severe impairment (Skakkebaek *et al.*, 1973; Baghdassarian *et al.*, 1975) with only two recorded cases of transmission of a YY gamete giving rise to XYY sons (Tzoneva-Maneva *et al.*, 1966; Sundequist and Hellstrom, 1969). The spermatogenic impairment in man is thought to be due, at least in part, to disruption of chromosome pairing (Chandley *et al.*, 1984). Although there are numerous examples stating that the second Y is absent in surviving germ cells of XYY men, Hulten and Pearson (1971), using a fluorescent staining technique have identified two Y chromosomes in a greater proportion of germ cells than had previously been estimated. It is probable that many XYY men have a mosaic germ line and that the degree of fertility is proportional to the number of normal XY cells in their testis tubules.

By contrast, XYY mice are usually sterile. Rathenberg and Muller (1973) and Evans *et al.* (1978) have reported that XYY mice show severe spermatogenic failure and oligospermia. Rathenberg and Muller (1973) observed predominantly Y-Y and X-Y bivalents with X and Y univalents at meiosis and more than 50% loss of spermatocytes between metaphase I and metaphase II. Whilst four XYY mice examined by Evans *et al.* (1978) showed all types of associations, five times more XY, Y than XYY cells were observed at MI. Only one of these males was transiently fertile, and even in this individual the sperm count was only 1% of normal (Evans *et al.*, 1978). More recently, Das and Behera (1984) found both XY and XYY spermatocytes in an XO/XY/XYY mosaic mouse. Whilst the 41, XYY cells exhibited all types of associations, there were three times the number of XY, Y to XYY spermatocytes. The fertility of this mouse was not determined since it was unmated, although it was reported to have normal looking sperm.

Three sex chromosomes at meiosis in XYY males may well disrupt normal pairing but the additional problem of two Y chromosomes, which implies a surfeit of Y-derived genes, may also cause genetic imbalance with deleterious consequences. Furthermore, these two problems need not necessarily be mutually exclusive. Burgoyne (1979) and Burgoyne and Biddle (1980) attempted to distinguish between these two possibilities by statistical analysis of the available meiotic XYY data, and they argued, in support of the pairing hypothesis, that the model of 'best fit' was one in which only MI trivalent configurations contributed to MII. Recent observations, however, in XO/XY/XYY mosaic mice (Palmer *et al.*, 1989b - in press) have shown that despite very high trivalent and Y-Y bivalent pairing at early pachytene, XYY cells were rapidly eliminated in the late

pachytene stages and virtually all were gone by MI. Hunt and Eicher (1989 - unpublished) have recently described some fertile XYY^{del} male mice (see Section 3.4). They attributed this fertility to the reduced amount of Y material, compared to XYY males, although in general the results were somewhat inconclusive. However, findings from a similar system using $XY\psi^m$ males with a mutated Y chromosome, ψ^m (Lovell-Badge, 1990 - in preparation), show that despite two normal sized Y chromosomes, these males can be fertile (Mahadevaiah and Burgoyne - unpublished). One possible explanation for such diversity could be the variation attributable to genetic background, which separate studies in our laboratory have shown affects the level of spermatogenic success and fertility in XYY mice (Mahadevaiah and Burgoyne - unpublished).

Since XYY mice have two copies of the Y short arm and three chromosomes it is interesting to compare pairing and fertility in this condition with that of $XYSxr^a$ mice which also have two copies of the Y short arm but only two chromosomes (see Section 3.2.1). $XYSxr^a$ mice were originally reported to have normal pairing at meiosis (Cattanach, 1975), but subsequent studies have demonstrated increased asynapsis (Winsor *et al.*, 1978; Chandley and Fletcher, 1980). Further studies have confirmed pairing irregularities and spermiogenic death in $XYSxr^a$ mice (Chandley and Speed, 1987) and Tease and Cattanach (1989) have shown that this is due to the Sxr^a fragment in some way interfering with correct chromosome alignment and pairing. Burgoyne and Baker (1984) suggested that the mosaic appearance of $XYSxr^a$ testes, initially described by Hannapel and Drews (1979), was due to regions of normal or defective pairing, the latter leading to cell degeneration. In support of this theory, is the fact that from breeding data, $XYSxr^a$ mice do not

produce more aneuploid offspring, which would be expected if the univalents arising from disrupted pairing segregated randomly and contributed to viable gametes.

Sex-reversed $XOSxr^a$ mice, with a univalent X chromosome, would appear to be an ideal system to investigate the consequences of lacking a pairing partner at meiosis. $XOSxr^a$ mice suffer spermatogenic disruption around the time of metaphase I and have high levels of degenerating secondary spermatocytes (Cattanach *et al.*, 1971; Cattanach, 1975). Surviving spermatids are mainly diploid (Levy and Burgoyne, 1986a), and any surviving spermatozoa have abnormal heads (see Sections 3.2.1 and 3.2.2). The disadvantage of the $XOSxr^a$ system as a suitable model, is the loss of the Y long arm. X^YO mice (Eicher *et al.*, 1983; Eicher and Washburn, 1986) (see Section 3.4), escape this problem, since they have virtually all of the Y chromosome attached to most of the X chromosome, yet remain univalent. In terms of the Miklos hypothesis, this would provide a unique test of the pairing model. Unfortunately, although some of these mice have been reported as fertile, there has so far been no systematic detailed study undertaken in these mice.

2.2.6 Conclusion

From the concept that the Y chromosome in mammals had the sole function of determining sex, the pendulum has swung far in the opposite direction and the Y chromosome is now realised to have an important contribution both in terms of genetic function and as a pairing partner for the X during male meiosis. This thesis attempts to further the understanding of the various functions of the Y in spermatogenesis; specifically:-

(a) To provide further details relating to the spermatogenesis gene *Spy*, by a quantitative analysis of spermatogenesis in XOSxr^b mice.

(b) To document details of the spermatogenic block in XOSxr^a males and the partial impairment in XYSxr^a males, by a quantitative analysis of spermatogenesis through puberty to adulthood.

(c) Thirdly, to try and ascertain to what extent the spermatogenic defects in XOSxr^a mice are due to the unpaired X chromosome or to the absence of the Y long arm, by providing a pairing partner for the XSxr^a chromosome, i.e. XSxr^aY^{del}.

3. BACKGROUND TO PROJECT

Before moving to the next chapter, it is deemed appropriate to describe a number of background aspects to this study.

3.1 Spermatogenesis

This thesis aims to demonstrate the Y chromosome involvement in spermatogenesis. It seems relevant, therefore, to briefly describe the spermatogenic stages in the developing mouse. Primordial germ cells, i.e. the embryonic germ cell lineage, are first identified by differential staining of the enzyme alkaline phosphatase which is highly expressed in the mesoderm around 8 days *post coitum* (dpc) (McLaren, 1985). The germ cells reside in the mesoderm at the base of the allantois having been derived from the epiblast (McLaren, 1985). When first identified, germ cells number less than 100 (McLaren, 1983a) but proliferate as they

migrate through the hindgut into the genital ridges overlying the mesonephric kidney by 10 - 11 dpc.

Sexual differentiation is first seen around 12 1/2 dpc in males when new, large, clear primordial Sertoli cells aggregate around the germ cells and form cords (Spiegelman and Bennett, 1973). The germ cells, now termed M-prospermatogonia (Multiplying prospermatogonia) (Hilscher *et al.*, 1974; Hilscher and Hilscher, 1978), undergo several more divisions but by around 14 1/2 - 16 1/2 dpc arrest at the G1 stage as T₁-prospermatogonia (Transitional prospermatogonia) (Hilscher and Hilscher, 1978; Hilscher, 1981b; McLaren, 1984). Resumption of mitosis, commencing the day after birth (20 1/2 dpc), gives rise to a second prospermatogonial stage T₂, and the daughter cells of this division are the undifferentiated A stem cells - the first spermatogonia (Hilscher *et al.*, 1974).

Some controversy surrounds the existence of T₂-prospermatogonia, and the mode of regeneration of undifferentiated stem cells, with some researchers believing that the daughter cells of the T₁ division are the stem cells (Clermont and Bustos-Obregon, 1968; Oakberg, 1971; Huckins and Oakberg, 1978; Hilscher, 1981a; Kluin and de Rooij, 1981). The stem cells have the dual function of establishing a self-replenishing system and proliferating to form the ongoing differentiating spermatogonia. Most evidence for the following six spermatogonial stages, A₁ - A₄, Intermediate and B, has been inferred from adult studies (Huckins, 1971; Clermont, 1972; Huckins and Oakberg, 1978). The final division of B spermatogonia gives rise to the interphase meiotic (preleptotene) cells around 27 1/2 dpc. Five meiotic prophase stages

follow: leptotene, zygotene, pachytene, diplotene and diakinesis, with the first pachytene cells seen around 32 1/2 dpc (Sung *et al.*, 1986). The metaphase I reduction division leading to MII secondary spermatocytes is first seen around 39 1/2 dpc. The development of rounded and then elongated (condensed) spermatids as described by Oakberg (1956), continues over the next two to three weeks so that by six weeks of age the young adult male is fully developed and has motile sperm. Fig. 1b shows an outline of the various germ cell divisions and stages of spermatogenesis in the mouse, from the foetus to the adult.

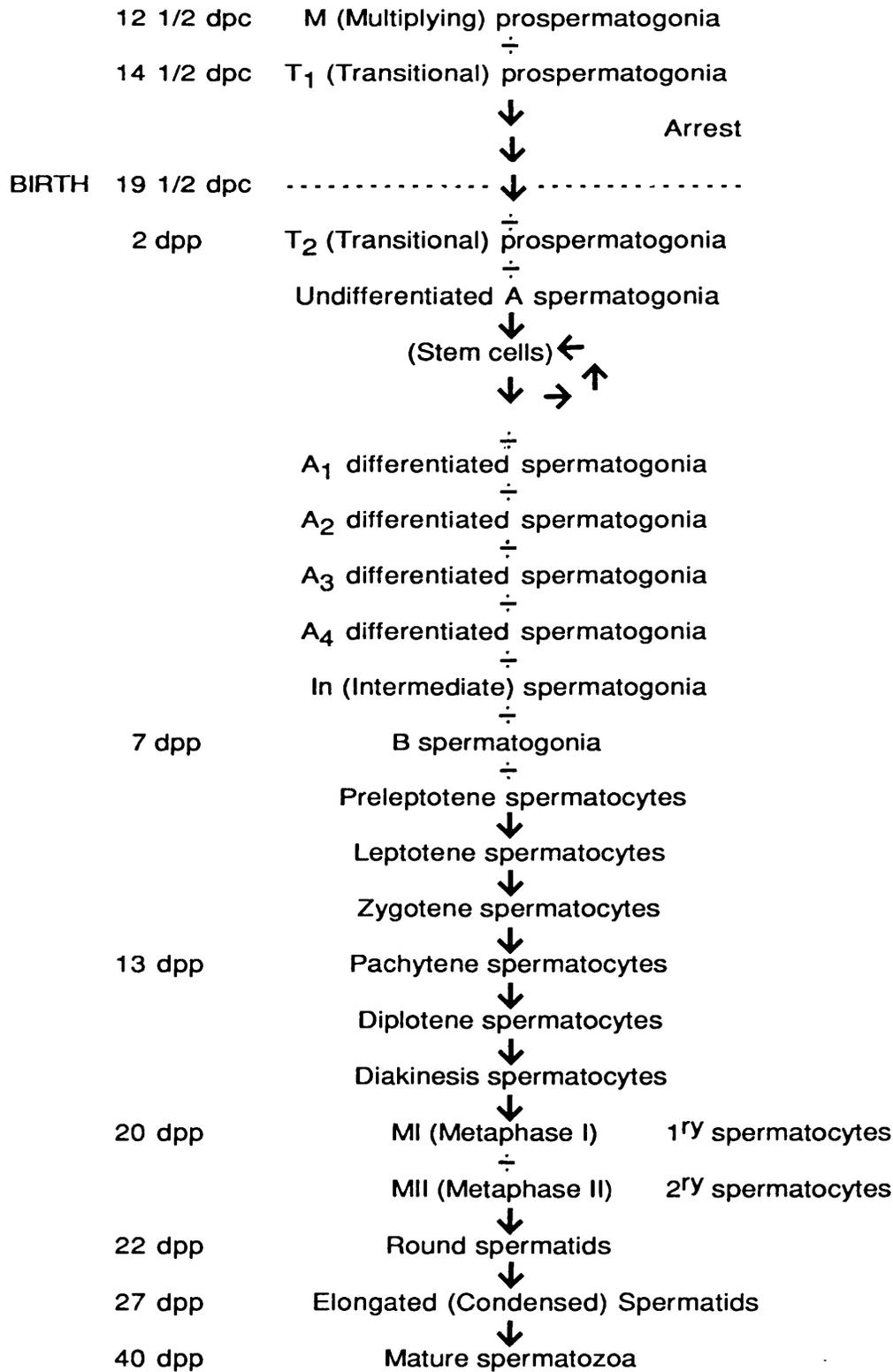


Fig. 1b. Outline of spermatogonial and meiotic stages in the developing mouse from foetus to adult.

3.2 Sex-Reversed (Sxr)

The term sex-reversed (Sxr) is used to describe an inherited form of sex reversal which causes XX and XO mice with Sxr to develop as phenotypic males (Cattanach *et al.*, 1971). Sex reversal is also now known in a number of other mammals including man (Cattanach, 1974; Chandley and Fletcher, 1980). With the recent discovery of Sxr variants, a new terminology was introduced with the original Sxr called Sxr^a (McLaren *et al.*, 1988) and this is the form used throughout this thesis.

3.2.1 Sxr^a

XXSxr^a adult mice were found to have small testes devoid of germ cells. The presence of two X chromosomes in male germ cells is incompatible with their survival beyond the T₂-prospermatogonial stage (Lyon, 1970; Burgoyne, 1978; McLaren, 1983b; Hilscher and Hilscher, 1989 - personal communication), so in order to investigate the effects of Y-chromosomal deficiencies, it is necessary to produce males with a monosomic X. Whilst having all stages of spermatogenesis XOSxr^a mice had reduced numbers of spermatids, which were often diploid, and few spermatozoa that were, in any case, abnormal although motile (Cattanach *et al.*, 1971; Levy and Burgoyne, 1986a; Burgoyne, 1987a).

Although the segregation of Sxr^a appeared to follow an autosomal dominant pattern of inheritance, extensive studies failed to map it to an autosome (Cattanach, 1975; Evans *et al.*, 1980; Chandley and Fletcher, 1980). In a series of experiments by Singh and Jones (Singh *et al.*, 1981; Jones and Singh, 1981; Singh and Jones, 1982), a Bkm probe

isolated from the sex-determining chromosome of the banded krait snake, was found to hybridise to male, but not female, DNA in the mouse. Although their detailed interpretation was incorrect, they did demonstrate that a part of the Y chromosome had transferred to the X in XXSxr^a mice. These molecular studies, coupled with cytogenetic evidence (Evans *et al.*, 1982), provided proof for the hypothesis that Sxr^a arose by a transposition of the testis-determining region to the distal region of the Y chromosome long arm beyond the pairing and exchange region, thus allowing crossing-over onto the X chromosome during male meiosis (Burgoyne, 1982; Eicher, 1982; Hansmann, 1982). Sex reversal in mice, therefore, provided the first real evidence of the postulated X-Y cross-over event. Recently it has been shown that the testis-determining region in the mouse is located on the Y short arm (Roberts *et al.*, 1988), so that transposition could most easily have occurred following intrachromosomal irregular fold-back pairing and self-synapsis of the Y chromosome (Bishop *et al.*, 1988; McLaren *et al.*, 1988).

The Y-derived Sxr^a fragment is H-Y antigen positive (*Hya*) (Bennett *et al.*, 1977; Simpson *et al.*, 1981) and clearly carries genes for testis determination and spermatogenesis. Most XYSxr^a mice are fertile, although fertility ranges from normal to sterile (Cattanach, 1975). Hannapel and Drews (1979) observed that in XYSxr^a testes there was a mosaic pattern of normal and defective spermatogenesis. They suggested that in regions that appeared normal only the Y chromosome was expressed, whereas in areas of defective spermatogenesis only Sxr^a was expressed (at this time, the origin of Sxr^a was not known). The interpretation by Burgoyne and Baker (1984), was that the mosaic appearance was due to normal areas with X-Y chromosome pairing, whilst

the defective areas were due to the high level of X-Y non-association caused by the Sxr^a fragment.

3.2.2 Sxr^b

A variant of Sxr, designated Sxr^b, was discovered when crossing a homozygous XSxr^aYSxr^a male with a T(X;16)16H (T16H) translocation female (McLaren *et al.*, 1984). The T16H/XSxr^a propositas proved to be H-Y antigen negative, unlike all previous T16H/XSxr^a females. It was not established whether loss of H-Y antigen expression was due to a mutation or a deletion. The progeny proved to be H-Y antigen negative which demonstrated that H-Y antigen expression was not the product of the testis-determining gene. It has recently been shown that XXSxr^b mice are also negative for the serologically determined antigen SDM (Goldberg, 1988). This finding finally excludes SDM from having any primary male sex-determining function either.

Molecular studies of the Sxr^a region have recently demonstrated that Sxr^b arose as a result of a partial deletion within Sxr^a and it is postulated to have occurred by an irregular intrachromosomal recombinant event (Roberts *et al.*, 1988).

Whilst XXSxr^b and XXSxr^a mice show the same histology, XOSxr^b present an entirely different picture to XOSxr^a (Burgoyne *et al.*, 1986; Burgoyne, 1987b). Disruption in spermatogenesis was seen to occur prior to meiosis in XOSxr^b mice, and it was hypothesised that a spermatogenesis gene (*Spy*), present in Sxr^a, was deleted in Sxr^b (Burgoyne *et al.*, 1986). As

a correlation existed between the absence of *Spy* and *Hya* expression, it was postulated that they could be one and the same.

3.3 XO Development

In human females, XO Turner's syndrome (Turner, 1938) individuals suffer phenotypic and gonadal disruption and sterility (Ford *et al.*, 1959). By contrast, XO female mice are viable and fertile (Welshons and Russell, 1959), although fecundity is reduced due to oocyte pool depletion (Lyon and Hawker, 1973; Burgoyne and Baker, 1981a) and litters are smaller with a higher mortality rate (Cattanach, 1962; Burgoyne and Biggers, 1976). XO female foetal development has been shown to be delayed by about 3 1/2 hours at 7 1/4 dpc - the earliest stage studied (Burgoyne *et al.*, 1983a) and XO mice have a lowered birthweight compared to controls (Burgoyne and Baker, 1981b; Burgoyne *et al.*, 1983b). It has been suggested that reduced post-natal growth rate may be connected to findings of altered metabolism and hypothyroidism (Deckers and van der Kroon, 1981).

The majority of studies to date have considered XO females with a paternal X (X^P), and a null-0 gamete from the mother. The paternal X undergoes preferential X-inactivation in extra-embryonic tissue in the early embryo (Harper *et al.*, 1982) probably due to differential methylation. It is possible, therefore, that XO females who derive their X chromosome maternally (X^mO) may suffer less deleterious effects than those described with a paternal X, (X^PO). Regardless of the causative factor, a residual 'XO effect' pertains. When XO male mice (e.g. such as $XOSx^a$, $XOSx^b$ and $XSx^{aY^{del}}$) are bred, consideration must be given to

the possibility of inherent developmental delay attributable to an 'XO effect'.

3.4 \underline{XY}^*

In the mouse, an opportunity exists to selectively breed from males with Y chromosomal rearrangements or partial deletions. One such structurally rearranged Y chromosome in the mouse is denoted Y^* (Eicher, 1982; Eicher *et al.*, 1983). Amongst the gametes, two translocation products are produced; one, symbolised Y^X , comprises a centromere and the pseudoautosomal region, now described as Y^{del} by Hunt and Eicher (1989 - unpublished), and this will be the form used throughout this thesis. The other product, symbolised X^Y , comprises almost the entire Y chromosome attached distally to most of the X chromosome (Eicher and Washburn, 1986). XX^Y males are sterile because the presence of two X chromosomes is incompatible with normal male germ cell development (Lyon, 1970). From matings of XY^* males with XO females, some X^YO male progeny were produced. These males have motile sperm and are sometimes fertile and type H-Y antigen positive, thus genetic information necessary for male determination and spermatogenesis is located within this attached Y region (Eicher and Washburn, 1986). XY^{del} mice are, of course, female since Y^{del} has lost the testis-determining region. A schematic showing the possible rearrangement for the Y^* chromosome is shown in Fig. 1c.

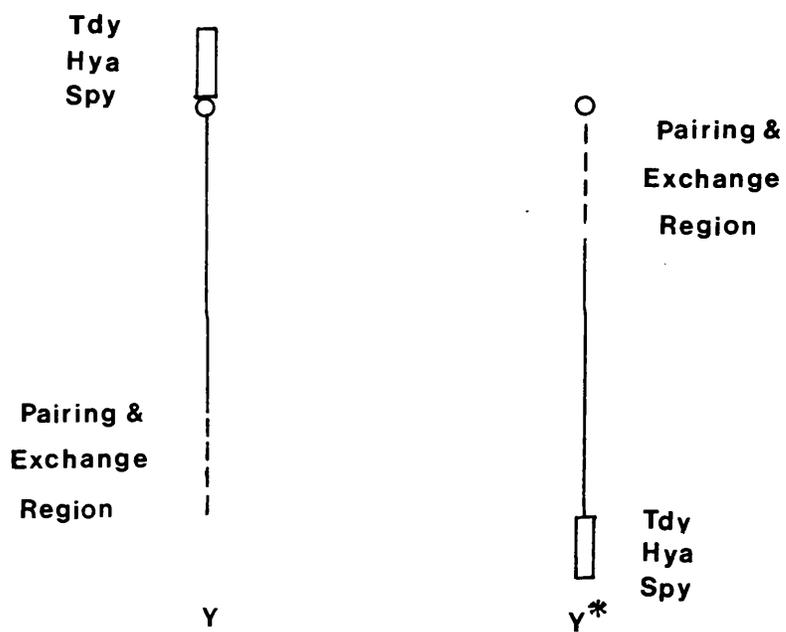


Fig. 1c. Schematic showing the proposed Y^* chromosomal rearrangement in the mouse.

CHAPTER 2

**A QUANTITATIVE ANALYSIS OF XOSxr^b TESTES DURING
THE FIRST TWO WEEKS AFTER BIRTH**

INTRODUCTION

An inherited form of sex reversal (Sxr^a) in mice, recently designated $Tp(Y)1Ct$, causes XX and XO individuals to develop as phenotypically normal males (Cattanach *et al.*, 1971; Cattanach, 1988). It is now known that Sxr^a originated by the Y short arm transposing, distal to the pairing and exchange region, to the tip of the Y long arm (McLaren *et al.*, 1988; Roberts *et al.*, 1988). This transposed Y short arm, regularly crosses-over onto the X chromosome at meiosis, such that XX, $XXSxr^a$, XY and $XYsxr^a$ progeny are produced in a 1:1:1:1 ratio (Burgoyne, 1982; Eicher, 1982; Evans *et al.*, 1982; Hansmann, 1982; Singh and Jones, 1982).

In addition to testis-determining information, Sxr^a types positive for transplantation H-Y antigen expression (Bennett *et al.*, 1977; Simpson *et al.*, 1981). A variant of Sxr^a , called Sxr^b , although retaining testis-determining information, has lost this H-Y antigen gene (McLaren *et al.*, 1984; Simpson *et al.*, 1986). This finding negated the widely accepted hypothesis of Wachtel *et al.* (1975), that H-Y antigen was the primary testis determinant. In that Wachtel *et al.* (1975) did not recognise two H-Y antigens (i.e. transplantation and serological SDM), this evidence only related to the transplantation H-Y antigen, but has recently been extended to include SDM (Goldberg, 1988).

Both $XXSxr^a$ and $XXSxr^b$ males are sterile. In $XXSxr$ males, the second X chromosome is reactivated in the foetal germ line at the same time as in XX females (McLaren and Monk, 1981), and two active X chromosomes are detrimental to the survival of male germ cells post-natally (Lyon, 1970; McLaren, 1983b), such that few daughter cells survive the T_1 -

prospermatogonial mitotic division (Hilscher and Hilscher, 1987 - personal communication). In order to investigate the effect of the Y-chromosomal loss in Sxr mice, it is therefore necessary to produce males with a univalent X chromosome, i.e. XOSxr^a or XOSxr^b.

XOSxr^b males have a severe spermatogenic impairment compared to XOSxr^a mice, with only a few germ cells occasionally reaching early meiotic prophase (Burgoyne *et al.*, 1986). The XO germ cells in an XO/XY/XYY mosaic male suffered a similar fate to XOSxr^b germ cells, despite a normal XY Sertoli cell environment (Levy and Burgoyne, 1986b). These findings led Burgoyne *et al.* (1986) to suggest that Sxr^a carries a spermatogenesis gene (*Spy*) which acts cell autonomously in the germ line, and that Sxr^b, which recent studies show arose by a deletion of DNA from Sxr^a (Bishop *et al.*, 1988; Mardon *et al.*, 1989), has lost *Spy*.

Handel (1987) stated that the only definitive evidence for the presence on the Y chromosome of a gene controlling spermatogonial proliferation would be specific information about the differentiation of spermatogenic cells in mice lacking portions of the Y chromosome. The purpose of the present study was to define the timing of the spermatogenic block in XOSxr^b mice by a quantitative analysis of germ cells in the first two weeks after birth, when the block first becomes apparent. It is clear that the spermatogenic block associated with the deletion in Sxr, clearly satisfies Handel's criterion.

MATERIALS AND METHODS

Mice

XYSxr^b males were mated with females heterozygous for the inversion In(X)1H. In(X)/X females produce some nullo-X eggs following crossing-over within the inversion (Evans and Phillips, 1975) and approximately 1 in 19 of the progeny from this cross have the XOSxr^b genotype (see Appendix 2a). The In(X)/X females were checked for vaginal plugs each morning, and coitus was presumed to have taken place at the midpoint of the previous dark cycle. Ages were calculated from conception, rather than birth, because it is known that the duration of pregnancy is affected by litter size. The majority of litters were born about 19 1/2 days *post coitum* (dpc), so that in what follows, this is equated with the day of birth. 157 litters were bred of which 59 included XOSxr^b males. Litters were processed from 19 1/2 dpc through 30 1/2 dpc (11 days *post partum*), 32 1/2 dpc (13 dpp) and 59 1/2 dpc (40dpp).

A similar breeding cross was set up to produce XOSxr^a males as a control for a possible 'XO effect'. Data from 35 litters are included in this study. The litters were processed at 19 1/2, 21 1/2 through 24 1/2, 27 1/2, 29 1/2, 31 1/2 and 33 1/2 dpc.

Body weights were recorded at autopsy. Following exclusion of 'runts' (Burgoyne *et al.*, 1983a), litters were evaluated, provided at least one XOSxr and one XY_±Sxr male was present. Both a qualitative and quantitative analysis demonstrated that XY and XYSxr testes were not

significantly different during the pre-meiotic stages (see Chapter 3), therefore XY and XYSxr males were not separately identified. 52 Sxr^b and 35 Sxr^a litters finally provided data.

Karyotyping

Mitotic spreads were prepared either by dissociating liver fragments (19 1/2 and 20 1/2 dpc) or by flushing out bone marrow cells (21 1/2 dpc onwards) in 0.004% colcemid in HEPES-buffered Eagle's minimal essential medium, and incubating at 32°C for 60 minutes (liver) or 15 minutes (bone marrow). Cells were then treated with 0.56% potassium chloride for 20 minutes followed by five changes of 3:1 methanol:glacial acetic acid fixative. The cells were then air-dried on slides and stained for 15 minutes in 2% Giemsa in pH 6.8 buffer. XOSxr males were identified by scoring at least 5 consecutive spreads with 39 chromosomes and no evidence of a Y chromosome. XY_±Sxr males were identified by 40 chromosomes, with a Y recognised by size and the presence of splayed arms (Ford, 1966).

Histology

Both testes from each male were weighed using a Cahn electrobalance, and then retained in Bouin's fixative awaiting the results of karyotyping. Testes from XOSxr and XY_±Sxr littermates were dehydrated through a series of alcohols and then cleared in cedar wood oil. The testes were embedded in paraffin wax, and serially sectioned at 3 µm. The sections were cleared with histoclear, rehydrated in alcohol and stained with haematoxylin and eosin, according to standard procedures.

Identification of Spermatogonia

Whilst the identification of meiotic cells is fairly straightforward since each stage has characteristics that are easy to recognise, the spermatogonial stages show more subtle differences. Their morphology has been characterised and described in adult rats and mice with reference to the adjacent meiotic stages, since there is a hierarchal order within the adult tubule from the basement membrane towards the lumen (Oakberg, 1956; Roosen-Runge, 1962; Monesi, 1962; Huckins, 1971; Oakberg, 1971; Huckins and Oakberg, 1978). The prospermatogonial stages in the prenatal and neonatal rat have been described by Hilscher *et al.* (1974) and Hilscher and Hilscher (1976).

A description of some of the problems of spermatogonial identification in the immature mouse together with the other references consulted, are described in Appendix 2b. Stage identification is outlined below:-

(a) T₁-prospermatogonia : large, round nucleus with one or two round 'blobs' of heterochromatin, often with a clear 'halo' around them. Usually located near the centre of the tubule. Example shown in Plate 2a.

(b) T₂-prospermatogonia : large, round nucleus with two or three irregular shaped 'blobs' of heterochromatin and no 'halo'. Often located at the periphery of the tubule adjacent to the basement membrane. Example shown in Plate 2b.

(c) Undifferentiated A spermatogonia : Large, usually oval, homogeneously granular nucleus with one small, round heterochromatic

'blob'. Location adjacent to the basement membrane, often lying horizontally. The first of the spermatogonial stages, these undifferentiated cells are believed to be the stem cell stock. Example shown in Plate 2c.

Five of the following six stages, all differentiating spermatogonia, lie mostly adjacent to the basement membrane, often with a clear visible ring of cytoplasm surrounding the nucleus. The nuclei progressively show a decrease in diameter and an increase in the amount of heterochromatisation.

(d) A_1 spermatogonia : Rounder and slightly smaller, with one or two small round 'blobs' of heterochromatin. Example shown in Plate 2d.

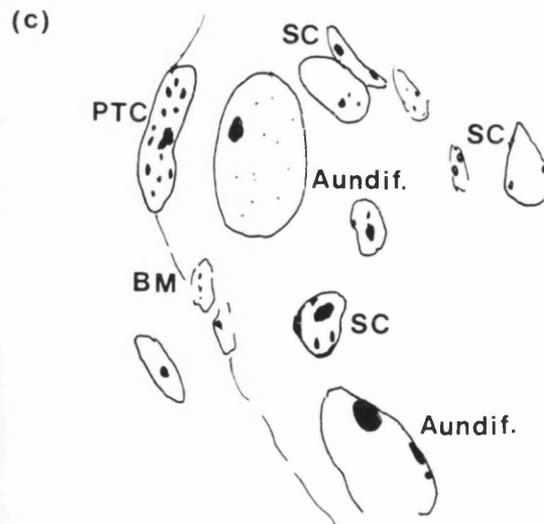
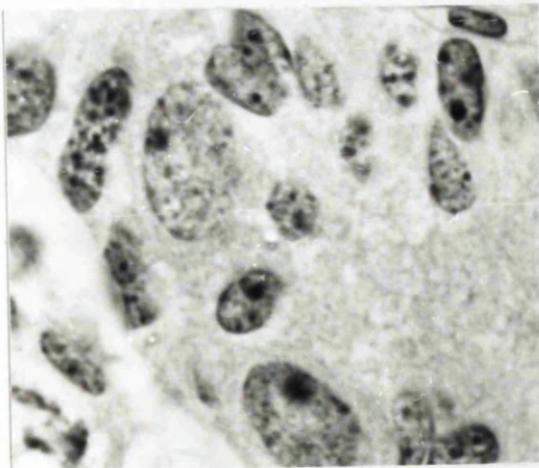
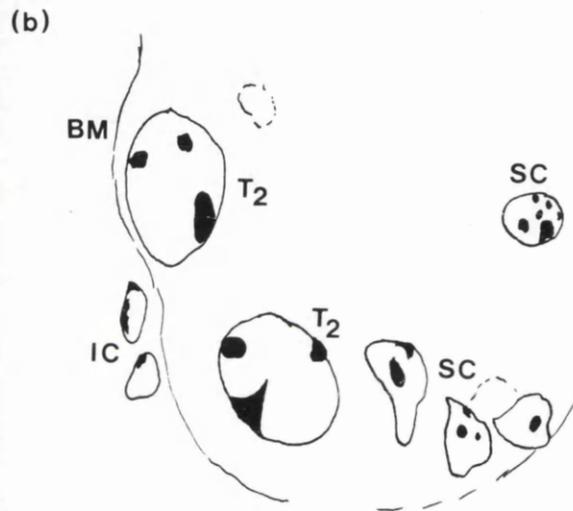
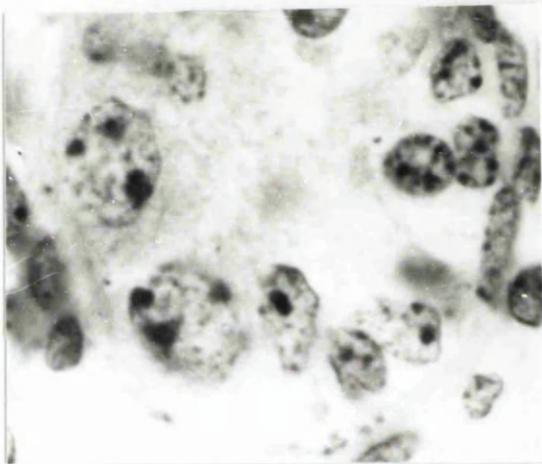
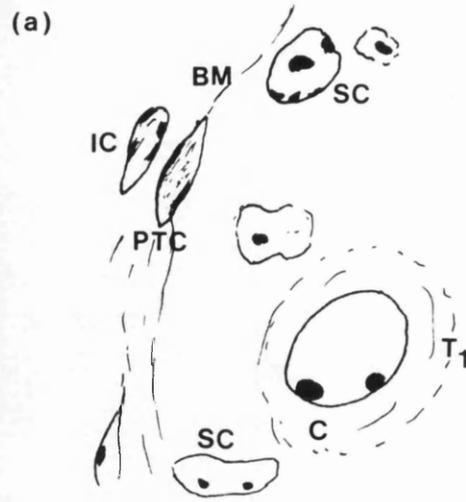
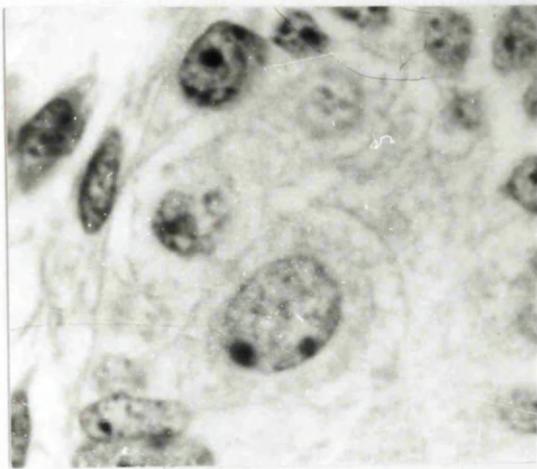
(e) A_2 spermatogonia : Round or oval, only slightly smaller with two to four round 'blobs' of heterochromatin. Example shown in Plate 2e.

(f) A_3 spermatogonia : Round or oval, smaller and with the first signs of a 'crust-like' ring around the nuclear membrane as well as two to four 'blobs' of heterochromatin. Example shown in Plate 2f.

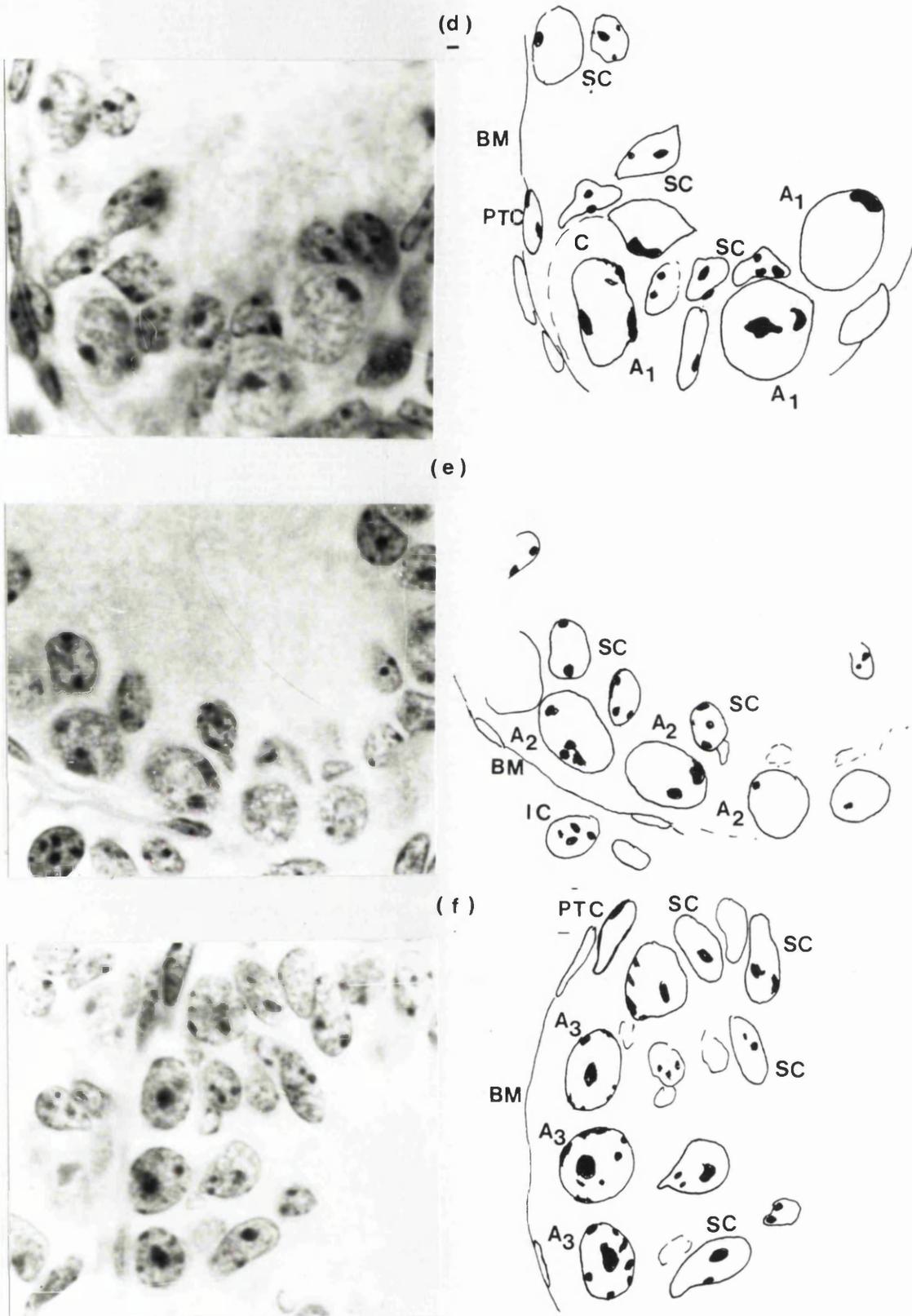
(g) A_4 spermatogonia : Round or oval, small, with a heavy 'crust' around the nuclear membrane and more than three 'blobs' of heterochromatin. Example shown in Plate 2g.

(h) Intermediate (In) spermatogonia : Smaller, round, heavily encrusted nuclear membrane intermingling with heterochromatic 'blobs', usually in small clusters at the periphery with a few towards the lumen. Example shown in Plate 2h.

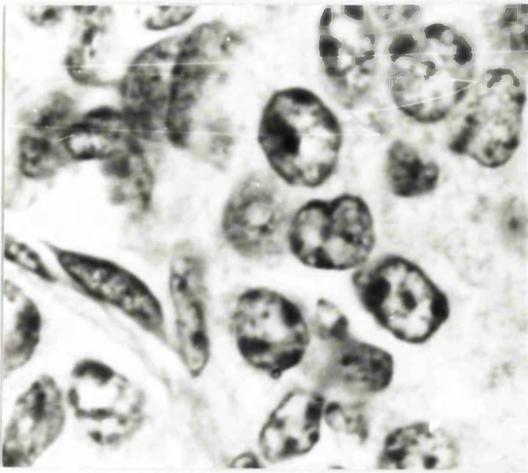
(i) B spermatogonia : Similar to the previous stage except even smaller, and present in larger clusters, mainly located in the centre of the lumen, with only a few near the periphery. Example shown in Plate 2i.



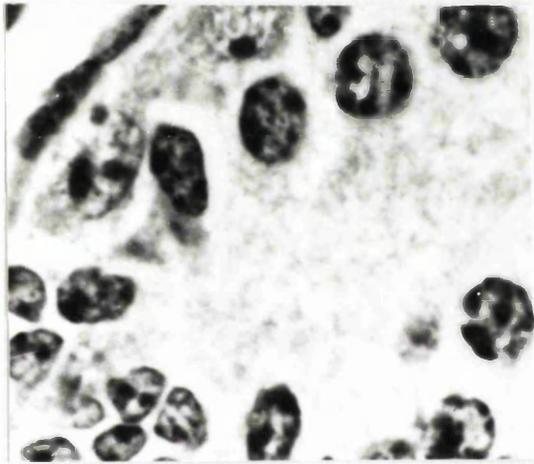
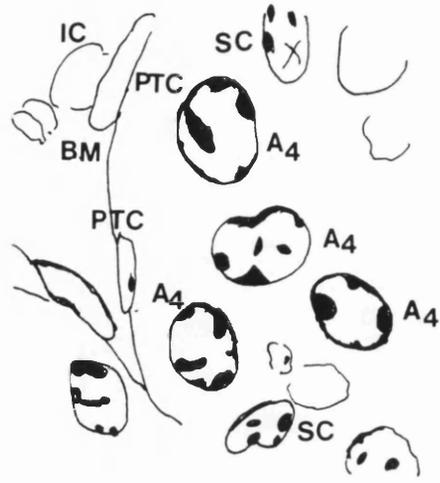
Plates 2a - c. Histological sections showing stages of (a) T_1 -prospermatogonia, (b) T_2 -prospermatogonia, and (c) undifferentiated A spermatogonia. BM = basement membrane; SC = Sertoli cell; PTC = pertitubular cell; IC = interstitial cell; C = cytoplasm. Magnification X1350.



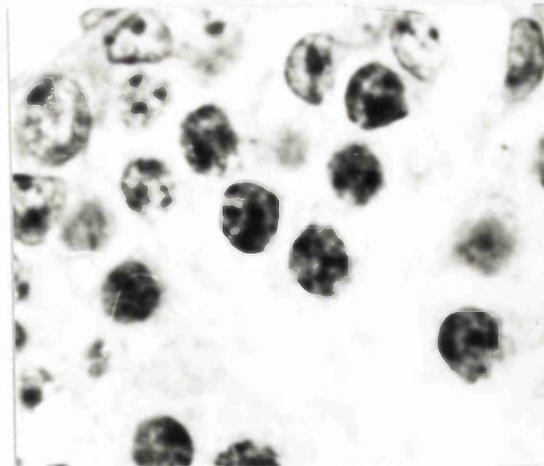
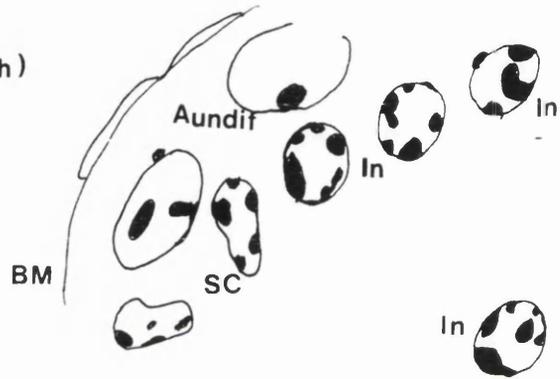
Plates 2d - f. Histological sections showing stages of (d) A₁ differentiated spermatogonia, (e) A₂ differentiated spermatogonia, and (f) A₃ differentiated spermatogonia. BM = basement membrane; SC = Sertoli cell; PTC = peritubular cell; IC = interstitial cell; C = cytoplasm. Magnification X1350.



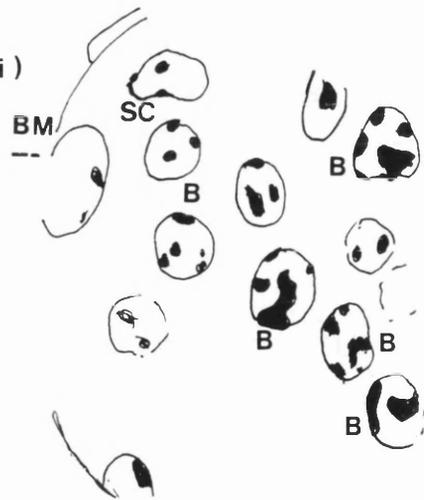
(g)



(h)



(i)



Plates 2g - i. Histological sections showing stages of (g) A4 differentiated spermatogonia, (h) Intermediate spermatogonia, and (i) B spermatogonia. BM = basement membrane; SC = Sertoli cell; PTC = peritubular cell; IC = interstitial cell; C = cytoplasm. Magnification X1350.

Quantitative Analysis

This analysis was carried out 'blind' with respect to genotype of the mice from which the sections were taken. The sampling was one tubule cross-section from every 20th section, or every 10th section for smaller testes, such that between 25 and 35 tubule cross-sections were analysed per testis. The procedure for selecting tubules for analysis was as follows: (a) A 0.25 mm square grid (R-4 grid, Graticules Ltd, Tonbridge, Kent) was 'stuck' to the bottom of the microscope slide with a film of water and a Chalkley grid (G52, Graticules Ltd) was inserted in the eyepiece. (b) When a section was selected, the square grid was focused under low power with a x10 objective and a square chosen at random. The central cross of the Chalkley grid was centered over the square and the section was brought back into focus. (c) The tubule cross-section adjacent to the central cross was analysed using x100 objective under oil immersion, provided it could be encompassed within the field of view.

This selection procedure ensures that all regions of the gonad have an equal chance of being sampled. Once a tubule was selected, all cells within the tubule cross-section were classified as to cell type, except dead or dying cells which were counted but could not be classified. Sertoli cells were scored as being in interphase or division. Gonia were scored as being in interphase or division, and were also classified as to stage, (i.e. T₁-prospermatogonia, T₂-prospermatogonia, undifferentiated A spermatogonia, differentiated A₁ or A₂ spermatogonia, differentiated A₃ or A₄ spermatogonia, Intermediate or B spermatogonia) using the criteria described in the preceding section. It was often difficult to assign divisions to specific spermatogonial stages and in these cases

they were classified according to the adjacent interphase stages in the same tubule. A category existed for cells that could not be classified. This group formed less than 0.5% of the germ cells scored and have been omitted from the analysis. It should be pointed out that these cell counts are crude counts, uncorrected for cell size and thickness of the sections.

RESULTS

The body weight data for the Sxr^b and Sxr^a litters are given in Table 2a. (i) and (ii). The best estimates for the body weights of the four genotypes (XOSxr^b, XY_±Sxr^b, XOSxr^a, XY_±Sxr^a) at the various ages studied are provided by the mean of litter means. In order to compare the two genotypes in each cross, mean weighted differences between these genotypes and the significance of these differences have been calculated from 'within litters' as described by Burgoyne *et al.* (1983b). From these mean weighted differences, it is clear that XOSxr^b mice are underweight when compared with XY_±Sxr^b mice. Despite the limited number of mice at each age, the difference is significant for 5/13 age groups, and pooling across age groups (the mean weighted differences are similar throughout the age range studied) gives an overall estimated weight deficit of -0.359 ± 0.059 g ($P < 0.005$). XOSxr^a mice are not significantly underweight when compared with XY_±Sxr^a mice (pooled mean weighted difference = 0.044 ± 0.069 g).

The body weight difference from the 'within litter' XY_±Sxr mean, for the two genotypes, are shown as histograms in Fig. 2a - b. The negative to positive ratio shows that XOSxr^a weights are fairly evenly distributed whereas the XOSxr^b weights show a heavy negative bias.

The testis weight data for the Sxr^b and Sxr^a litters are given in Table 2b. (i) and (ii). The XOSxr^a testes, Table 2b (ii), are not underweight when compared with XY \pm Sxr^a littermates, but XOSxr^b testes, Table 2b. (i), are significantly underweight for 9/13 of the ages studied. Since XOSxr^b mice are underweight, this testis weight deficit could simply be a reflection of the overall reduction in body weight. The XOSxr^b testis weights were therefore corrected by dividing by individual body weight and multiplying by the mean XY \pm Sxr^b body weight for the relevant litters. The mean weighted XOSxr^b to XY \pm Sxr^b differences for these corrected testis weights are plotted in Fig. 2c. XOSxr^b testes are significantly underweight by 23 1/2 dpc and the weight deficit rapidly increases thereafter.

The reason for the reduced testis weight in XOSxr^b mice is apparent in Fig. 2d, which gives the mean number of germ cells and Sertoli cells per tubule cross-section in XOSxr^b and XY \pm Sxr^b mice, throughout the period studied. As expected, there is a marked increase in the number of germ cells per tubule cross-section in XY \pm Sxr^b mice, but by contrast there is no increase in XOSxr^b mice. There is no deficiency of Sertoli cells in XOSxr^b mice. Indeed the mitotic index for Sertoli cells during the period 19 1/2 - 32 1/2 dpc was found to be very similar in XOSxr^b (0.85%) and XY \pm Sxr^b (0.90%). The mitotic index for Sertoli cells drops to less than 0.3% after 24 1/2 dpc in both genotypes. Clearly, the testis weight deficiency in XOSxr^b mice is due to germinal failure.

In view of the normal numbers of Sertoli cells in XOSxr^b mice, in the more detailed analysis of the germ cell deficiency that follows, germ cell numbers are expressed per 100 Sertoli cells, rather than per tubule cross-section.

In Fig. 2 e - l, germ cell numbers are plotted against age for the various classes of germ cells identified in the scoring procedure. The numbers of T_1 -prospermatogonia are indistinguishable in $XOSxr^b$ and $XY_{\pm}Sxr^b$ mice. However, $XOSxr^b$ mice clearly have fewer T_2 -prospermatogonia than the controls and pooling over the period 20 1/2 - 24 1/2 dpc reveals that $XOSxr^b$ have only 39% of the control value. By contrast, $XOSxr^a$ mice have 91% of the control value. Since T_2 -prospermatogonia are assumed to be the progenitors of the undifferentiated A spermatogonia, a deficit of undifferentiated A spermatogonia is expected in $XOSxr^b$ mice, and is indeed observed ($XOSxr^b$ is 54% of $XY_{\pm}Sxr^b$). Similarly, there is the expected deficit of differentiating A_1/A_2 spermatogonia ($XOSxr^b$ is 42% of $XY_{\pm}Sxr^b$). The number of A_3/A_4 spermatogonia, however, is reduced much more than expected ($XOSxr^b$ is 7% of $XY_{\pm}Sxr^b$) and there are no Intermediate or B spermatogonia.

Examples of histological sections are shown for $XOSxr^b$, $XY_{\pm}Sxr^b$ and $XOSxr^a$ testes in Plates 2j - l. Plate 2j. shows (i) $XOSxr^b$, (ii) $XY_{\pm}Sxr^b$ and (iii) $XOSxr^a$ testes at 24 1/2 dpc. There is virtually no difference between $XOSxr^b$ testes at this stage, except perhaps slightly less evidence of active germ cell division. Three days later, at 27 1/2 dpc, $XOSxr^b$ testes already show signs of the mitotic germ cell block, Plate 2k (i), compared to (ii) $XY_{\pm}Sxr^b$ and (iii) $XOSxr^a$ testes that already have the first wave of B spermatogonia and a few preleptotene cells. By 29 1/2 dpc, $XOSxr^b$ germ cells are virtually depleted and many tubules are Sertoli cells only, Plate 2l (i), whilst (ii) $XY_{\pm}Sxr^b$ and (iii) $XOSxr^a$ tubules show active mitosis and increased numbers of late differentiating spermatogonia, preleptotene and leptotene cells.

This pattern of germ cell deficiency in XOSxr^b mice is largely accounted for by observations on mitotic index (Fig. 2m). That is to say, there is a shortage of dividing T₁-prospermatogonia accounting for the drop in the number of T₂-prospermatogonia; a reduced frequency of divisions among A₁/A₂ spermatogonia accounting for the much more severe shortage of A₃/A₄ spermatogonia; and no dividing A₃/A₄ spermatogonia accounting for the absence of In/B spermatogonia.

During the scoring procedure, the gonial cells with the morphological characteristics of A₁ and A₂ spermatogonia were pooled, although it is assumed that they are distinct generations of spermatogonia as in the adult (Appendix 2a). When the mitotic index of A₁/A₂ spermatogonia is plotted against age (Fig. 2n), there is no marked shortage of divisions in XOSxr^b mice until 25 1/2 dpc, raising the possibility that it is the A₂, rather than the A₁, spermatogonia that are affected.

If A₁/A₂ spermatogonia rarely divide to give A₃ or A₄, but the undifferentiated A spermatogonia continue to divide, one might expect a 'piling up' of A₁/A₂ stages. This is not observed, implying that the cells that fail to divide are degenerating. This is supported by observations on the germ cell degeneration index (Fig. 2o), which has been calculated on the assumption that all the dying cells observed were germ cells. The degeneration index is very low in XOSxr^b and XY_±Sxr^b mice. Nevertheless, from 26 1/2 dpc onwards, XOSxr^b mice clearly have more degenerating cells than controls, which is consistent with the increased degeneration of A₁/A₂ spermatogonia. It is tempting to suggest that the increased degeneration index in XOSxr^b mice at 22 1/2 dpc is similarly due to the death of T₁-prospermatogonia that failed to divide.

Although no Intermediate or B spermatogonia were scored during the quantification, very rare patches of these spermatogonia, and also early meiotic stages can be found in 32 1/2 dpc and adult (59 1/2 dpc) XOSxr^b testes. They occur without the normal hierarchy of stages, and in small patches, as if an occasional A₃/A₄ spermatogonium divides and the products proceed via the usual stages up to early pachytene.

Days post coitum	No. of Mice		Mean \pm s.e.m. body weights (g)*		Mean \pm s.e.m. weighted $XOSxr^b - XY_{\pm}Sxr^b$ difference (g)	Significance of $XOSxr^b - XY_{\pm}Sxr^b$ difference (P)
	$XOSxr^b$	$XY_{\pm}Sxr^b$	$XOSxr^b$	$XY_{\pm}Sxr^b$		
19 1/2	3	5	1.570 \pm 0.03	1.693 \pm 0.08	- 0.120 \pm 0.046	NS
20 1/2	3	9	1.917 \pm 0.10	2.200 \pm 0.10	- 0.284 \pm 0.070	< 0.005
21 1/2	4	12	2.065 \pm 0.15	2.175 \pm 0.10	- 0.120 \pm 0.180	NS
22 1/2	7	16	2.436 \pm 0.12	2.862 \pm 0.11	- 0.496 \pm 0.160	< 0.005
23 1/2	11	14	3.297 \pm 0.24	3.645 \pm 0.30	- 0.237 \pm 0.135	NS
24 1/2	6	19	3.876 \pm 0.16	4.240 \pm 0.13	- 0.347 \pm 0.197	0.05 - 0.025
25 1/2	5	19	4.094 \pm 0.19	4.932 \pm 0.26	- 0.842 \pm 0.108	< 0.005
26 1/2	3	8	5.130 \pm 0.66	5.500 \pm 0.79	- 0.330 \pm 0.266	NS
27 1/2	4	8	5.290 \pm 0.25	5.525 \pm 0.16	- 0.324 \pm 0.144	< 0.005
28 1/2	3	7	6.385 \pm 0.11	6.500 \pm 0.10	- 0.115 \pm 0.194	NS
29 1/2	6	12	6.618 \pm 0.66	6.675 \pm 0.47	- 0.219 \pm 0.284	NS
30 1/2	4	6	7.505 \pm 0.71	8.025 \pm 0.67	- 0.498 \pm 0.332	NS
32 1/2	4	6	8.060 \pm 0.50	8.563 \pm 0.53	- 0.483 \pm 0.407	NS
Pooled mean weighted difference					- 0.359 \pm 0.059	< 0.005

* Mean of litter means

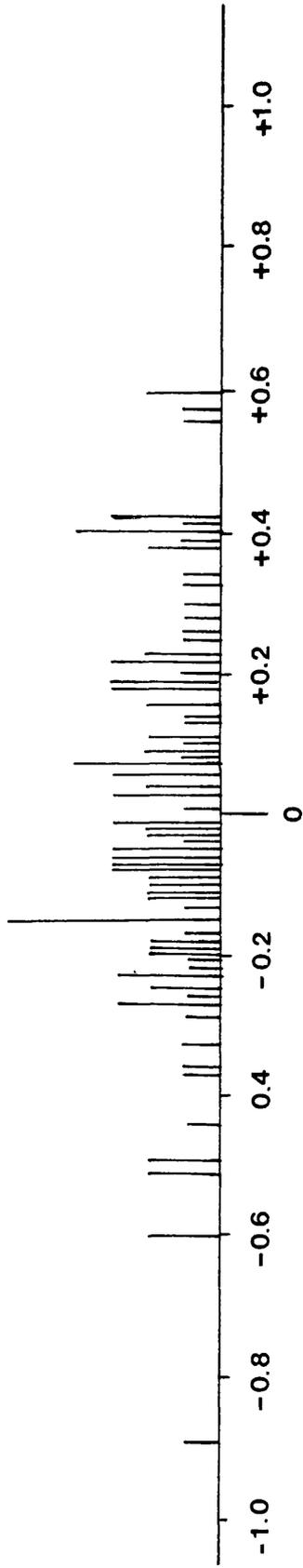
TABLE 2a (i) Mean body weights for $XOSxr^b$ and $XY_{\pm}Sxr^b$ mice and the estimated differences between them for the period 19 1/2 - 32 1/2 dpc.

Days post coitum	No. of Mice		Mean \pm s.e.m. body weights (g)		Mean \pm s.e.m. weighted XOSxr ^a - XY \pm Sxr ^a difference (g)	Significance of XOSxr ^a - XY \pm Sxr ^a difference (P)
	XOSxr ^a	XY \pm Sxr ^a	XOSxr ^a	XY \pm Sxr ^a		
19 1/2	3	6	1.467 \pm 0.04	1.450 \pm 0.01	+ 0.010 \pm 0.050	NS
21 1/2	4	6	2.727 \pm 0.27	2.927 \pm 0.01	- 0.241 \pm 0.103	0.05 - 0.025
22 1/2	5	14	3.142 \pm 0.43	3.148 \pm 0.45	- 0.003 \pm 0.102	NS
23 1/2	4	12	3.448 \pm 0.14	3.465 \pm 0.25	+ 0.004 \pm 0.082	NS
24 1/2	4	12	4.123 \pm 0.31	4.213 \pm 0.17	- 0.130 \pm 0.219	NS
27 1/2	4	10	5.868 \pm 0.40	6.273 \pm 0.54	- 0.404 \pm 0.162	0.025 - 0.010
29 1/2	6	17	6.658 \pm 0.45	6.305 \pm 0.32	+ 0.529 \pm 0.230	0.025 - 0.010
31 1/2	4	15	7.918 \pm 0.38	8.285 \pm 0.47	- 0.367 \pm 0.275	NS
33 1/2	4	12	9.145 \pm 0.62	9.295 \pm 0.33	- 0.092 \pm 0.157	NS
Pooled mean weighted difference					- 0.044 \pm 0.069	NS

* Mean of litter means.

TABLE 2a (ii) Mean body weights for XOSxr^a and XY \pm Sxr^a mice and the estimated difference between them for the period 19 1/2 - 33 1/2 dpc.

$XY \pm S_{xr}b$



$XOS_{xr}b$

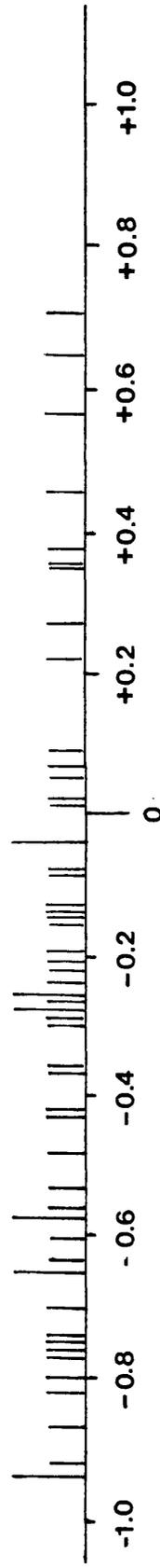
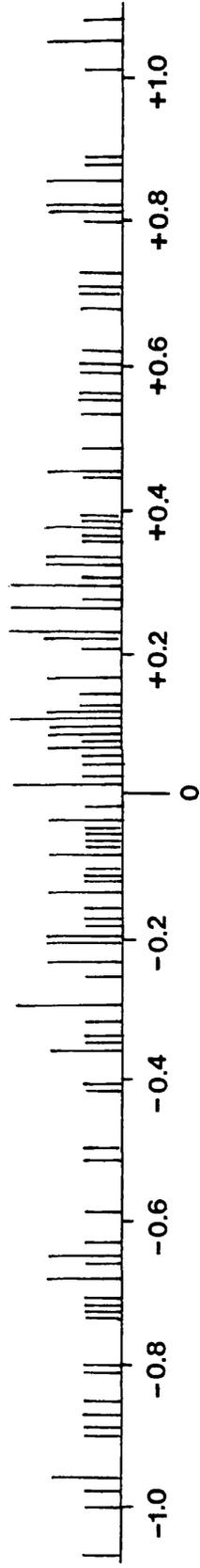


Fig. 2a. Histogram showing the $XOS_{xr}b$ deviation (within litters) from the $XY \pm S_{xr}b$ body weight mean

$XY \pm S_{Xr}^a$



XOS_{Xr}^a

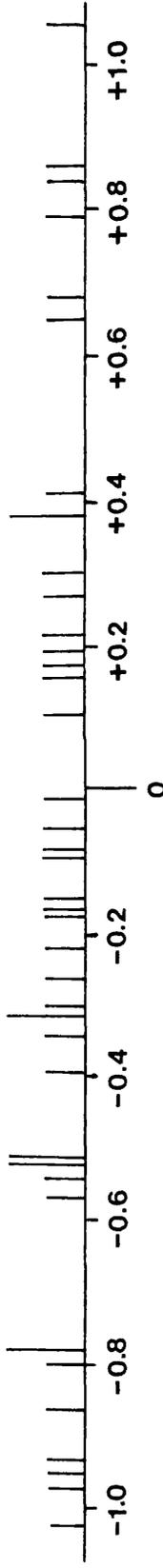


Fig. 2 b. Histogram showing the XOS_{Xr}^a deviation (within litters) from the $XY \pm S_{Xr}^a$ body weight mean

Days <i>post</i> <i>coitum</i>	No. of Mice		Mean \pm s.e.m. testis weights (mg)*		Mean \pm s.e.m. weighted XOSxr ^b -XY+Sxr ^b difference (mg)	Significance of XOSxr ^b -XY+Sxr ^b difference (P)
	XOSxr ^b	XY+Sxr ^b	XOSxr ^b	XY+Sxr ^b		
19 1/2	3	5	0.883 \pm 0.01	0.960 \pm 0.26	- 0.063 \pm 0.019	0.05 - 0.025
20 1/2	3	9	0.980 \pm 0.17	1.167 \pm 0.18	- 0.185 \pm 0.167	NS
21 1/2	4	12	1.183 \pm 0.10	1.260 \pm 0.02	- 0.091 \pm 0.163	NS
22 1/2	7	16	1.440 \pm 0.10	1.782 \pm 0.17	- 0.380 \pm 0.167	0.025 - 0.010
23 1/2	11	14	2.190 \pm 0.14	2.622 \pm 0.20	- 0.373 \pm 0.117	< 0.005
24 1/2	6	19	2.528 \pm 0.13	2.942 \pm 0.05	- 0.402 \pm 0.268	NS
25 1/2	5	19	2.734 \pm 0.18	4.148 \pm 0.22	- 1.396 \pm 0.239	< 0.005
26 1/2	3	8	3.580 \pm 0.18	4.207 \pm 0.25	- 0.606 \pm 0.405	NS
27 1/2	4	8	3.708 \pm 0.27	5.003 \pm 0.23	- 1.232 \pm 0.255	< 0.005
28 1/2	3	7	3.385 \pm 0.68	4.555 \pm 0.48	- 1.114 \pm 0.383	0.025 - 0.010
29 1/2	6	12	4.555 \pm 0.40	6.248 \pm 0.41	- 1.974 \pm 0.613	< 0.005
30 1/2	4	6	4.615 \pm 0.16	7.685 \pm 1.80	- 3.617 \pm 0.896	< 0.005
32 1/2	4	6	6.027 \pm 0.19	10.347 \pm 0.64	- 4.356 \pm 0.293	< 0.005

* Mean of litter means

TABLE 2b (i) Mean testis weights for XOSxr^b and XY+Sxr^b mice and the estimated difference between them for the period 19 1/2 - 32 1/2 dpc.

Days post coitum	No. of Mice		Mean \pm s.e.m. testis weights (mg)*		XY \pm Sxr ^a	Mean \pm s.e.m. weighted XOSxr ^a -XY \pm Sxr ^a difference (mg)	Significance of XOSxr ^a -XY \pm Sxr ^a difference (P)
	XOSxr ^a	XY \pm Sxr ^a	XOSxr ^a	XOSxr ^a			
19 1/2	3	6	0.837 \pm 0.02	0.757 \pm 0.08	+ 0.068 \pm 0.106	NS	
21 1/2	4	6	1.863 \pm 0.18	1.940 \pm 0.10	- 0.097 \pm 0.091	NS	
22 1/2	5	14	2.108 \pm 0.33	2.104 \pm 0.30	- 0.036 \pm 0.105	NS	
23 1/2	4	12	2.608 \pm 0.24	2.563 \pm 0.26	+ 0.078 \pm 0.188	NS	
24 1/2	4	12	2.818 \pm 0.22	3.030 \pm 0.27	- 0.226 \pm 0.228	NS	
27 1/2	4	10	4.938 \pm 0.56	4.898 \pm 0.40	+ 0.059 \pm 0.156	NS	
29 1/2	6	17	6.358 \pm 0.69	5.655 \pm 0.38	+ 0.907 \pm 0.192	< 0.005	
31 1/2	4	15	8.898 \pm 1.20	9.323 \pm 1.15	- 0.435 \pm 0.620	NS	
33 1/2	4	12	12.688 \pm 2.87	12.343 \pm 1.05	+ 0.691 \pm 0.944	NS	

* Mean of litter means.

TABLE 2b (ii) Mean testis weights for XOSxr^a and XY \pm Sxr^a mice and the estimated difference between them for the period 19 1/2 - 33 1/2 dpc.

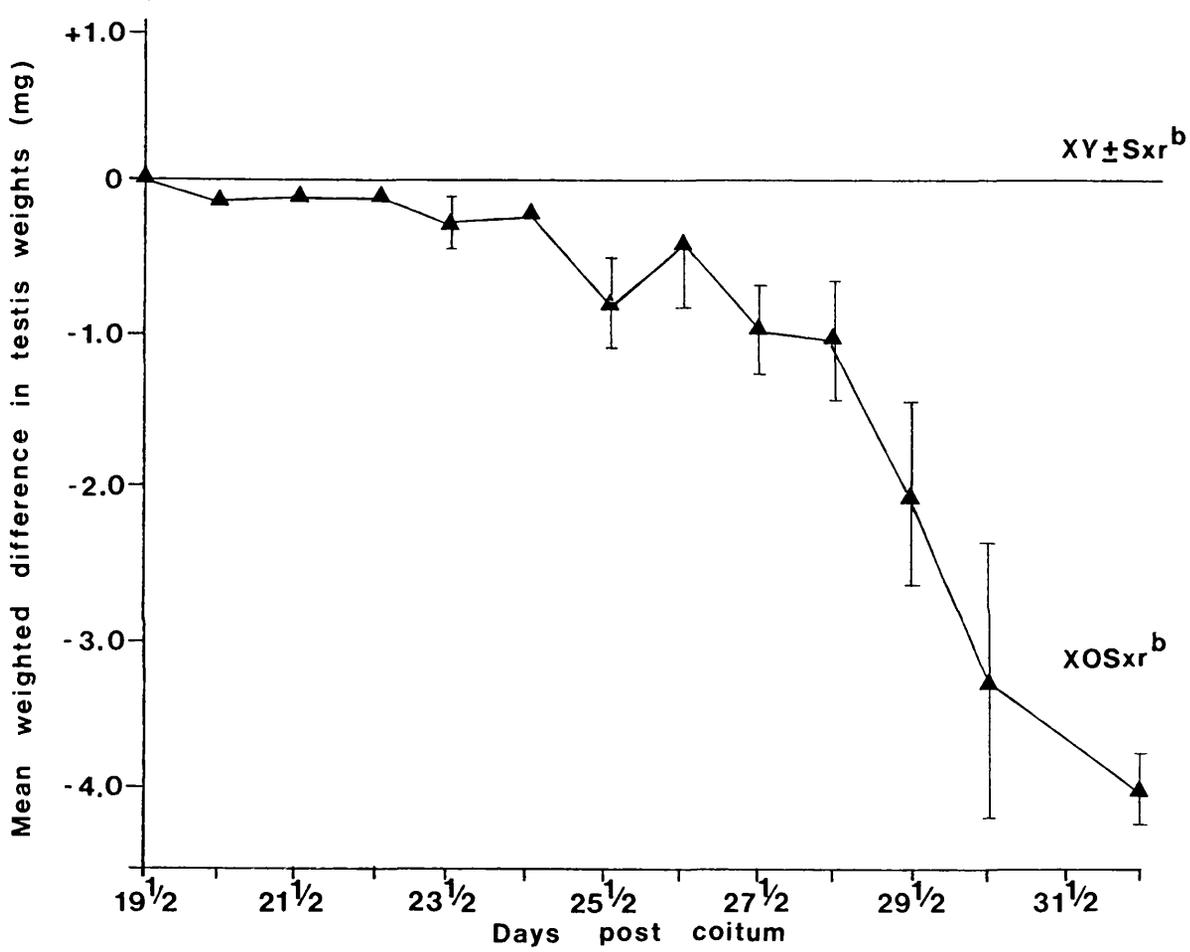


Fig. 2c. Mean weighted difference in testis weights (mg) (corrected for body weights) for XOSxr^b and XY±Sxr^b mice for the period 19 1/2 - 32 1/2 dpc. Where error bars are shown, the differences are significant (t-test, 1-tailed).

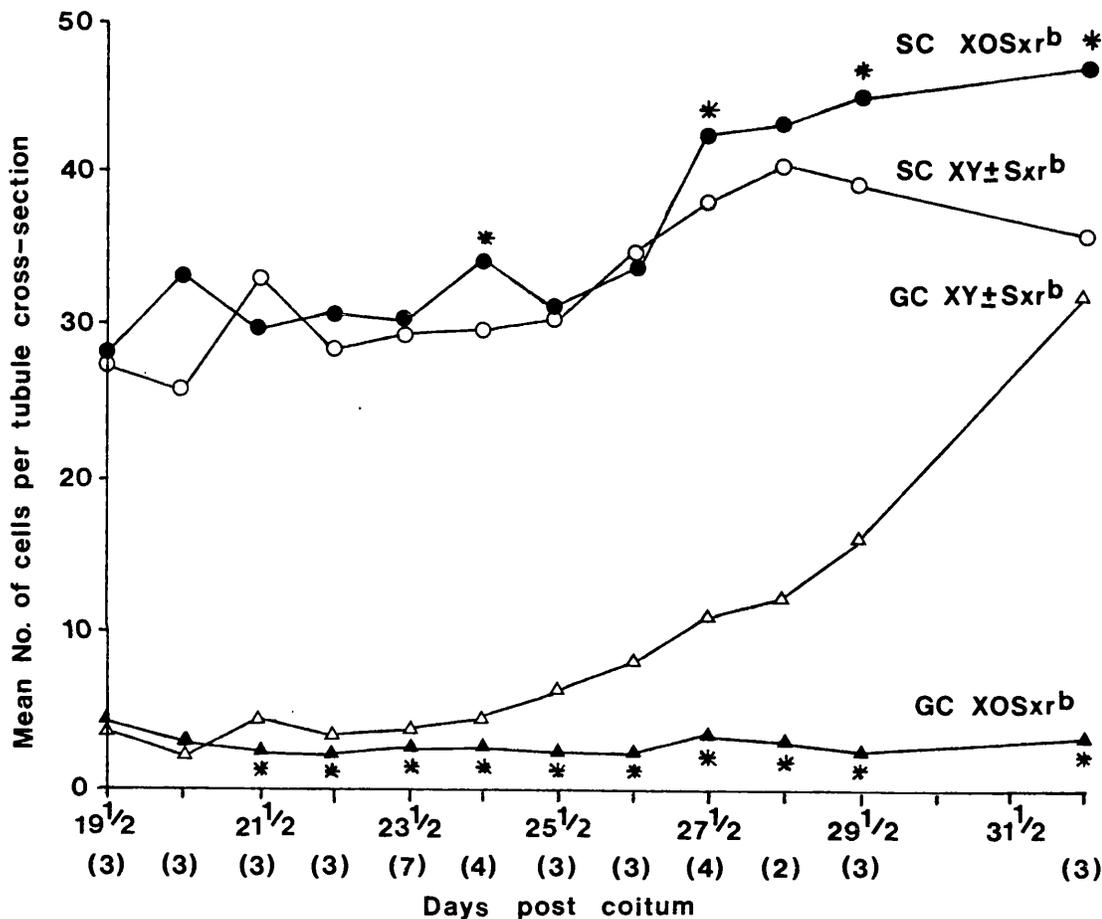


Fig. 2d. Mean number of Sertoli cells (SC) and germ cells (GC) per tubule cross-section in XOSxr^b and XY±Sxr^b mice for the period 19 1/2 - 32 1/2 dpc. The numbers in parentheses are the numbers of litters scored at each age. Asterisks indicate XOSxr^b points which are significantly different from controls (t-test, 2-tailed). The significantly higher number of Sertoli cells in XOSxr^b tubules at 29 1/2 and 32 1/2 dpc is a scoring artifact : at these ages some large tubule cross-sections from the controls had to be excluded because they would not fit in the field of view, resulting in an underestimate of the numbers of Sertoli cells and germ cells for controls at these ages.

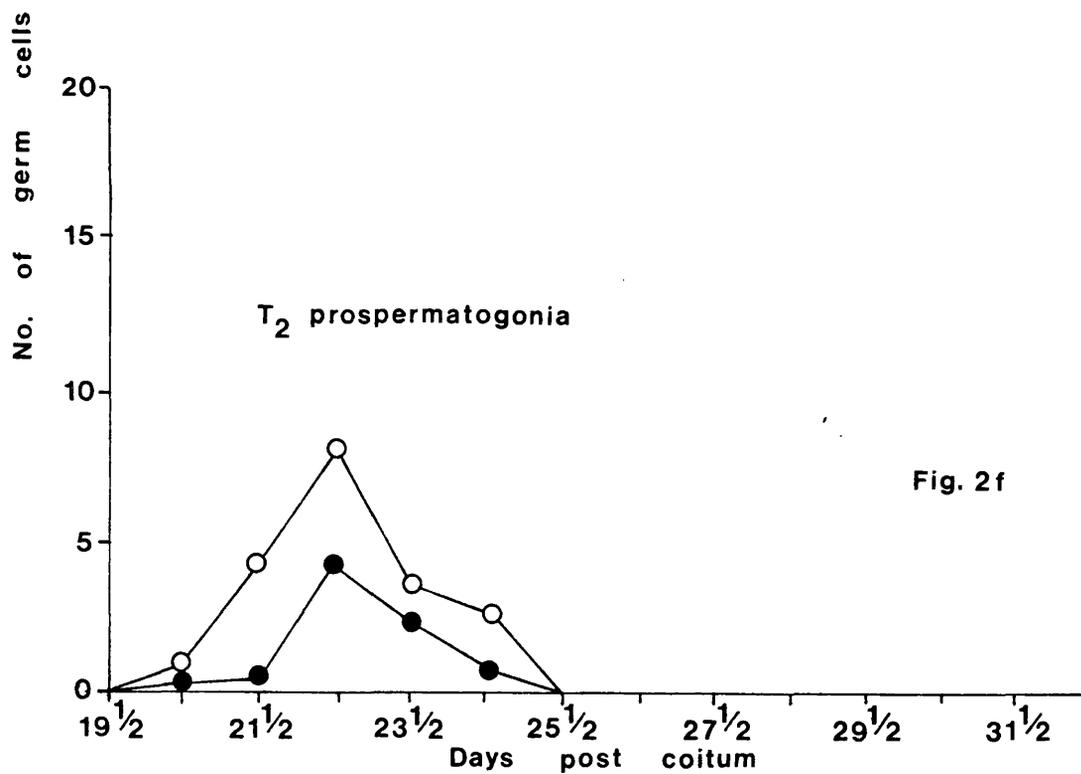
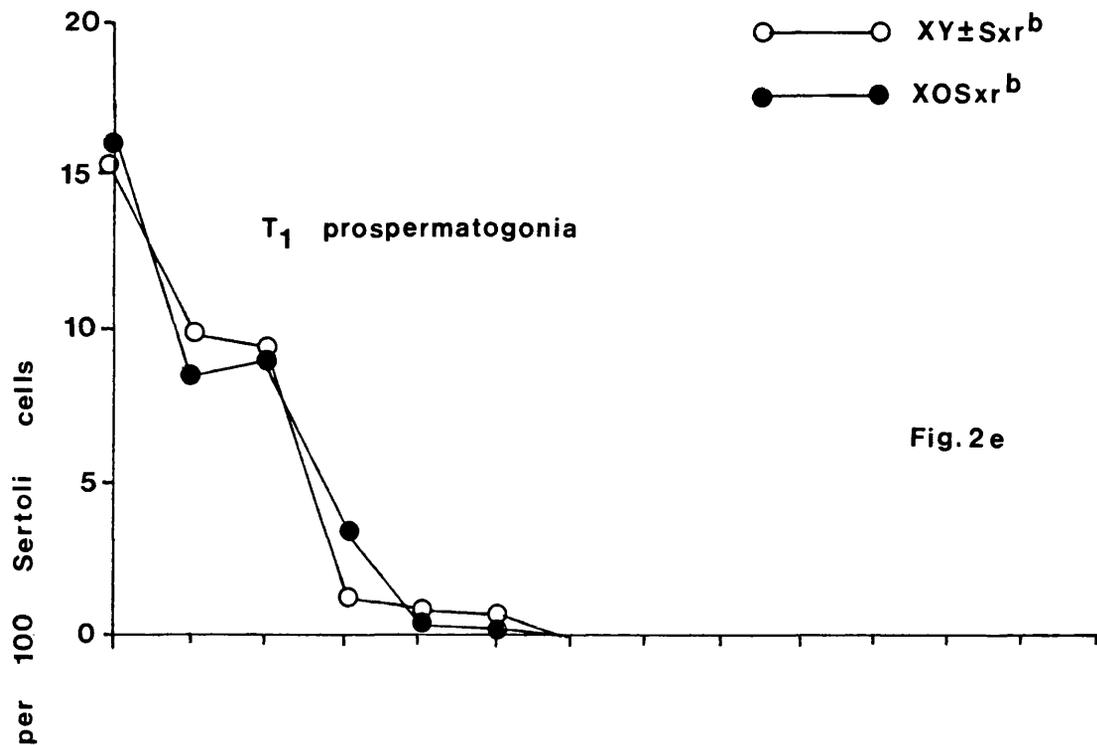


Fig. 2e - f. Number of germ cells per 100 Sertoli cells for the (e) T₁-prospermatogonia, and (f) T₂-prospermatogonia stages in XOSxr^b and XY±Sxr^b testes during the period 19 1/2 - 32 1/2 dpc.

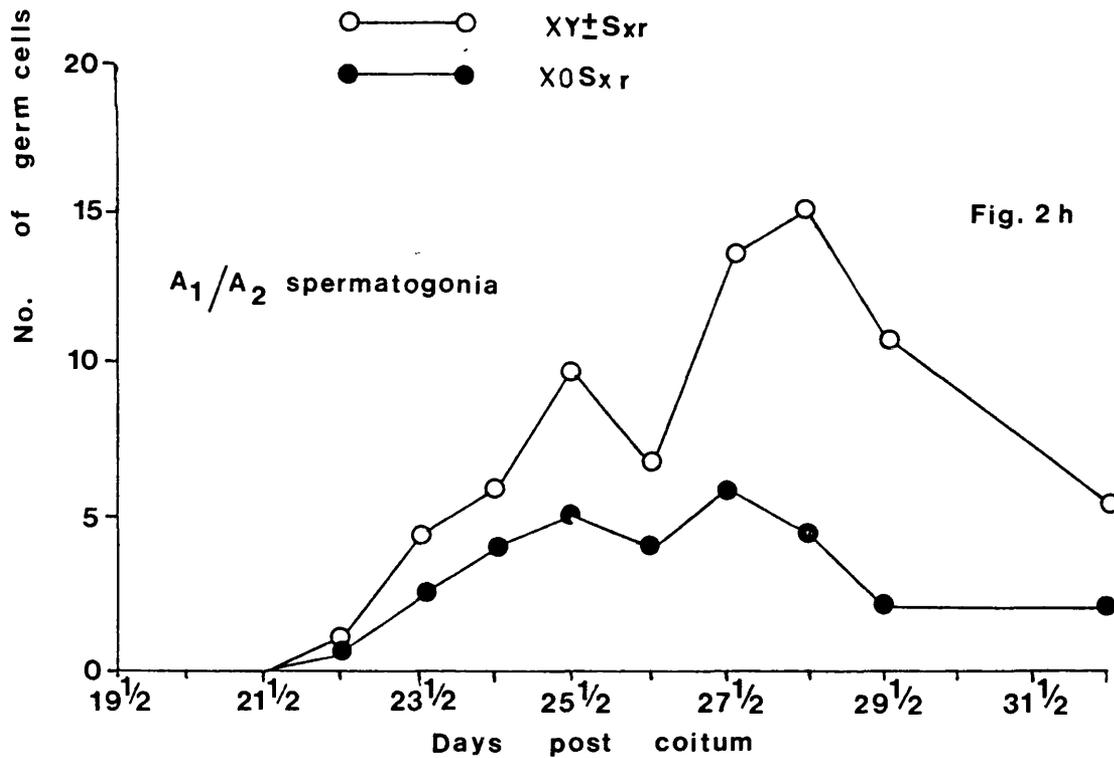
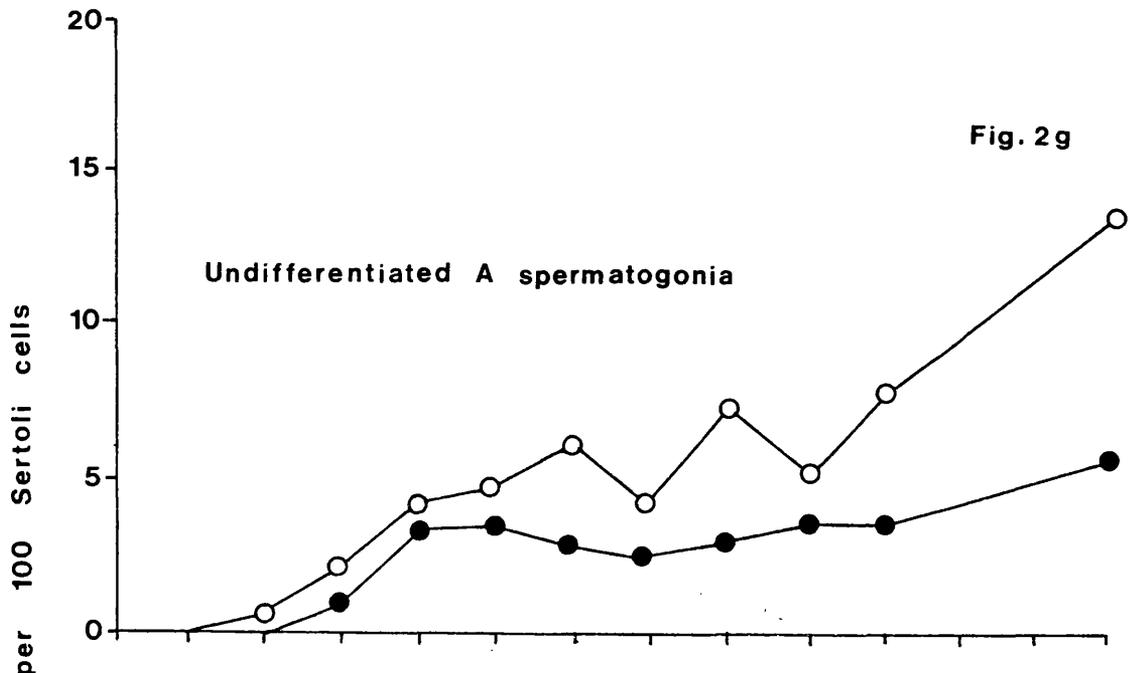


Fig. 2g-h. Number of germ cells per 100 Sertoli cells for (g) undifferentiated A spermatogonia, and (h) A₁/A₂ spermatogonia stages in X0Sxr^b and XY±Sxr^b testes during the period 19 1/2 - 32 1/2 dpc.

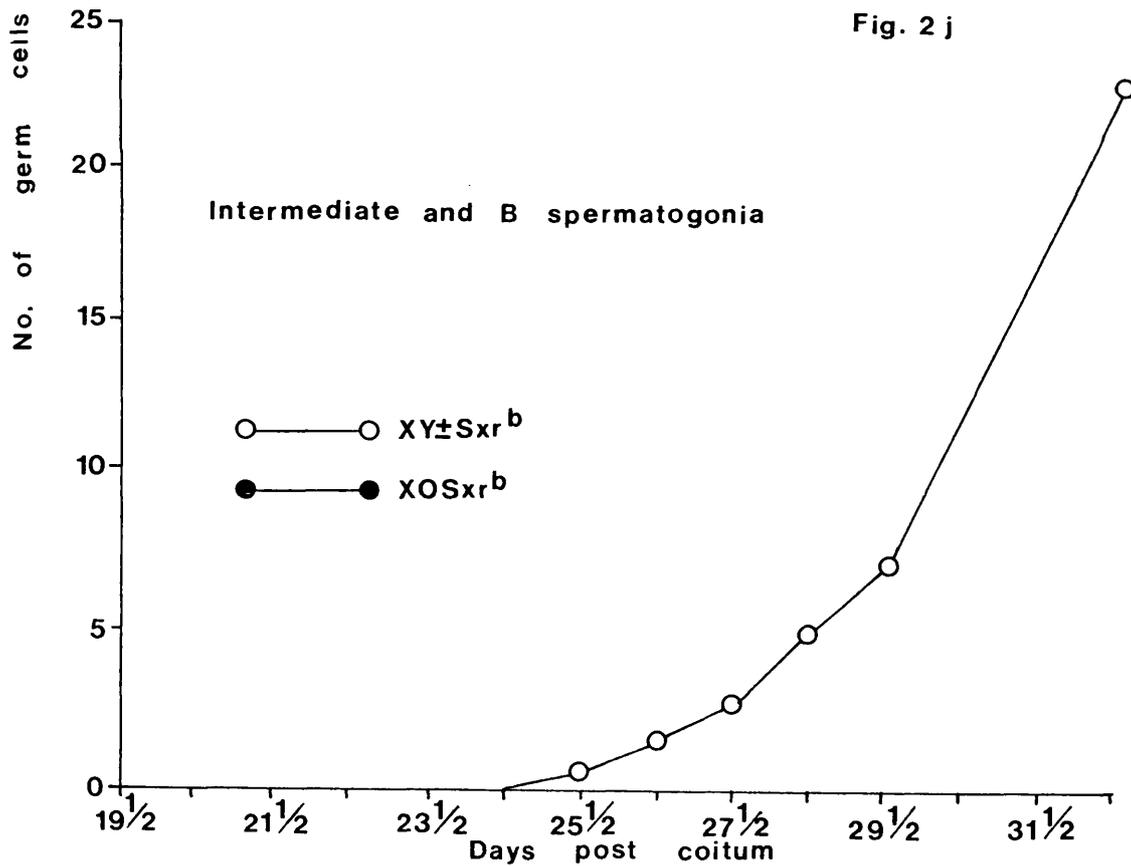
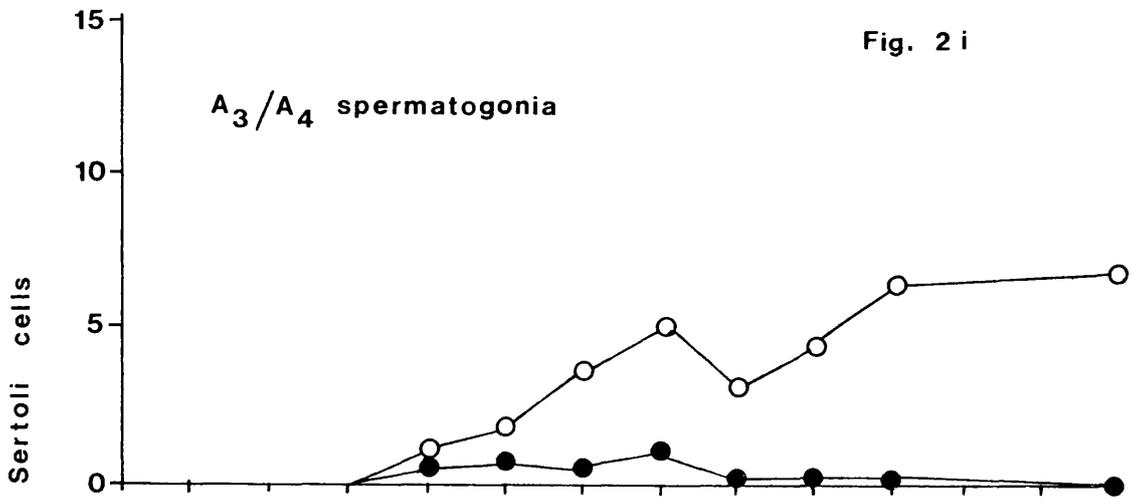


Fig. 2i-j. Number of germ cells per 100 Sertoli cells for (i) A_3/A_4 spermatogonia, and (j) Intermediate and B spermatogonia stages in $XOSxr^b$ and $XY\pm Sxr^b$ testes for the period 19 1/2 - 32 1/2 dpc.

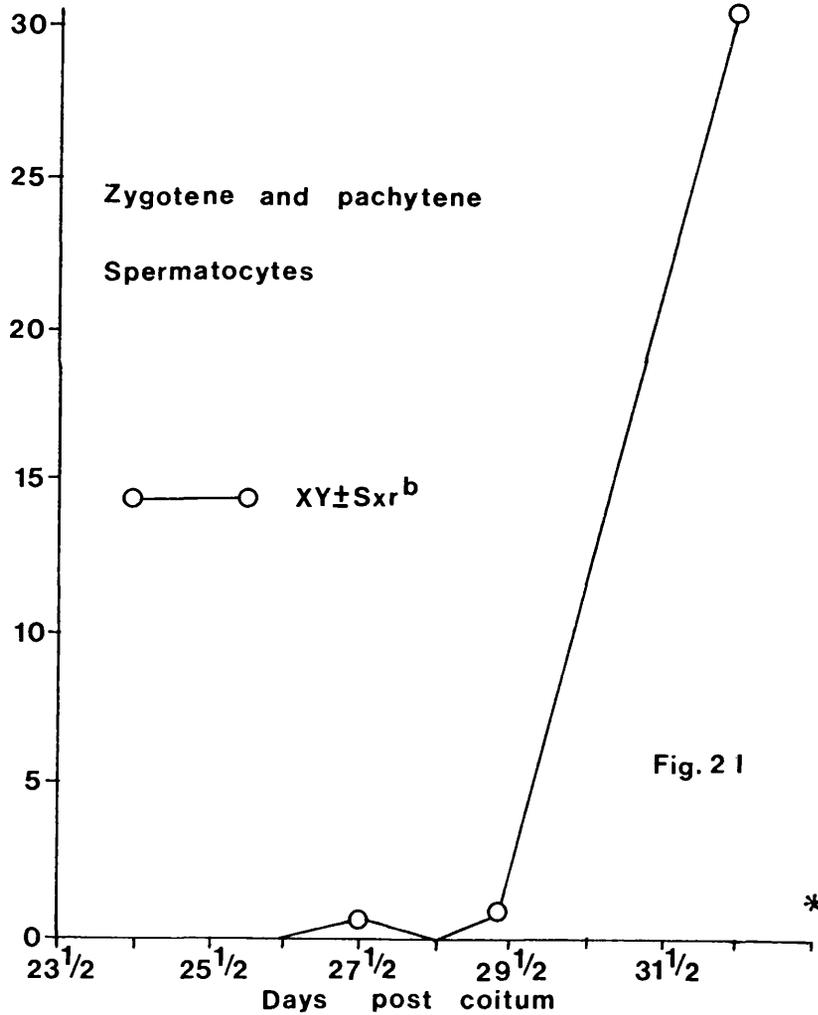
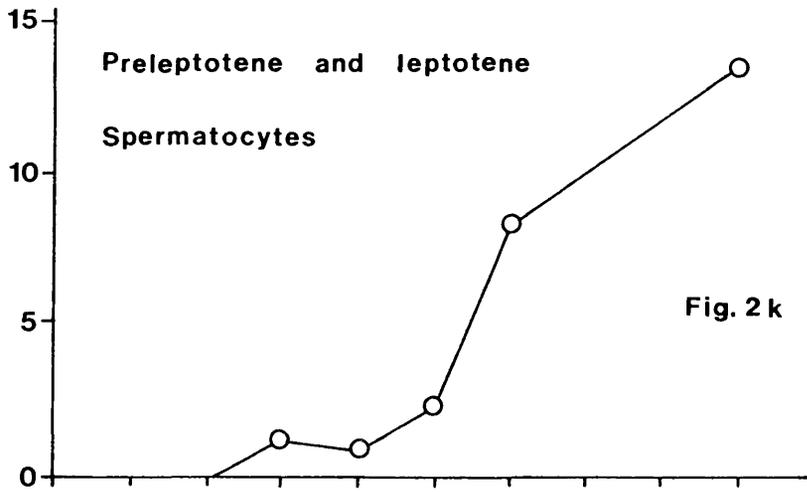


Fig. 2k-l. Number of germ cells per 100 Sertoli cells for (k) Preleptotene/leptotene spermatocyte, and (l) Zygotene/pachytene spermatocyte stages in $XOSxr^b$ and $XY_{\pm}Sxr^b$ testes during the period 19 1/2 - 32 1/2 dpc. The asterisk denotes occasional $XOSxr^b$ zygotene or pachytene cells.

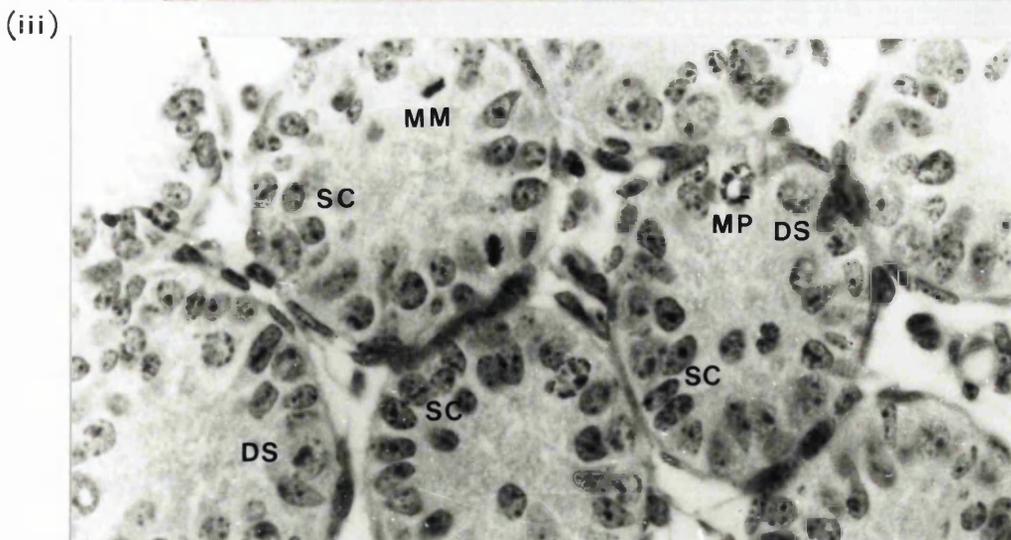
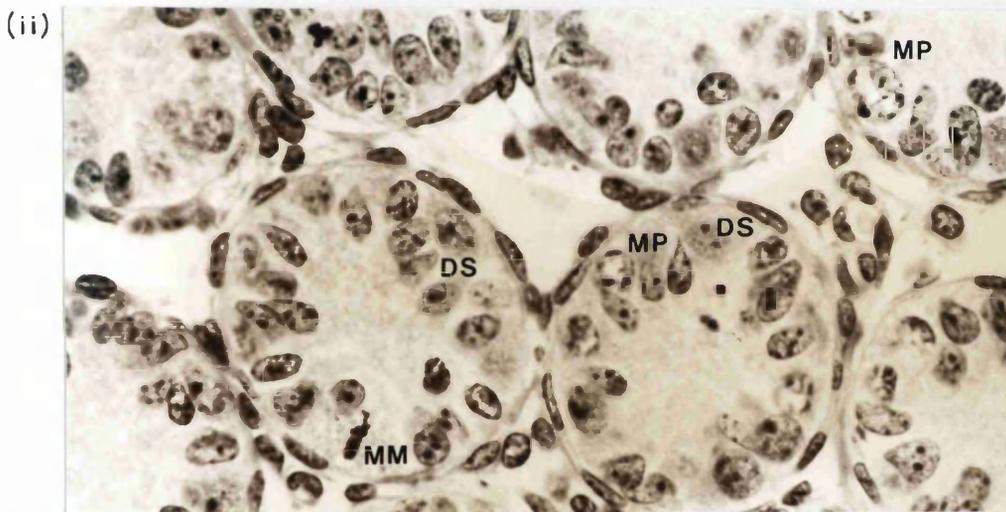
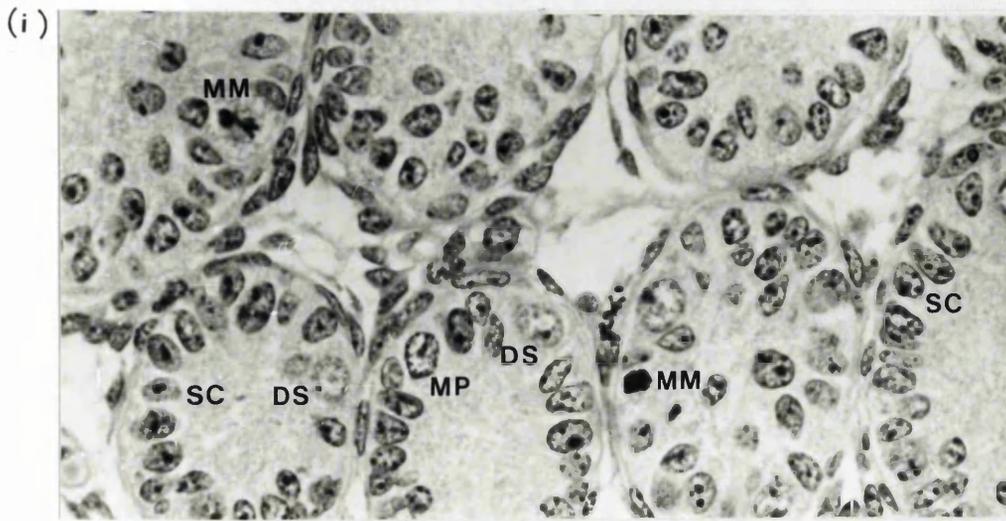


Plate 2j. Histological sections at 3 μ m of (i) XOSxr^b, (ii) XY \pm Sxr^b and (iii) XOSxr^a testes at 24 1/2 dpc (5 dpp). SC = Sertoli cell; MP = mitotic prophase; MM = mitotic metaphase; DS = differentiating spermatogonia. Magnification X700.

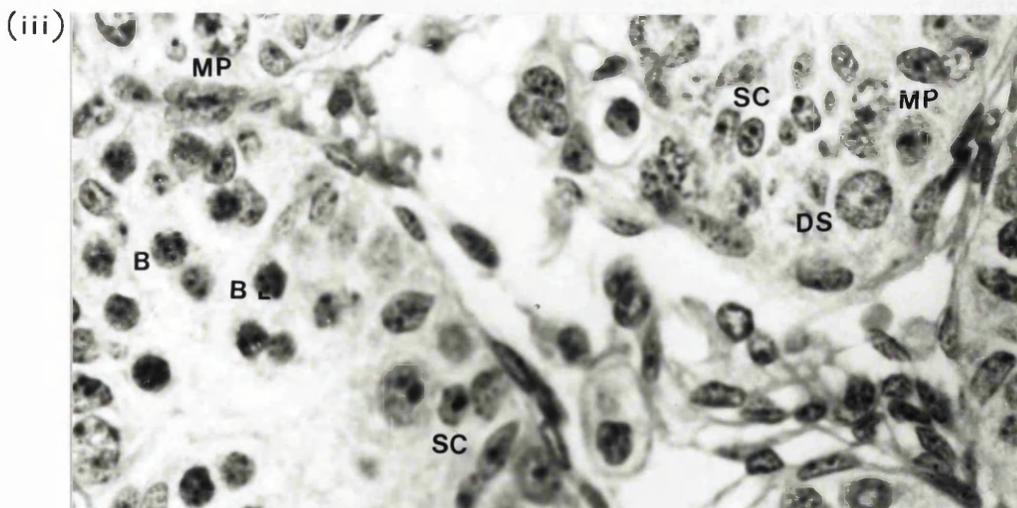
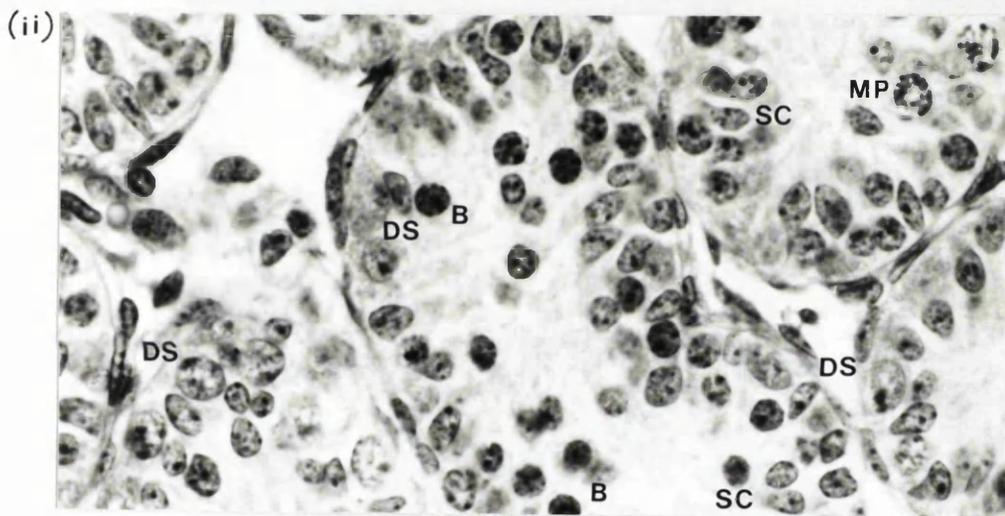
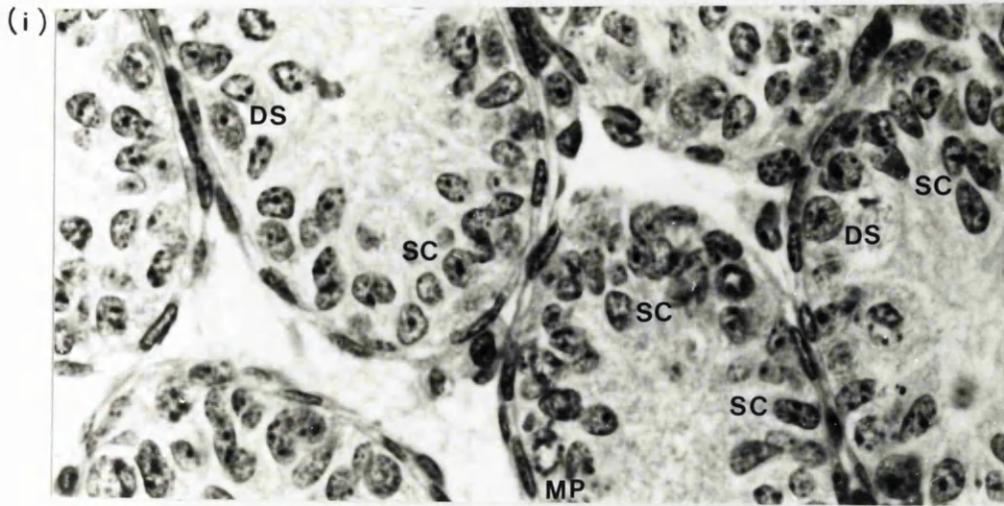


Plate 2k. Histological sections at $3\ \mu\text{m}$ of (i) XOSxr^b , (ii) $\text{XY}\pm\text{Sxr}^b$ and (iii) XOSxr^a testes at $27\ 1/2\ \text{dpc}$ (8 dpp). SC = Sertoli cell; MP = mitotic prophase; MM = mitotic metaphase; DS = differentiating spermatogonia; B = B spermatogonia. Magnification X700.

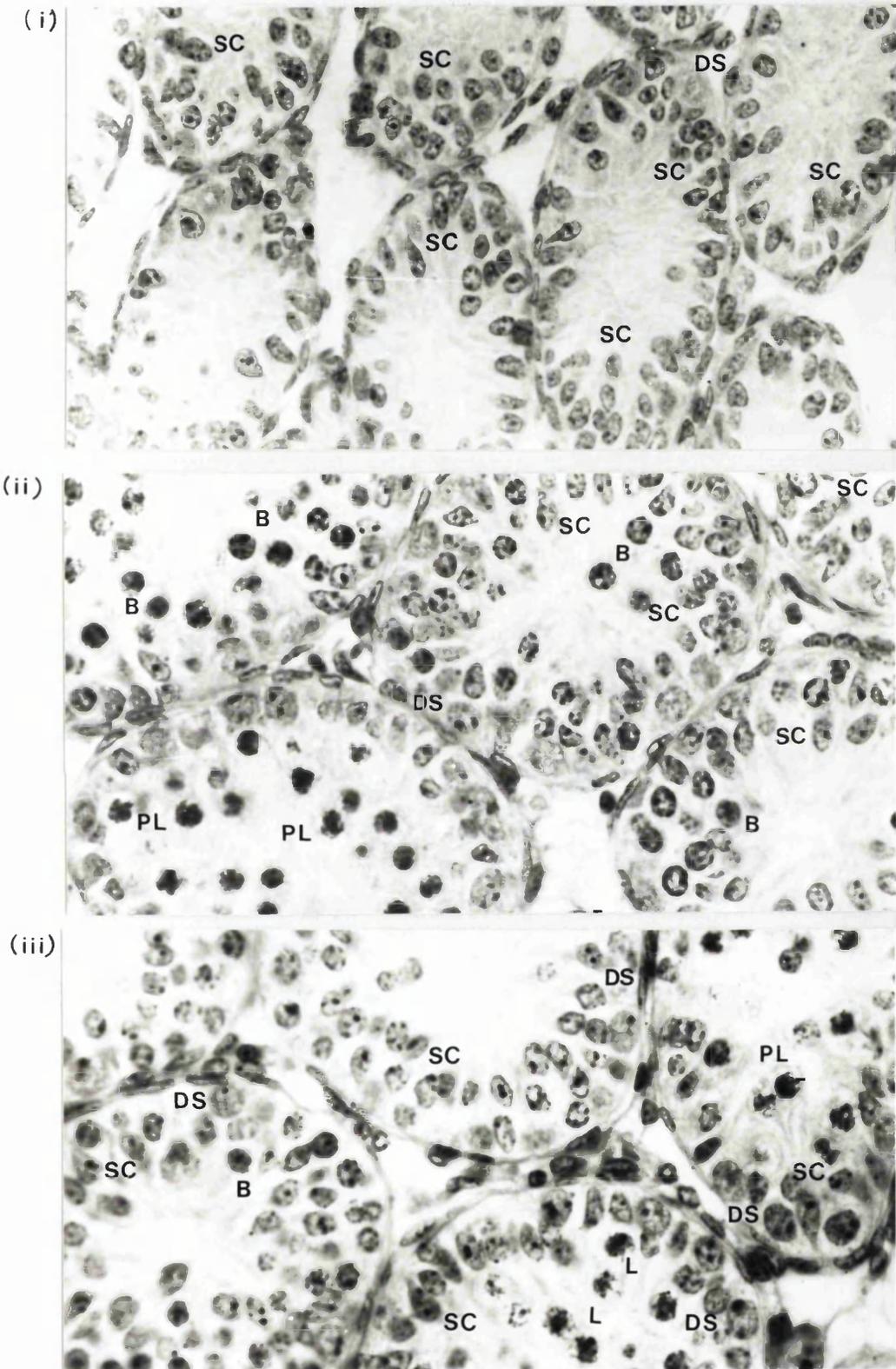


Plate 21. Histological sections at 3 μ m of (i) XOSxr^b, (ii) XY±Sxr^b and (iii) XOSxr^a testes at 29 1/2 dpc (10 dpp). SC = Sertoli cell; PM = mitotic prophase; MM = mitotic metaphase; DS = differentiating spermatogonia; B = B spermatogonia; PL = preleptotene; L = leptotene. Magnification X700.

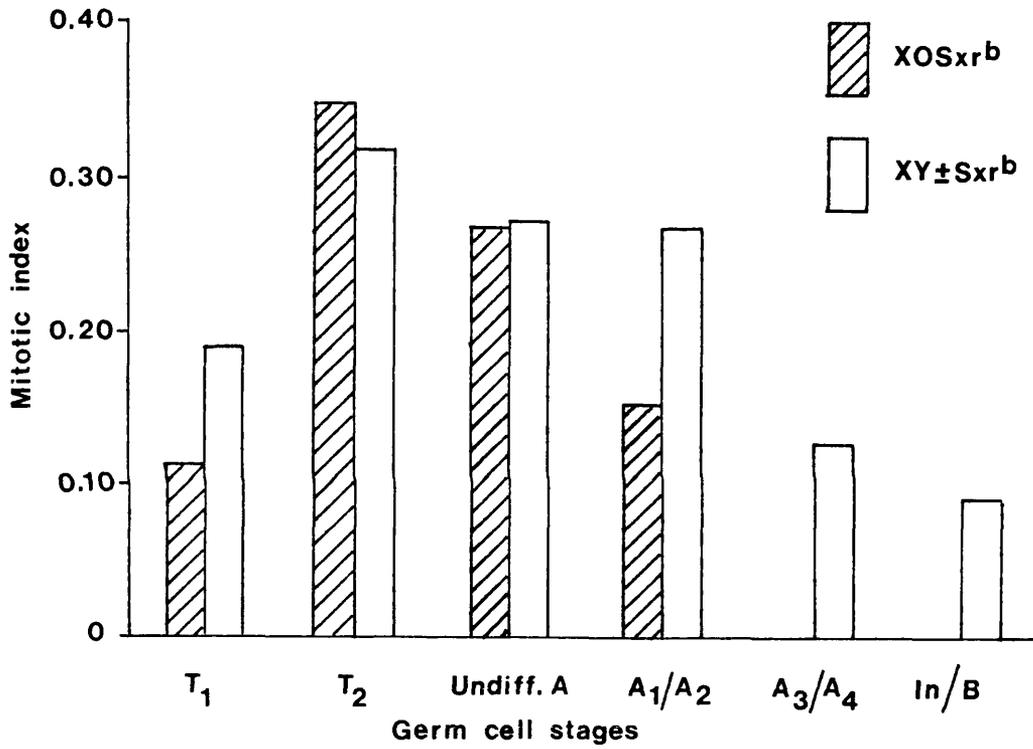


Fig. 2m. Histogram showing the mitotic index according to germ cell stage of XOSxr^b and XY±Sxr^b mice.

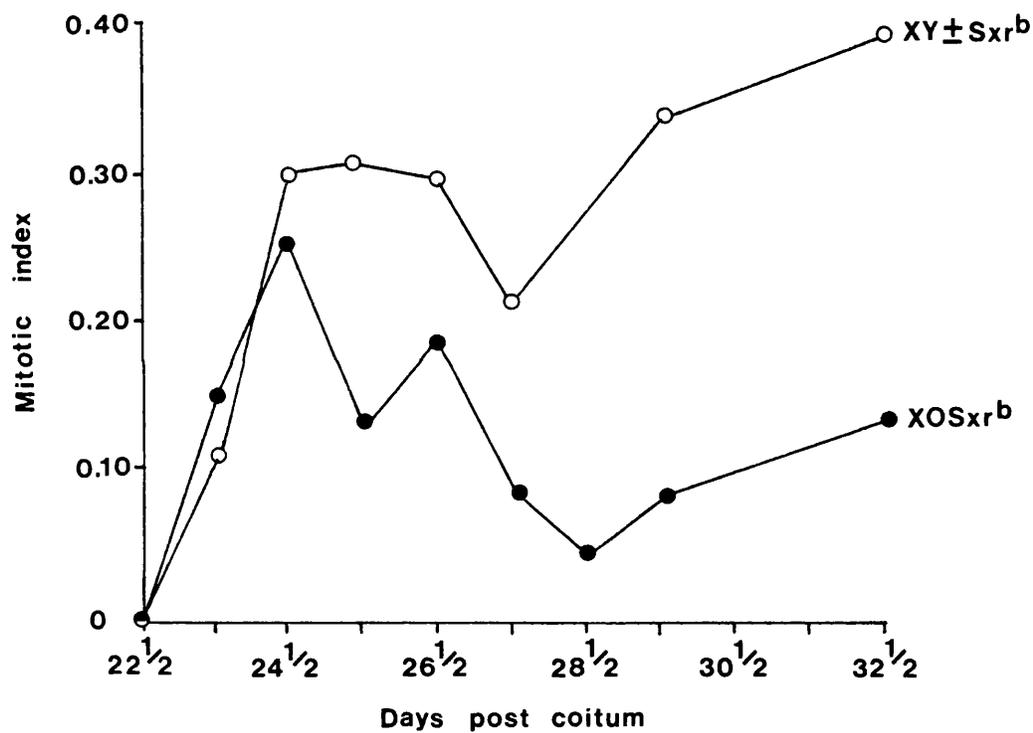


Fig. 2n. Mitotic index of A₁/A₂ spermatogonia in XOSxr^b and XY±Sxr^b mice during the period 19 1/2 - 32 1/2 dpc.

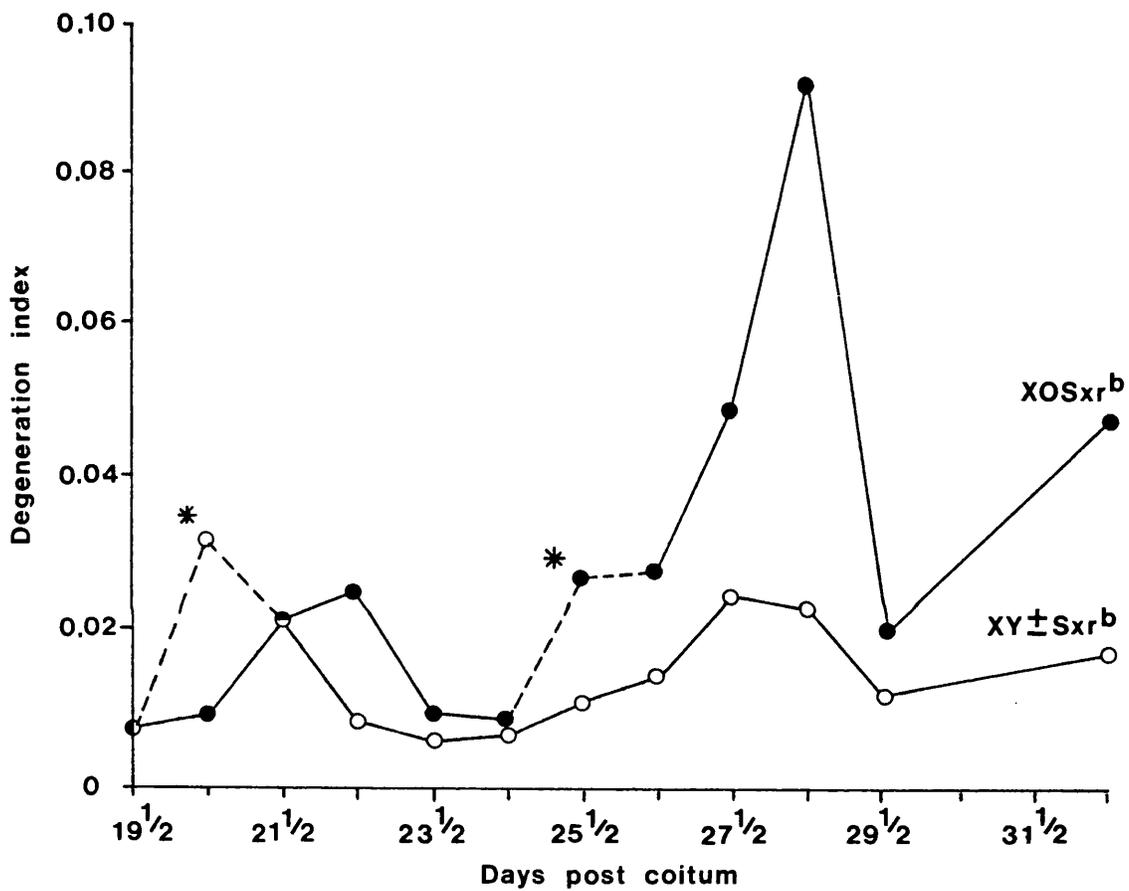


Fig. 20. The germ cell degeneration index in XOSxr^b and XY±Sxr^b mice for the period 19 1/2 - 32 1/2 dpc was calculated on the assumption that all dying cells were germ cells. The two points marked with an asterisk are artificially high, in that only one of the males at each of these points showed an elevated degeneration index.

DISCUSSION

The present results show that XOSxr^b testes have normal numbers of germ cells at birth, but become severely deficient in germ cells in the ensuing two weeks. During the same period, the numbers of Sertoli cells remain normal. These findings are consistent with the view of Burgoyne *et al.* (1986) that the spermatogenic failure in XOSxr^b mice is due to the loss of a gene (*Spy*) that acts cell autonomously in the germ line.

The quantitative analysis of the germ cell deficiency in XOSxr^b mice firstly revealed a reduction in mitotic activity among T₁-prospermatogonia, which resulted in a shortage of T₂-prospermatogonia, and consequently a reduced pool of undifferentiated A spermatogonia. However, mitotic activity among the undifferentiated A spermatogonia, which includes the spermatogonial stem cells, was found to be normal.

It was during the early differentiating spermatogonial stages that the spermatogenic block occurred, with mitotic failure rapidly leading to an almost complete absence of Intermediate and B spermatogonia and subsequent meiotic stages. The proliferation block resulted in less than half the total number of differentiating A spermatogonia by 25 1/2 dpc with a further rapid decline to less than a quarter of A spermatogonia by 29 1/2 dpc compared to the XY_±Sxr^b littermates.

XO female mice are developmentally retarded in early pregnancy (Burgoyne *et al.*, 1983a) and are significantly underweight post-natally (Burgoyne *et al.*, 1983b). It was anticipated that XOSxr^b mice would also be underweight at birth, and this proved to be the case. Unexpectedly, however, a significant body weight difference was observed between

XOSxr^b and XOSxr^a mice. The XOSxr^a mice were originally included in this study, to act as a control for the 'XO effect' in XOSxr^b mice, but this was obviously negated by the body weight findings.

Coincidentally, the genetic basis for the early developmental advantage of XY over XX embryos (Tsunoda *et al.*, 1985; Seller and Perkins-Cole, 1987) was being investigated in our laboratory, concurrently with this study of XOSxr^b mice. It was thought that an explanation for the post-natal weight difference between XOSxr^b and XOSxr^a mice might be due to a postulated growth and development factor (dubbed *Gdy*) on the Y, responsible for accelerating growth at the foetal stage, and this factor might be present on Sxr^a but missing from Sxr^b. A separate study of XXSxr^a and XXSxr^b foetal body weights subsequently proved this postulate to be incorrect. Additionally, further study in our laboratory now suggests that the XO developmental retardation seen in early pregnancy may be due to the X chromosome's paternal derivation, which is known to be preferentially inactivated, rather than to X univalence *per se*. Regardless of the causative factor, an 'XO effect' appears to retard growth in XOSxr^b mice and seems to be ameliorated in XOSxr^a mice, at least post-natally.

The reason for this growth differential remains unresolved. It is known that the Y-derived Sxr^b chromosomal fragment in XYSxr^b mice originated from a different Y background than the normal intact Y chromosome (which derives from the R^{III} background - the same as the Y of the Sxr^a chromosomal fragment in XYSxr^a), so there could be some incompatibility

related to the 'Y background' that might be a causative factor. Alternatively, a growth gene, first expressed neonatally, could be present in Sxr^a but not in Sxr^b.

The deletion of Y-chromosomal material involved in the generation of Sxr^b has thus removed genetic information required for H-Y antigen expression (McLaren *et al.*, 1984) and for spermatogenesis (Burgoyne *et al.*, 1986). The gene controlling spermatogenesis (*Spy*) and the gene for H-Y antigen expression (*Hya*) might be one and the same (Burgoyne *et al.*, 1986).

At the molecular level, it has been shown that one of the two mouse homologues to the human *ZFY* (a Y-chromosomal gene that encodes a zinc finger protein), *Zfy-2*, which is present along with *Zfy-1* in Sxr^a, has been deleted from Sxr^b (Roberts *et al.*, 1988; Mardon *et al.*, 1989; Nagamine *et al.*, 1989). Because *Zfy-1* and *Zfy-2* were strongly transcribed in normal adult mouse testes, probably in germ cells (Mardon and Page, 1989; Nagamine *et al.*, 1989), *Zfy-2* was an obvious candidate for *Spy*. Recent evidence demonstrates that *Zfy-1*, but not *Zfy-2*, is expressed in differentiating embryonic mouse testes and that neither are expressed in mutant W^e/W^e mouse testes which lack germ cells entirely (Koopman *et al.*, 1989). A separate study is currently underway to determine *Zfy-2* expression in XOSxr^a testes during the critical post-natal period, demonstrated in XOSxr^b mice, when the spermatogenesis gene is expressed (Koopman *et al.* - unpublished).

As to the function of the 'spermatogenesis gene' *Spy*, we have clearly shown that the spermatogenic failure seen in XOSxr^b mice is due to a

failure in proliferation during the differentiating A spermatogonial stages, and so by definition, *Spy* is important for the survival/proliferation of these spermatogonial stages. Whether the deficiency of T₁-prospermatogonial divisions of XOSxr^b mice is also a consequence of the deletion of *Spy*, or whether it is due to the deletion of a gene separate from *Spy*, remains to be determined.

CHAPTER 3

A QUANTITATIVE ANALYSIS OF SPERMATOGENESIS THROUGHOUT PUBERTY IN XOS_{xr}^a AND XYS_{xr}^a MICE

Dr S Darling provided assistance at the latter end of this project for some of the DNA Southern blot analysis. 105 results were obtained by the writer, of which 78 were used in the final data, and 37 results by Dr Darling, of which 35 were used in the final data.

INTRODUCTION

In the previous study, the loss of the spermatogenesis gene in $XOSxr^b$ mice resulted in a premeiotic block in germ cell proliferation. By contrast, $XOSxr^a$ mice have active spermatogenesis up to and including prophase of meiosis, some surviving spermatids and occasionally sperm which are defective (Cattanach *et al.* 1971; Cattanach, 1975).

Hannapel and Drews (1979) showed many degenerating primary spermatocytes at late pachytene and metaphase I in the $XOSxr^a$ tubular patches derived from loss of an X chromosome in $XXSxr^a$ mice. The spermatids that survived in these tubules appeared to have very large nuclei, i.e. diploid (Hannapel *et al.*, 1980). Burgoyne and Baker (1984) found similar large spermatids in $XOSxr^a$ testes and postulated that these large diploid spermatids were a manifestation of the spermatogenic arrest in these mice, and that this spermatogenic arrest was a consequence of X chromosome univalence, as suggested by Miklos (1974).

Spermatid DNA content measured for both round and elongated spermatids in $XOSxr^a$ mice demonstrated that two classes of spermatids existed, haploid and diploid. The majority of mice analysed did have a high percentage of diploid spermatids, with the diploid component ranging between 34 - 94% (Levy and Burgoyne, 1986a).

A significant correlation has been found between high frequency of cells with univalent sex chromosomes at diakinesis/metaphase I in human male meiosis and low numbers of cells at metaphase II (Chandley *et al.*, 1976). At pachytene, studies have shown that the univalent XSxr^a chromosome may self-pair and form a ring, balloon or hairpin in a proportion of cells (Chandley and Fletcher, 1980; Mahadevaiah *et al.*, 1988). It was suggested that the only cells capable of forming haploid spermatids in XOSxr^a mice were those that had non-homologously self-synapsed at pachytene, but experiments to test this were inconclusive (Levy, 1986).

XYSxr^a males have also been the subject of considerable analysis. Whilst most XYSxr^a males are fertile, they may occasionally be sterile (Cattanach, 1975). They show increased levels of X-Y dissociation at pachytene and MI (Winsor *et al.*, 1978; Evans *et al.*, 1980; Chandley and Fletcher, 1980) and the sterile males appear to have the highest level of dissociation. Self-synapsis of the YSxr^a chromosome in the form of a balloon shape has been described (Chandley and Speed, 1987; Mahadevaiah *et al.*, 1988) and it was suggested that this might be the underlying cause of the univalence, but recent evidence suggests that univalence and subsequent self-pairing is a consequence of, rather than the cause of, disruption to pairing (Tease and Cattanach, 1989). XYSxr^a testes have been shown by Hannapel and Drews (1979) to exhibit a mosaic pattern of normal and defective tubules. They suggested that normal spermatogenesis was controlled by the Y chromosome and that the tubules showing spermatogenic breakdown at pachytene and MI were controlled by the Sxr^a fragment. (At this time it was thought that Sxr^a was located on an autosome). Burgoyne and Baker (1984), however, attributed the defective

spermatogenesis to the increased incidence of X-Y separation at meiosis, once again invoking Miklos' (1974) model to explain the spermatocyte loss.

The study described in this chapter provides a quantitative analysis of the spermatogenic defects in pubertal XOSxr^a and XYSxr^a mice throughout the first meiotic wave. The objective was to see whether the spermatogenic defects were restricted to the period following meiotic pairing, as is required by Miklos' model (1974).

MATERIALS AND METHODS

Mice

XOSxr^a (and XYSxr^a) mice were produced as described in Chapter 2, Materials and Methods, except the fathers were XYSxr^a (rather than XYSxr^b). Approximately 1 in 16 of the progeny from the cross were of the XOSxr^a genotype. 168 litters were bred of which 55 litters included XOSxr^a and XY males. Litters were processed on alternate days from 27 1/2 days *post coitum* through to 37 1/2 dpc, then daily at 38 1/2, 39 1/2 and 40 1/2 dpc and again on alternate days from 41 1/2 dpc through to 49 1/2 dpc. Following the exclusion of runts, 54 litters were subjected to DNA analysis and 35 litters finally provided data.

Karyotyping and Histology

Both karyotyping and histology were according to the methods already described in Chapter 2, Materials and Methods.

Quantitative analysis

This analysis was carried out in a similar manner to that described in Chapter 2, Materials and Methods, with the following modifications. Sampling was one tubule cross-section from every 20th, 30th, 40th or 50th section depending upon testis size, such that 20 tubule cross-sections were analysed per testis. The square grid was focused under low power with a x10 or x4 objective, and oil immersion was carried out under x100 or x50 objective, the demarcation point for higher powers being testes up to 33 1/2 dpc and lower powers for testes from 35 1/2 dpc and older.

Between 27 1/2 dpc and 33 1/2 dpc inclusive, once a tubule was selected, all cells within the tubule cross-section were classified as described in Chapter 2, Materials and Methods. With the larger testes from 35 1/2 dpc to 49 1/2 dpc, the selected tubule was randomly divided into quadrants, using the Chalkley grid (G52, Graticules Ltd) inserted in the eyepiece. All cells falling within two adjacent quadrants (i.e. half the tubule) were classified.

Southern blot analysis

In the past, XYs_{xr}^a and XY littermates have been identified either by testis weights or test matings, but the age of the mice in this project precluded these methods. A 1.8kb genomic sequence has been cloned, and the probe, designated SX1, detects an additional homologous sequence in XYs_{xr}^a compared to XY genomic DNA on Southern blots. (pSX1 was a gift from Dr C Bishop of the Institute Pasteur, Paris). The isolation of DNA from

tails and the Southern blot hybridisation procedure using the pSX1 probe are described in detail in Appendix 3a, but are briefly outlined below.

Approximately 1.5 cm of tail was digested overnight at 50°C with proteinase K. The DNA was then purified away from the proteins and other molecules by several phenol extractions. The DNA was subsequently precipitated, washed and dried, before being resuspended in TE buffer and stored at 4°C. DNA concentrations and impurities were calculated from O.D. spectrophotometer readings at 260 and 280 nm. Ten micrograms of DNA were digested with the restriction enzyme *Eco* R1 at 37°C. The resulting DNA fragments were then ordered, according to size, by electrophoresis over a 16 - 20 hour period in a 0.8% agarose gel. The technique of Southern blotting was used to transfer the DNA fragments from the gel onto Hybond-N membrane filters using a 20 x SSC transfer buffer (Southern, 1975). The membrane-bound DNA was then cross-linked under ultra violet irradiation for 5 minutes. Although this procedure originally gave good results, problems were later encountered with poor transfer of the higher molecular weight DNA fragments. Despite an intensive period of trouble-shooting, the problems were not entirely resolved (see Appendix 3a) but some improvement followed the substitution of Hybond-N positive membranes and an alkali transfer buffer.

The filters were probed with pSX1 insert, labelled with ³²P isotope, by random priming and hybridised overnight at 68°C. Filters were washed in 2 x SSC followed by a high stringency wash at 0.1 x SSC for 15 minutes, all at 68°C. Finally, the filters were exposed to Fuji RX100 film with intensifying screens and stored at -70°C for 24 - 72 hours.

RESULTS

The body weight data is presented as the estimated mean of litter means for the three genotypes, XYSxr^a , XOSxr^a and XY in Table 3a (i) and (ii). As described in the Results section of Chapter 2, the mean weighted differences and the significance of these differences have been calculated from 'within litters' as described by Burgoyne *et al.* (1983b). No significant difference is demonstrated at any of the age groups in the XYSxr^a - XY comparison, Table 3a (i). In the XOSxr^a - XY comparison, Table 3a (ii), there is a significant difference in only 2/14 age groups. These results are plotted in Fig. 3a. Although the negative point plotted at 33 1/2 dpc is significant in Table 3a (ii), it is thought this might be spurious, since the points on either side are positive. However, the negative point plotted at 41 1/2 dpc is significant and forms part of a consistent negative trend over a number of days, and appears, therefore, to be a meaningful body weight loss although this cannot be explained at this age.

Testis weight data for the three genotypes is given in Table 3b (i) and (ii). XYSxr^a testes, Table 3b (i), are underweight for the majority of age groups from 33 1/2 dpc but this is not significant until 45 1/2 dpc. The XOSxr^a testis weights, Table 3b (ii), are consistently underweight from 33 1/2 dpc and significantly so at 41 1/2 dpc and from 47 1/2 dpc. These results are plotted in Fig. 3b.

In order to investigate the reduction in testis weights, the total number of germ cells per day are pooled and adjusted per 100 Sertoli cells for XY, XYSxr^a and XOSxr^a mice (Fig. 3c). There is no consistent difference in germ cell numbers in either XYSxr^a or XOSxr^a compared to XY until 40 1/2 dpc, at which point the numbers are consistently lower in both, with

XOSxr^a more severely affected than XYSxr^a. Clearly, the reduction in testis weights in both XOSxr^a and XYSxr^a mice is a direct consequence of either a deficiency in germ cell proliferation and/or an increase in germ cell degeneration.

When the number of germ cells per 100 Sertoli cells is plotted against cell type for the three genotypes (Fig. 3d), it is clear that XYSxr^a males, have an earlier germ cell reduction (approximately 25%) at the pachytene stage, and an approximate 50% reduction at subsequent stages. By contrast, there is a severe, almost total, lack of haploid spermatids in XOSxr^a mice.

A detailed analysis of XY, XYSxr^a and XOSxr^a germ cells at each of the pachytene to spermatozoa stages during the period under review is represented graphically in Figs. 3e - i. At pachytene, there are overall less germ cells in XYSxr^a mice from 38 1/2 dpc onwards whereas the number of XOSxr^a germ cells at pachytene closely matches that of XY (Fig. 3e). The reduction in germ cells in XYSxr^a testes is seen throughout the meiotic and post-meiotic stages and additionally there is an approximate 24 hour delay prior to the first appearance of diplotene/diakinesis/MI cells (Fig. 3f). In XOSxr^a mice, pooling diplotene/diakinesis and MI data shows a decreased number of XOSxr^a germ cells at all ages from 39 1/2 dpc compared to XY mice (Fig. 3f). Diploid spermatids are present in both XYSxr^a and XY mice but represent less than 10% of the haploid number whereas in XOSxr^a testes, few spermatids survive and of those that do, approximately one third are diploid (Fig. 3g). From Figs. 3h - i, it can be seen that there are less than half the number of haploid spermatids (Fig. 3h) and elongated spermatids/spermatozoa (Fig. 3i) in XYSxr^a compared to XY testes.

Examples of testis histology are shown for XY, XYSxr^a and XOSxr^a tubules in Plates 3a - c. The XYSxr^a tubules show small patches of germ cell degeneration at the onset of pachytene, Plate 3a (ii), but there is no obvious degeneration in XOSxr^a mice, Plate 3a (iii) or in the control XY litter, Plate 3a (i). By the first appearance of condensed spermatids around 47 1/2 dpc, tubules in XYSxr^a mice show a mosaic appearance of dying MI cells and surviving elongated spermatids/spermatozoa, Plate 3b (ii) and 3c (ii), compared to the block at MI in XOSxr^a tubules, Plate 3b (iii) and 3c (iii). By contrast, XY tubules have elongated spermatids/spermatozoa and no sign of degeneration, Plate 3b (i) and 3c (i).

The mitotic indices of XY = 0.15, XYSxr^a = 0.14 and XOSxr^a = 0.16, confirm that there is no reduction in germ cell proliferation prior to meiosis in XYSxr^a or XOSxr^a and the spermatogonial degeneration index of 0.01 for all three genotypes confirms that there is no excess pre-meiotic cell death in XYSxr^a or XOSxr^a testes either. The meiotic and post-meiotic degeneration index shows an earlier degeneration by 37 1/2 dpc in XYSxr^a , compared to XY, and cell death throughout the period (Fig. 3j), whereas the degeneration index for XOSxr^a males shows an exponential rise in dying cells from 39 1/2 dpc compared to normal XY and XYSxr^a mice.

When the degeneration index is plotted against germ cell stage for XOSxr^a mice, virtually all cell death occurs at diplotene/diakinesis/MI stages, although cells that survive until the spermatid stage then degenerate (Fig. 3k). There is increased germ cell death at pachytene in XYSxr^a mice which could account for the reduced numbers of pachytene cells shown in Fig. 3e. Further degeneration occurs at MI. Additionally, diploid spermatids in XYSxr^a tubules have a higher degeneration index than XY.

Days post coitum	No. of Mice		Mean \pm s.e.m. body weights (g)*		Mean \pm s.e.m. weighted $\text{XYSxr}^a\text{-XY}$ difference (g)	Significance of $\text{XYSxr}^a\text{-XY}$ difference (P)
	XYSxr^a	XY	XYSxr^a	XY		
** 27 1/2	1	2	6.230 \pm 0.00	6.050 \pm 0.13	+0.18 \pm 0.23	NS
29 1/2	8	4	6.320 \pm 0.58	6.433 \pm 0.38	-0.16 \pm 0.37	NS
31 1/2	7	2	7.945 \pm 0.49	7.470 \pm 0.41	+0.13 \pm 0.46	NS
** 33 1/2	2	1	9.190 \pm 0.48	10.340 \pm 0.00	-1.15 \pm 0.83	NS
** 35 1/2	3	2	9.260 \pm 0.50	8.610 \pm 0.93	+0.65 \pm 0.95	NS
37 1/2	4	4	9.300 \pm 0.87	8.925 \pm 0.38	+0.13 \pm 0.59	NS
** 38 1/2	1	1	11.060 \pm 0.00	11.270 \pm 0.00	-0.21 \pm 0.00	NS
39 1/2	11	4	11.313 \pm 0.73	11.115 \pm 0.57	+0.16 \pm 0.56	NS
40 1/2	4	5	12.888 \pm 0.34	13.685 \pm 0.72	-0.76 \pm 1.32	NS
** 41 1/2	3	3	10.783 \pm 0.00	10.673 \pm 0.00	+0.11 \pm 0.80	NS
** 43 1/2	1	3	19.100 \pm 0.00	15.910 \pm 1.43	+3.19 \pm 2.86	NS
** 45 1/2	2	1	17.770 \pm 0.35	16.330 \pm 0.00	+1.44 \pm 0.60	NS
47 1/2	4	7	20.160 \pm 1.55	19.800 \pm 1.21	+0.25 \pm 0.85	NS
49 1/2	2	3	21.775 \pm 0.44	22.585 \pm 0.24	-0.71 \pm 0.72	NS

* Mean of litter means
 ** Denotes only 1 litter at this age
 *** 0.00 indicates no standard error possible

TABLE 3a (i) Mean body weights for XYSxr^a and XY mice and the estimated difference between them for the period 27 1/2 - 49 1/2 dpc.

Days post coitum	No. of Mice		Mean \pm s.e.m. body weights (g) *		Mean \pm s.e.m. weighted XOSxr ^a -XY difference (g)	Significance of XOSxr ^a -XY difference (P)
	XOSxr ^a	XY	XOSxr ^a	XY		
27 1/2	2	3	5.295 \pm 0.51	5.510 \pm 0.54	-0.22 \pm 0.17	NS
29 1/2	5	4	7.007 \pm 0.40	6.433 \pm 0.38	+0.65 \pm 0.38	NS
31 1/2	2	2	7.570 \pm 0.73	7.650 \pm 0.59	-0.08 \pm 0.00	
33 1/2	2	5	8.495 \pm 0.57	10.130 \pm 0.21	-1.46 \pm 0.15	< 0.005
35 1/2	2	2	7.790 \pm 0.37	8.610 \pm 0.93	-0.82 \pm 0.99	NS
37 1/2	2	4	9.260 \pm 0.58	8.925 \pm 0.38	+0.30 \pm 0.75	NS
38 1/2	2	2	12.025 \pm 1.16	11.855 \pm 0.59	+0.17 \pm 0.00	
39 1/2	5	4	10.743 \pm 0.89	11.115 \pm 0.57	-0.49 \pm 0.85	NS
40 1/2	4	5	11.405 \pm 1.56	13.685 \pm 0.72	-2.27 \pm 1.32	NS
41 1/2	4	7	10.763 \pm 1.87	13.593 \pm 2.24	-2.79 \pm 0.84	0.025 - 0.010
43 1/2	2	6	16.860 \pm 0.38	17.925 \pm 2.02	-1.07 \pm 1.65	NS
45 1/2	1	1	17.140 \pm 0.00	16.330 \pm 0.00	+0.81 \pm 0.00	
47 1/2	6	9	20.500 \pm 1.27	20.36 \pm 1.02	+0.06 \pm 0.76	NS
49 1/2	3	5	24.083 \pm 0.96	23.330 \pm 0.76	+0.65 \pm 2.30	NS

* Mean of litter means
 ** Denotes only 1 litter at this age
 *** 0.00 indicates no standard error possible

TABLE 3a (ii) Mean body weights for XOSxr^a and XY mice and the estimated difference between them for the period 27 1/2 - 49 1/2 dpc.

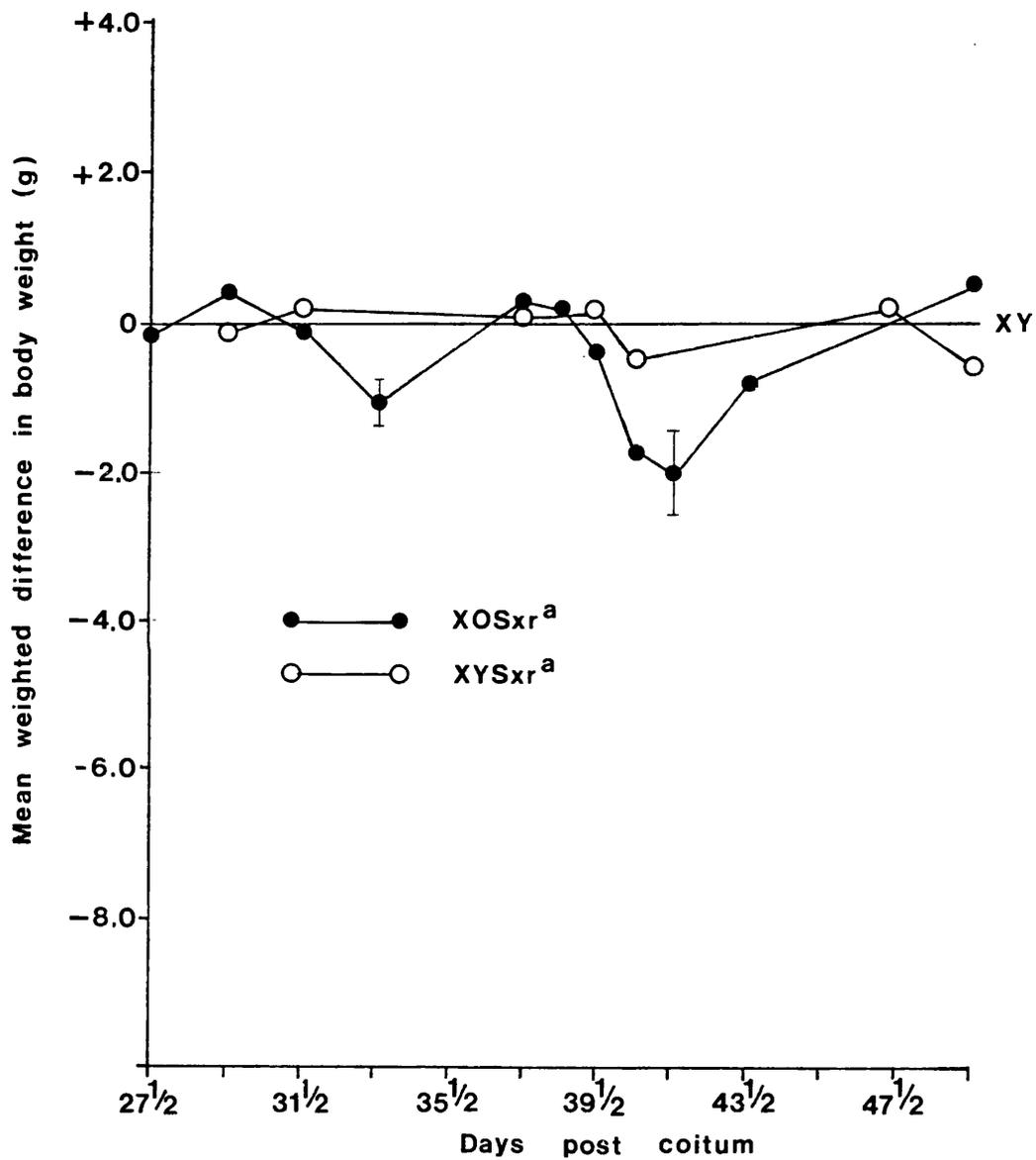


Fig. 3a. Mean weighted difference in body weights for XY, XYSxr^a and XOSxr^a mice for the period 27 1/2 - 49 1/2 dpc. Where error bars are shown, the differences are significant (t-test, 1-tailed).

Days post coitum	No. of Mice		Mean \pm s.e.m. testis weights (mg)*		Mean \pm s.e.m. weighted difference (g)	Significance of difference (P)
	XY	XY	XY	XY		
** 27 1/2	1	2	4.890 \pm 0.00	5.050 \pm 0.32	- 0.16 \pm 0.55	NS
29 1/2	8	4	5.830 \pm 0.37	6.052 \pm 0.36	- 0.26 \pm 0.31	NS
31 1/2	7	2	7.542 \pm 0.53	7.120 \pm 0.40	+ 0.45 \pm 1.14	NS
** 33 1/2	2	1	11.890 \pm 0.93	13.059 \pm 0.00	- 1.16 \pm 1.61	NS
** 35 1/2	3	2	13.650 \pm 1.49	13.033 \pm 1.24	+ 0.62 \pm 2.13	NS
37 1/2	4	4	12.885 \pm 2.05	16.080 \pm 2.22	- 3.29 \pm 1.78	NS
** 38 1/2	1	1	16.728 \pm 0.00	23.238 \pm 0.00	- 6.51 \pm 0.00	
39 1/2	11	4	20.370 \pm 1.36	27.890 \pm 2.37	- 7.74 \pm 1.73	< 0.005
40 1/2	4	5	26.256 \pm 0.58	31.514 \pm 3.71	- 5.73 \pm 6.36	NS
** 41 1/2	3	3	21.030 \pm 0.00	23.499 \pm 0.00	- 2.46 \pm 3.35	NS
** 43 1/2	1	3	49.260 \pm 0.00	40.770 \pm 4.87	+ 8.49 \pm 9.74	NS
** 45 1/2	2	1	33.362 \pm 0.14	35.330 \pm 0.00	- 1.97 \pm 0.24	0.05 - 0.025
47 1/2	4	7	48.400 \pm 9.26	55.230 \pm 1.75	- 9.46 \pm 3.07	0.025 - 0.010
49 1/2	2	3	44.673 \pm 7.96	61.522 \pm 0.49	-17.94 \pm 2.75	0.05 - 0.025

* Mean of litter means
 ** Denotes only 1 litter at this age
 *** indicates no standard error possible

TABLE 3b (i) Mean testis weights for XY^{Sxr^a} and XY mice and the estimated difference between them for the period 27 1/2 - 49 1/2 dpc.

Days post coitum	No. of Mice		Mean \pm s.e.m. testis weights (mg)*		XY	Mean \pm s.e.m. weighted XOSxr ^a - XY difference (g)		Significance of XOSxr ^a - XY difference (P)
	XOSxr ^a	XY	XOSxr ^a	XY		XOSxr ^a	XY	
27 1/2	2	3	5.023 \pm 1.38	4.390 \pm 0.66		+ 0.73 \pm 0.42	NS	
29 1/2	5	4	6.830 \pm 0.71	6.055 \pm 0.36		+ 1.03 \pm 0.41	0.05 - 0.025	
31 1/2	2	2	7.090 \pm 0.82	7.125 \pm 0.40		- 0.03 \pm 0.00	NS	
33 1/2	2	5	9.632 \pm 2.28	13.720 \pm 0.67		- 3.71 \pm 0.33	< 0.005	
35 1/2	2	2	9.765 \pm 1.43	13.032 \pm 1.24		- 3.27 \pm 1.89	NS	
37 1/2	2	4	16.230 \pm 0.59	16.080 \pm 2.22		- 0.40 \pm 2.95	NS	
38 1/2	2	2	25.120 \pm 1.93	25.442 \pm 2.21		- 0.32 \pm 0.00	NS	
39 1/2	5	4	23.380 \pm 1.43	27.890 \pm 2.37		- 4.94 \pm 3.93	NS	
40 1/2	4	5	26.485 \pm 5.78	31.512 \pm 3.71		- 5.18 \pm 6.36	NS	
41 1/2	4	7	25.090 \pm 5.80	37.345 \pm 11.55		- 11.82 \pm 3.28	0.010 - 0.005	
43 1/2	2	6	31.500 \pm 5.61	46.862 \pm 6.09		- 10.34 \pm 5.71	NS	
45 1/2	1	1	34.228 \pm 0.00	35.330 \pm 0.00		- 1.11 \pm 0.00		
47 1/2	6	9	39.390 \pm 2.89	53.452 \pm 2.17		- 14.46 \pm 4.22	0.010 - 0.005	
49 1/2	3	5	40.810 \pm 0.18	63.350 \pm 1.85		- 22.69 \pm 7.50	0.05 - 0.025	

* Mean of litter means

** Denotes only 1 litter at this age

*** 0.00 indicates no standard error possible

TABLE 3b (ii) Mean testis weights for XOSxr^a and XY mice and the estimated difference between them for the period 27 1/2 - 49 1/2 dpc.

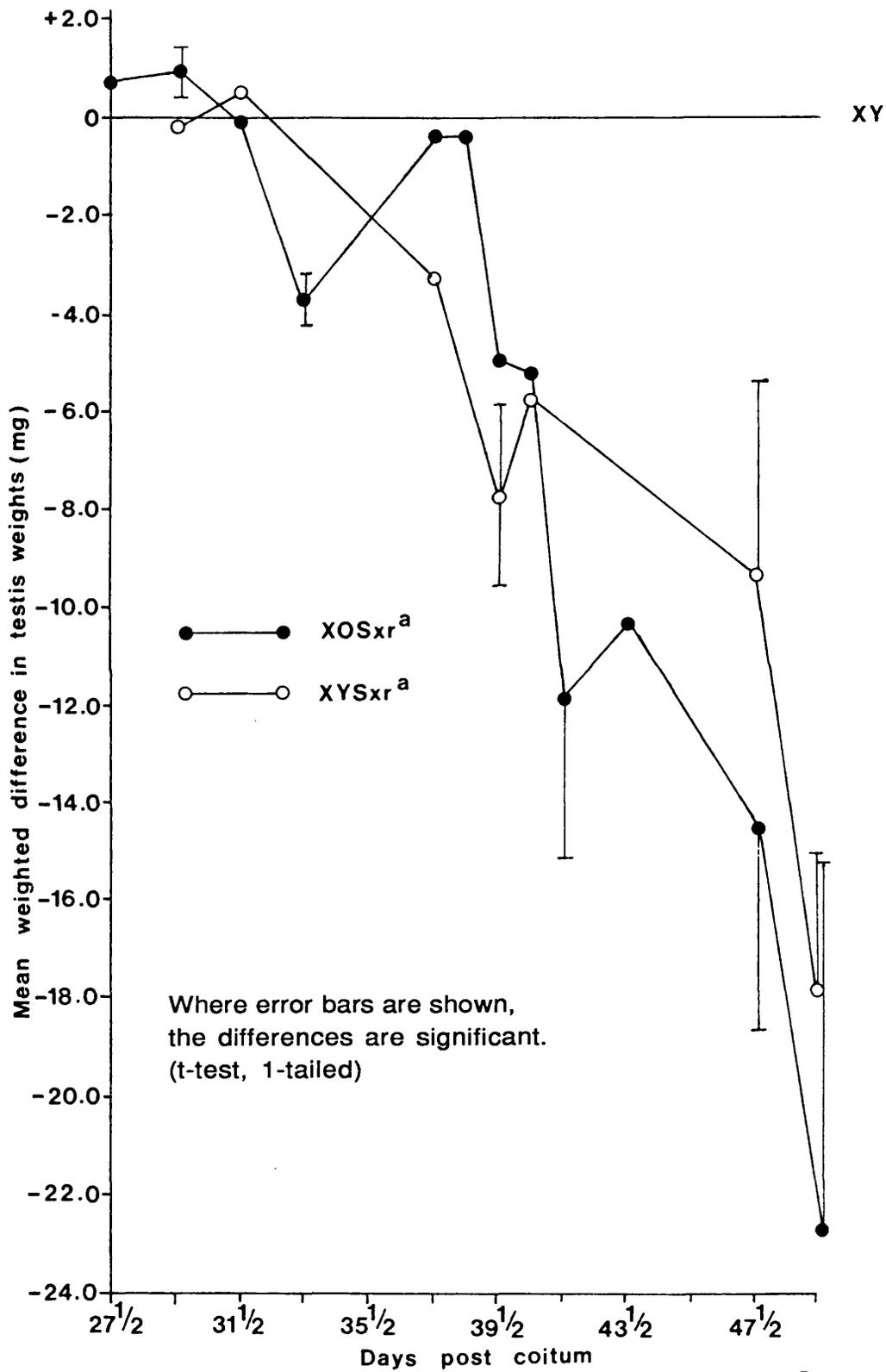


Fig. 3b. Mean weighted differences in testis weights for XY, XYSxr^a and XOSxr^a mice for the period 27 1/2 - 49 1/2 dpc.

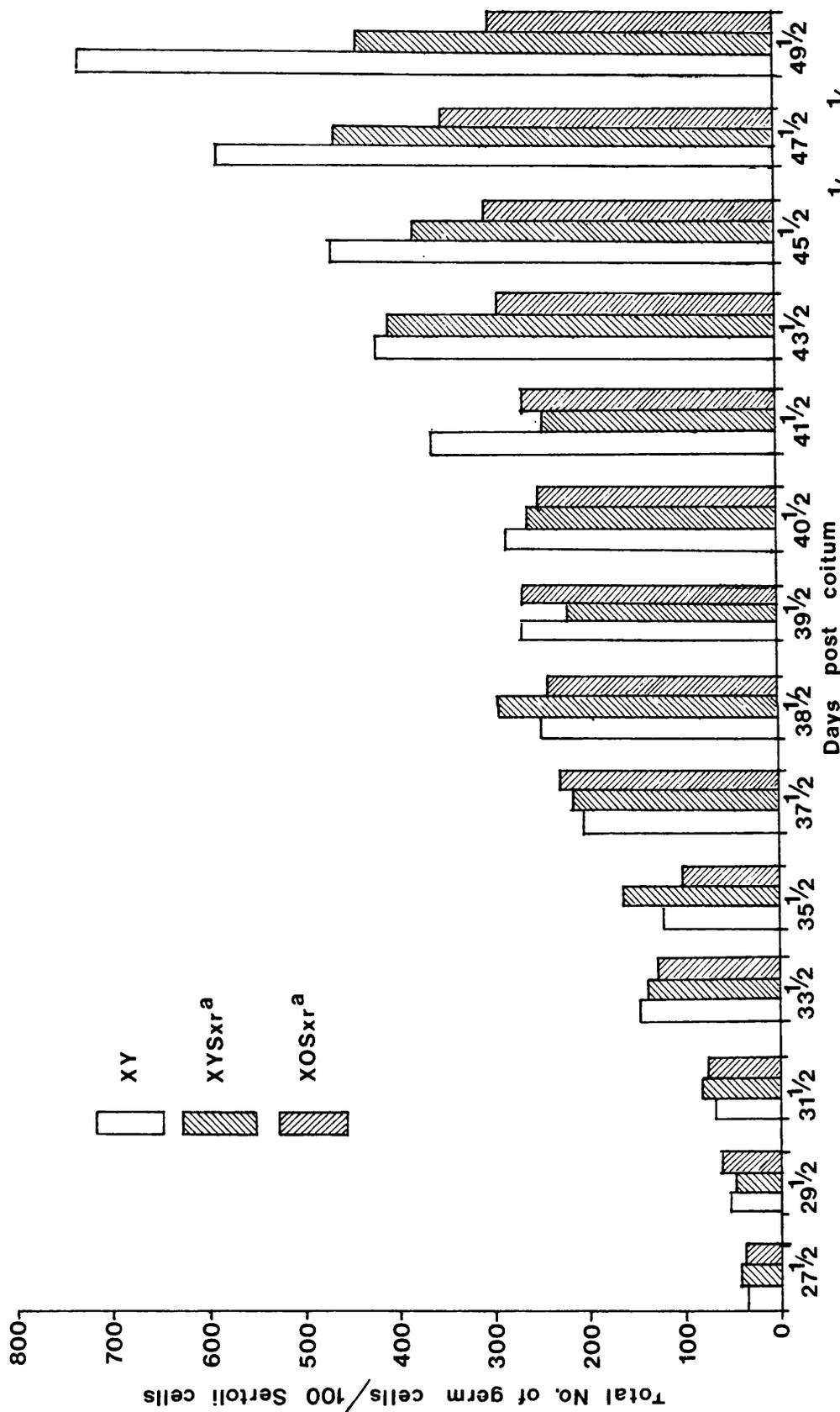


Fig. 3c Histogram of the total No. of germ cells per 100 Sertoli cells during the period 27 1/2 - 49 1/2 dpc

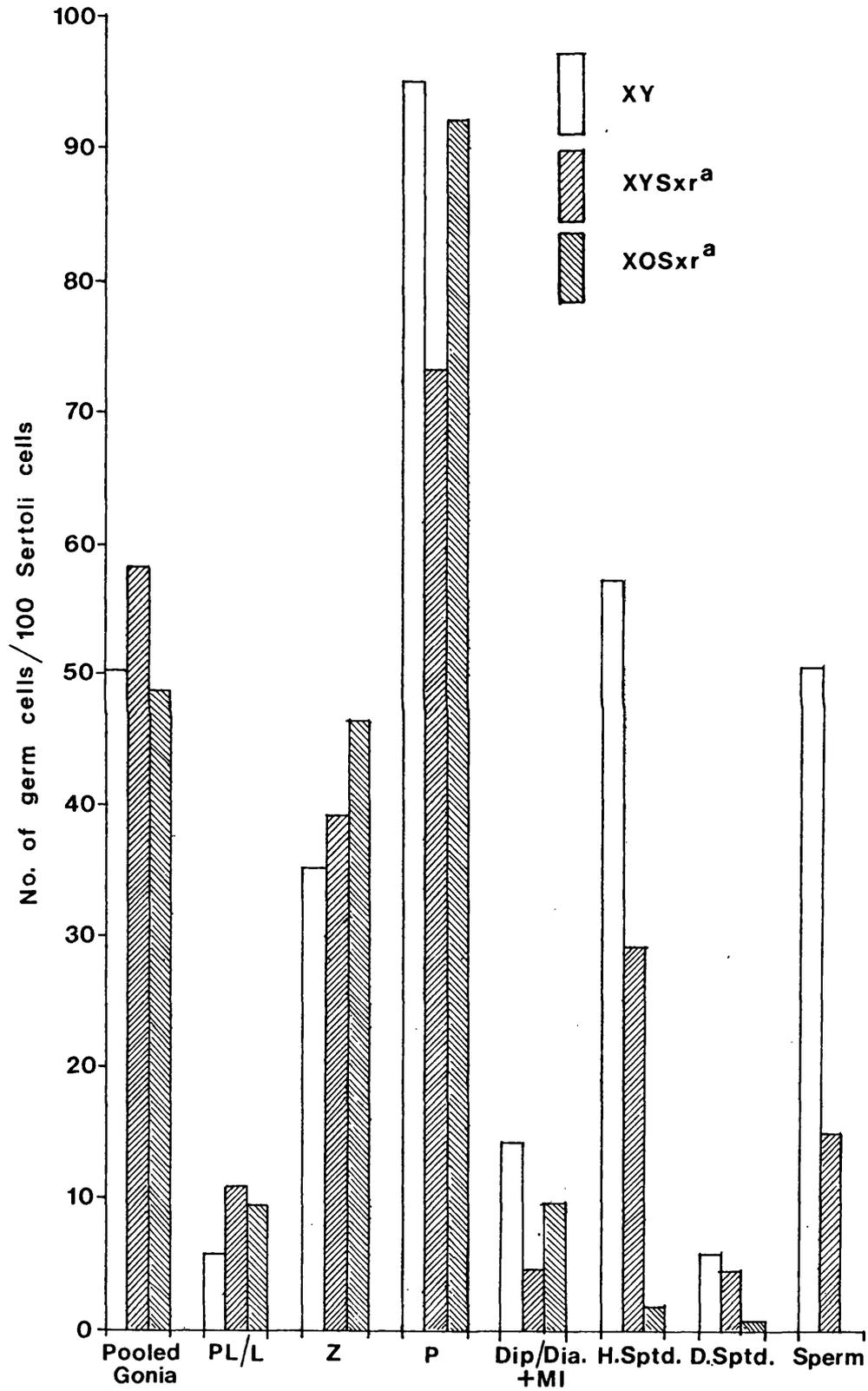


Fig. 3d. Histogram showing the total number of germ cells per 100 Sertoli cells in XY, XYSxr^a and XOSxr^a testes according to germ cell stage.

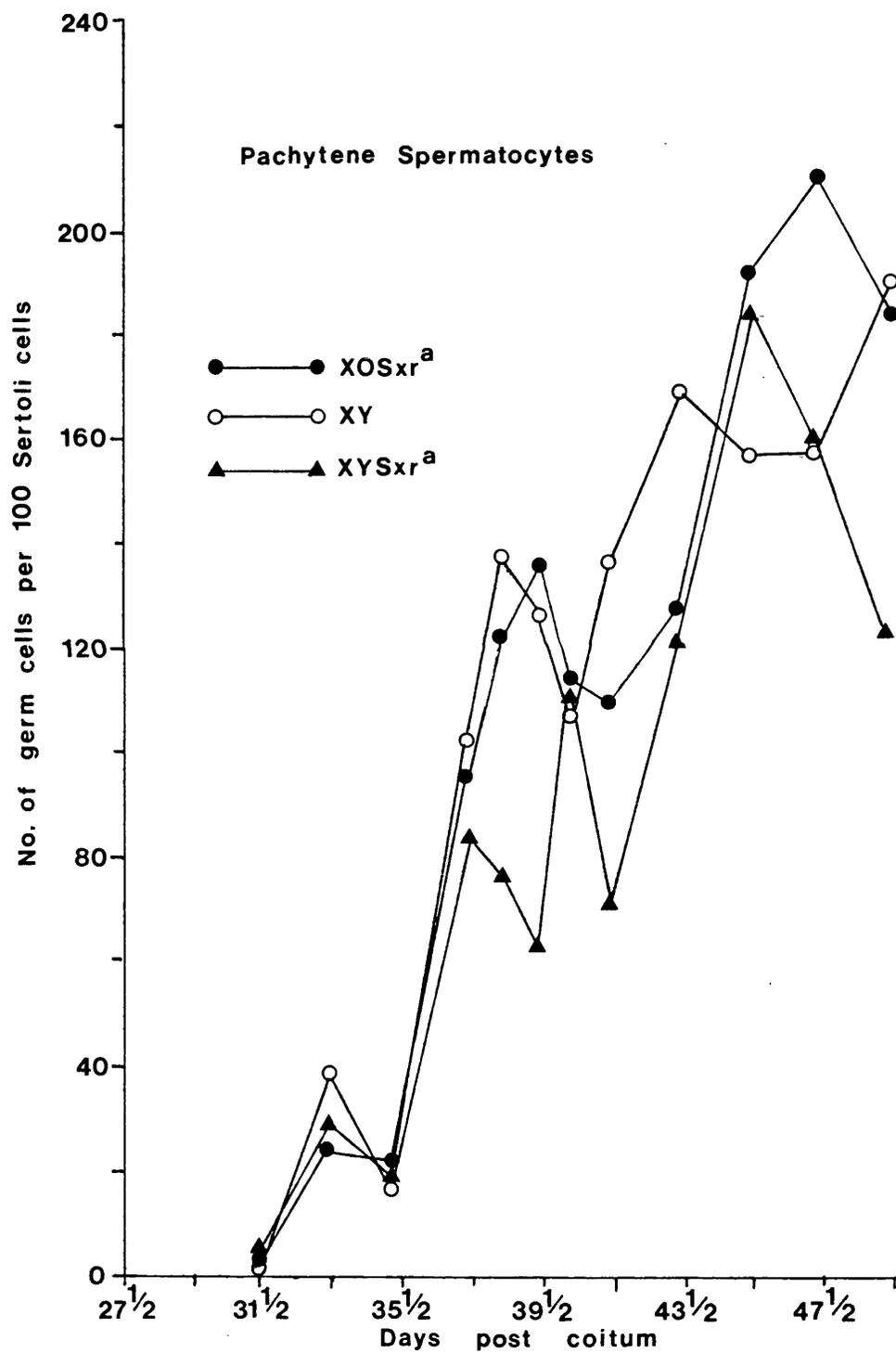


Fig. 3e. Total number of pachytene spermatocytes per 100 Sertoli cells in XY, XYSxr^a and XOSxr^a testes during the period 27 1/2 - 49 1/2 dpc.

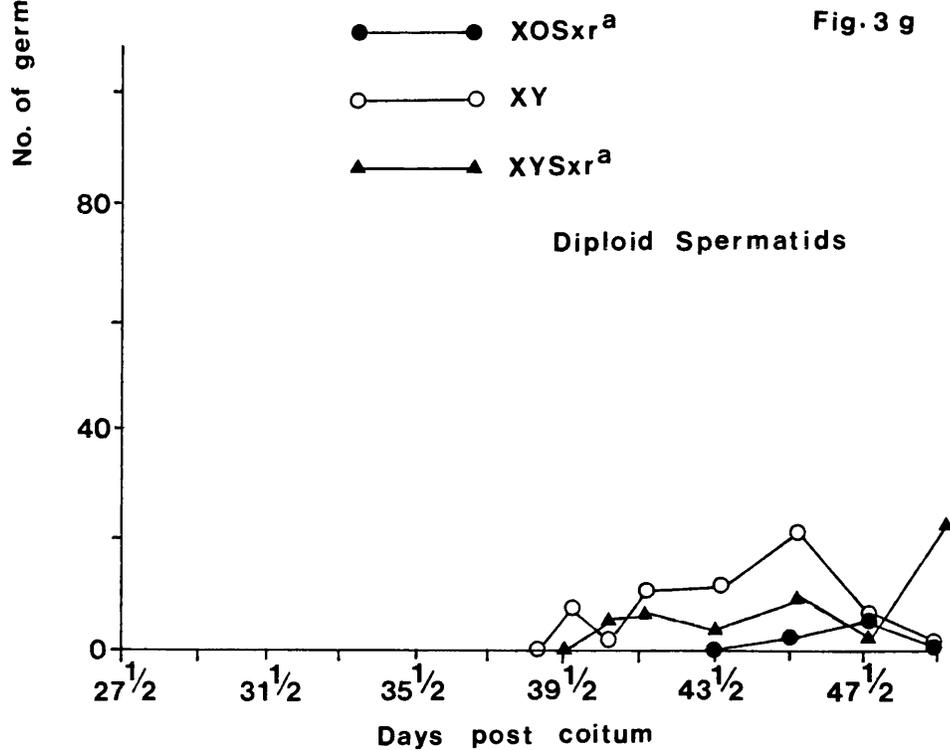
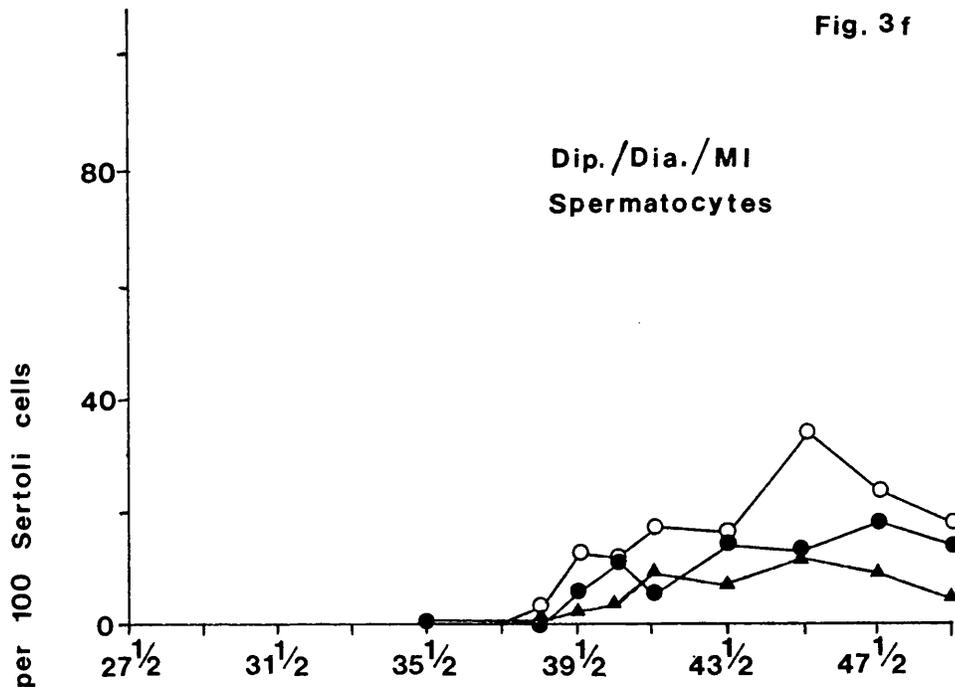


Fig. 3f-g. Total number of (f) pooled diplotene/diakinesis/MI spermatocytes, and (g) diploid spermatids per 100 Sertoli cells in XY, XYSxr^a and XOSxr^a testes during the period 27 1/2 - 49 1/2 dpc.

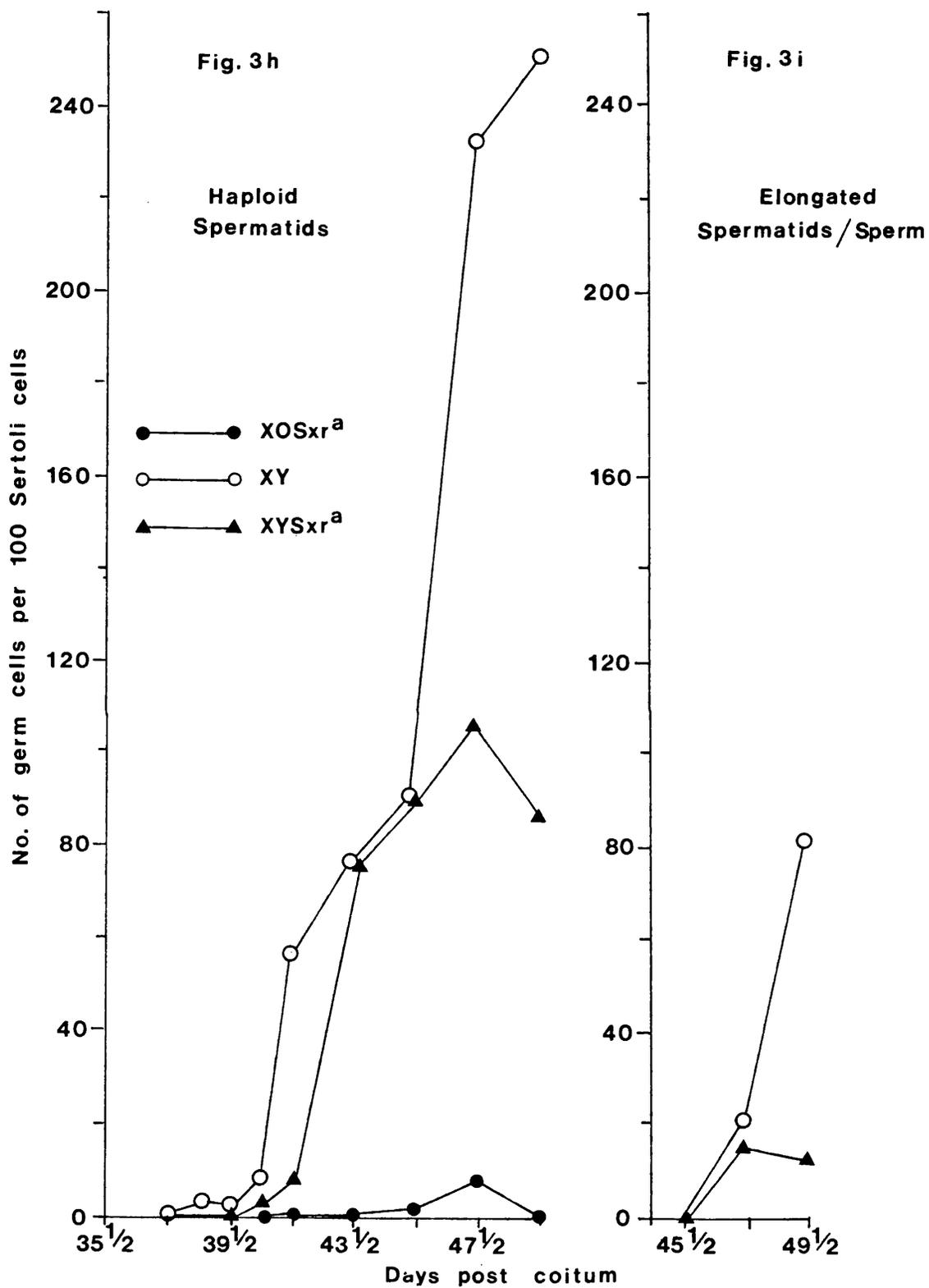


Fig. 3h-i. Total number of (h) haploid spermatids, and (i) condensed spermatids/spermatozoa per 100 Sertoli cells in XY, XYSxr^a and XOSxr^a testes during the period 27 1/2 - 49 1/2 dpc.

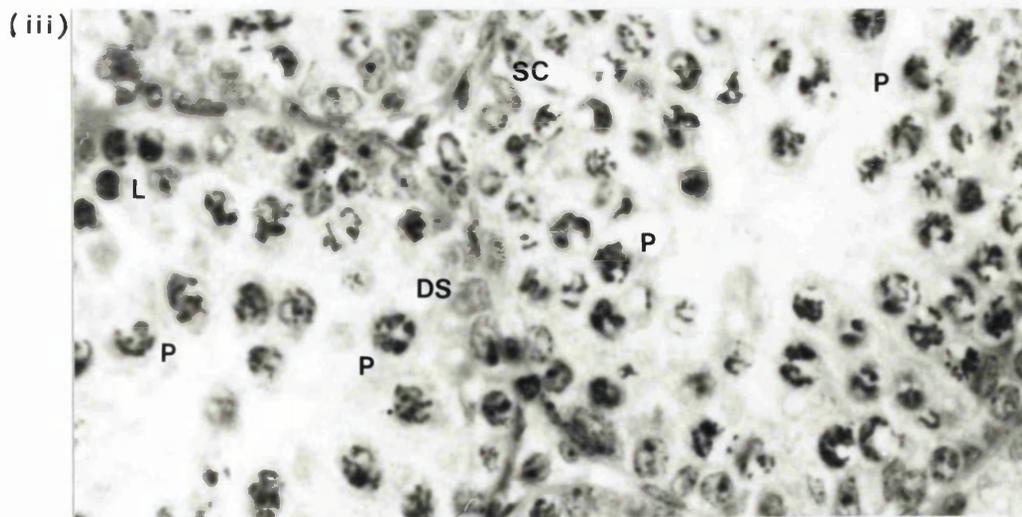
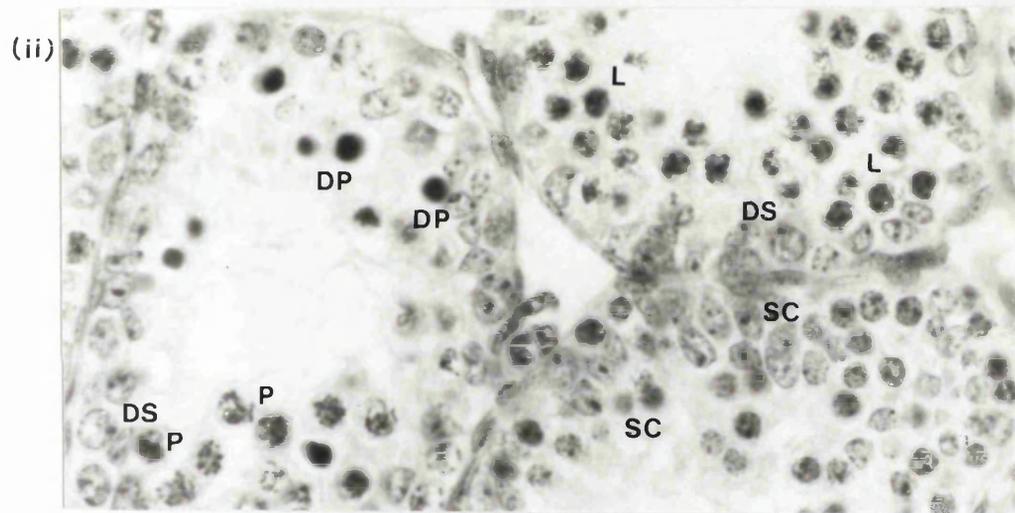
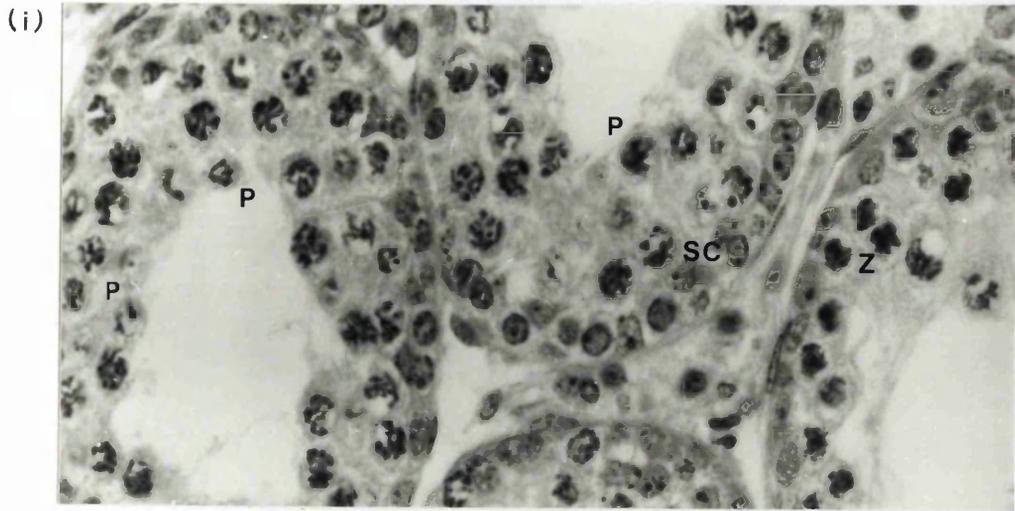


Plate 3a. Histological sections showing the pachytene stage in (i) XY, (ii) XYSxr^a, and (iii) XOSxr^a testes at 33 1/2 dpc. SC = Sertoli cell; DS = differentiating spermatogonia; L = leptotene; Z = zygotene; P = pachytene; DP = degenerating pachytene. Magnification X700.

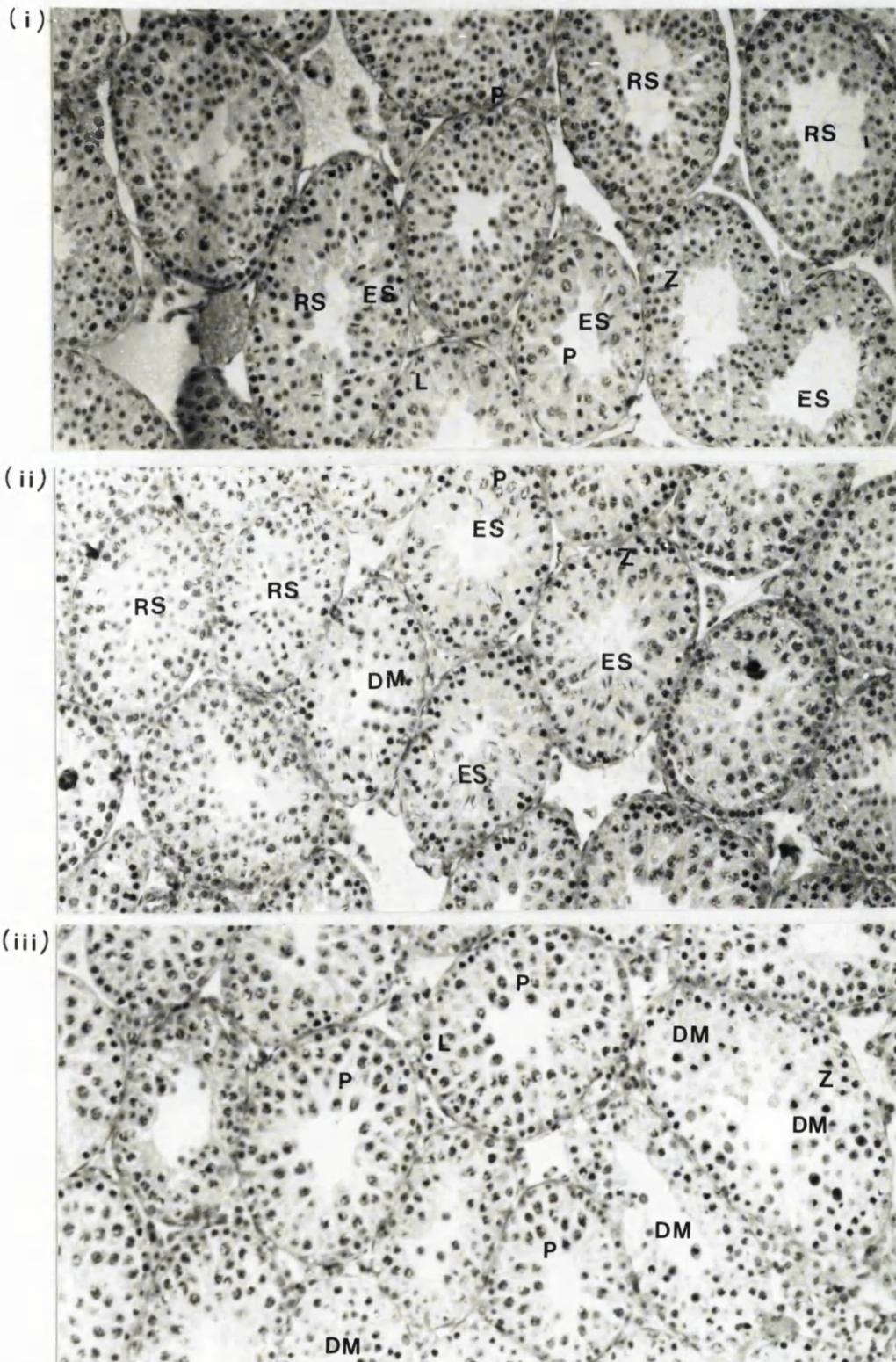


Plate 3b. Histological sections showing condensed spermatids/spermatozoa stages in (i) XY, (ii) XYSxr^a, and (iii) XOSxr^a testes at 47 1/2 dpc. SC = Sertoli cell; DS = differentiating spermatogonia; L = leptotene; Z = zygotene; P = pachytene; M = metaphase I; DM = degenerating metaphase I; RS = round spermatid; ES = elongated spermatid. Magnification X180.

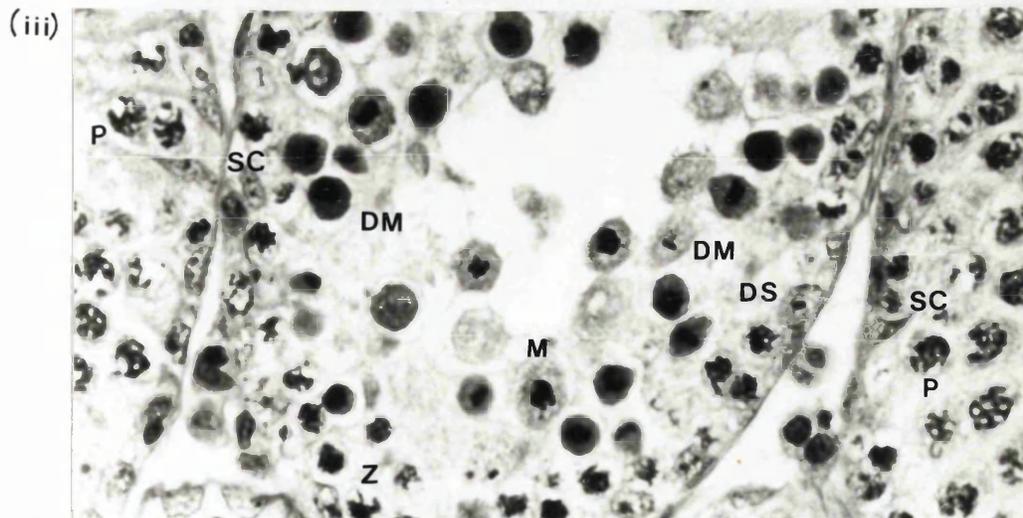
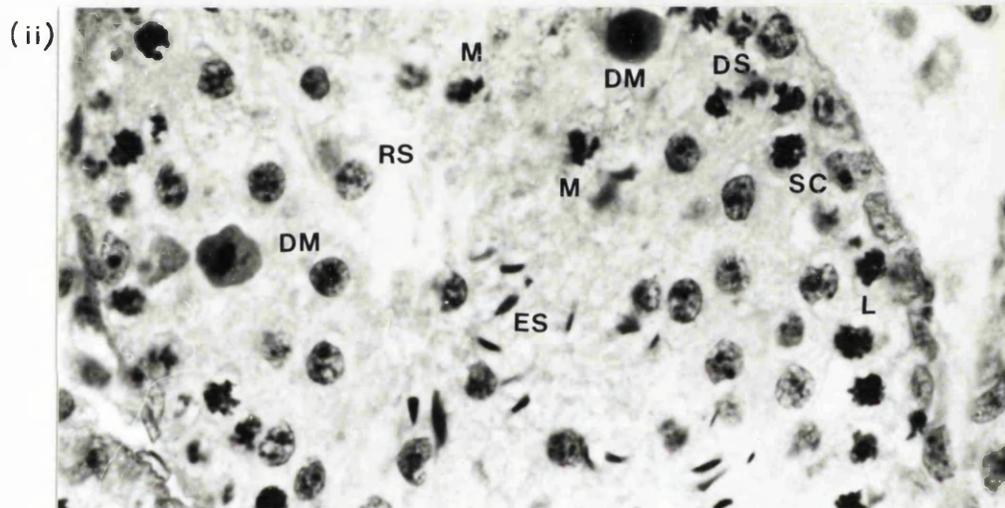
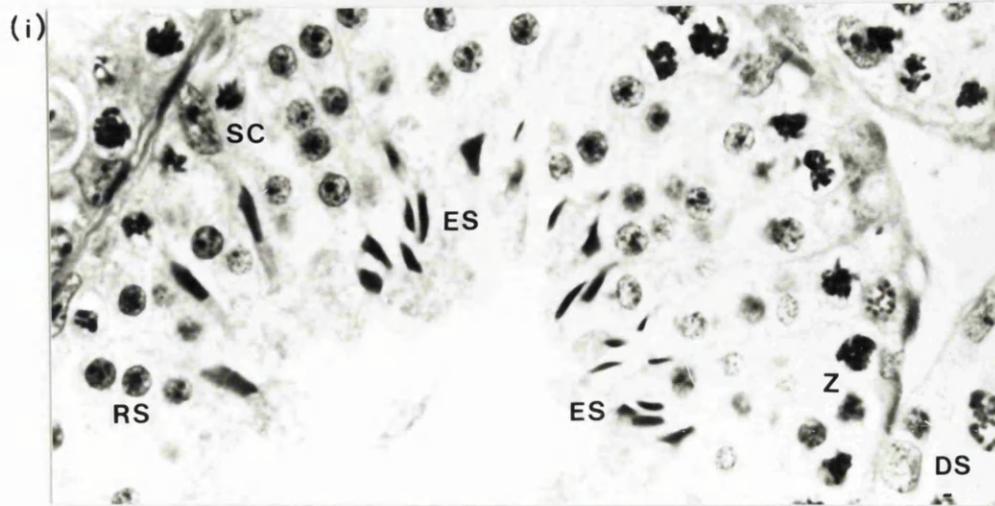


Plate 3 c. Histological sections showing condensed spermatid/spermatozoa stages in (i) XY, (ii) XYsxa, and (iii) XOSxa testes at 47 1/2 dpc. SC = Sertoli cell; DS = differentiating spermatogonia; L = leptotene; Z = zygotene; P = pachytene; M = metaphase I; DM = degenerating metaphase I; RS = round spermatid; ES = elongated spermatid. Magnification X700.

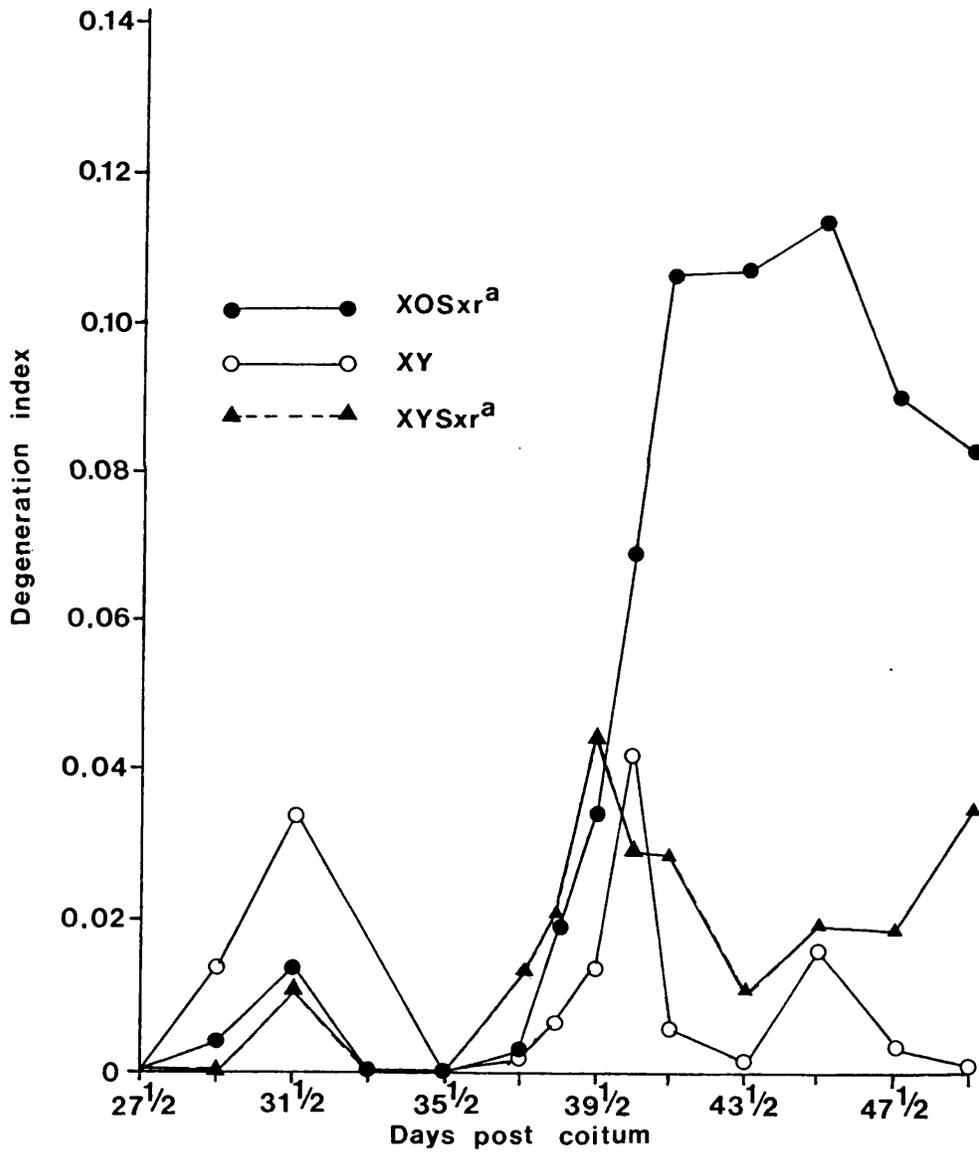


Fig. 3j. Germ cell degeneration index of meiotic and post-meiotic germ cells in XY, XYSxr^a and XOSxr^a mice during the period 27 1/2 - 49 1/2 dpc.

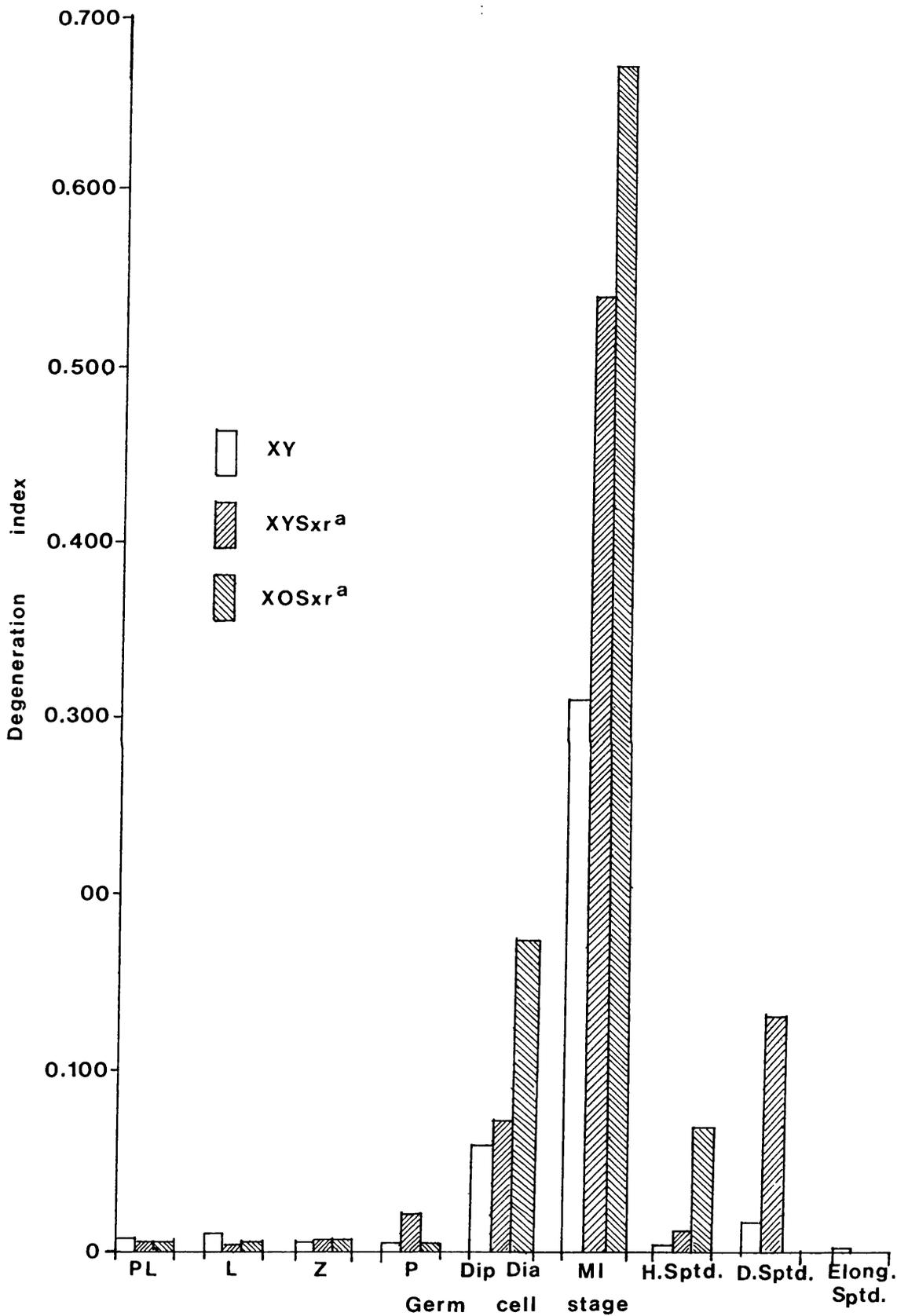


Fig. 3k. Histogram showing the degeneration index of meiotic and post-meiotic germ cells in XY, XYSxr^a and XOSxr^a mice according to germ cell stage.

DISCUSSION

The present results confirm the findings of Hannapel and Drews (1979) that the total spermatogonial number in $XY\text{Sxr}^a$ mice is not reduced compared to XY. They demonstrate further that this finding is consistent at all stages, from undifferentiated A through to B spermatogonia and extends to the early meiotic prophase stages up to, and including, zygotene. Additionally, the results show conclusively that a similar situation pertains in $XO\text{Sxr}^a$ mice. The quantitative analysis also demonstrates that the breakdown during meiosis and the subsequent degeneration begins earlier in $XY\text{Sxr}^a$ than $XO\text{Sxr}^a$ males.

Let us first consider the comparison of $XY\text{Sxr}^a$ to XY littermates. The first appearance of pachytene cells in $XY\text{Sxr}^a$ is the same as XY and initially the cell number is the same. $XY\text{Sxr}^a$ mice show a one day delay before the first appearance of diplotene/diakinesis/MI and this 24 hour delay is sustained throughout the subsequent stages. In addition, degeneration commences earlier in $XY\text{Sxr}^a$ tubules, and by diplotene/diakinesis/MI and spermatid stages there are approximately half the number of $XY\text{Sxr}^a$ cells compared to XY with degenerating and surviving cells in the same tubule. By the condensed spermatid stage, this number has fallen to less than 50%.

The picture that emerges from the $XO\text{Sxr}^a$ comparison with XY is somewhat different. There is clearly no difference between these sibs in terms of the first appearance of pachytene or the total number of germ cells at this stage. Germ cells at diplotene/diakinesis/MI stages in XY testes are first seen at 37 1/2 dpc, whereas in $XO\text{Sxr}^a$ mice they appear to be delayed by one day. Any cells surviving to the spermatid stage in $XO\text{Sxr}^a$ mice are delayed by four days. In addition to this delay, there is marked

degeneration. These results clearly indicate meiotic failure around diplotene/diakinesis/MI in XOSxr^a mice. The degeneration index indicates that most of the cells, blocked at MI, eventually degenerate and the few cells that 'slip through the net' then degenerate as haploid or diploid spermatids.

The impairment in XYSxr^a mice is therefore earlier, at pachytene, but although there is a one day delay, there is no drastic block like that seen in XOSxr^a mice, and those cells that segregate at MI (approximately half), continue through to round spermatids, the majority of which elongate to spermatozoa.

The pairing-abnormal spermatid development hypothesis (Miklos, 1974)

A hypothesis, derived from extensive data on abnormal chromosome behaviour and abnormal spermatid development in *Drosophila melanogaster* was proposed by Miklos in 1974 which linked disruption in chromosome pairing to sterility in a number of other species. The original concept of pairing sites on the X and Y chromosome and their association during male meiosis was introduced by Cooper (1964). Miklos expanded this concept and postulated that specific pairing sites existed on both autosomes and sex chromosomes and that interaction between homologous pairing sites was essential for normal spermiogenic development. Failure of the pairing sites to become 'saturated' during meiosis would lead to arrest and post-meiotic abnormality.

How does the XOSxr^a and XYSxr^a spermatogenic breakdown observed in this study compare to the hypothesis proposed by Miklos? Clearly, there is no germ cell degeneration prior to pachytene, so all cell loss occurs post-pairing in accordance with Miklos' model. However, the timing of cell loss differs between XOSxr^a and XYSxr^a, so can this finding too be accounted for by the model? In XOSxr^a testes, all germ cells have a single unpaired univalent chromosome and, according to the model, this implies a number of 'unsaturated' pairing sites with cell loss seen at MI. Conversely, in XYSxr^a mice, the testes are mosaic for normal and defective spermatogenesis and the onset of cell loss is at pachytene. It is assumed that when the X and Y chromosomes pair, all subsequent post-meiotic stages proceed normally through to functional sperm. However, high levels of X-Y non-association have been described in XYSxr^a mice (Winsor *et al.*, 1978; Evans *et al.*, 1980; Chandley and Fletcher, 1980) and here there are two unpaired univalents, which implies double the number of 'unsaturated' pairing sites. It is suggested, therefore, that the discrepancy in the stage of initial cell loss seen in XYSxr^a and XOSxr^a testes can be accounted for by the Miklos theory.

In 1979 and 1980, Burgoyne and Biddle undertook a statistical analysis of the available data on XYY mice. They concluded that only trivalents gave rise to post-meiotic products and that all cells with univalents blocked between MI and MII. Most germ cells in XOSxr^a testes are seen to block at MI (Cattanach *et al.*, 1971) although some spermatids are found. The majority of these spermatids are diploid (Burgoyne and Baker, 1984; Levy and Burgoyne, 1986a), consistent with the proposal that diploid spermatids resulted from omission of the MII reduction division (Burgoyne and Baker, 1984), and in keeping with the Burgoyne and Biddle (1980)

predictions. The finding of haploid spermatids in XOSxr^a mice, however, was at variance with their conclusions. It was suggested by Levy (1986) and Burgoyne (1987a) that self-synapsis of the single XSxr^a chromosome (Chandley and Fletcher, 1980) might result in satisfaction of some, or all, of the pairing sites leading to spermatid formation, in accordance with Miklos' model. Although Mahadevaiah *et al.* (1988) argued against this possibility because the XOSxr^a component in some XXSxr^a mice appeared to give rise to normal-looking sperm without self-synapsis, reference is made, in this respect, to the following chapter.

It is known that variation in genetic background can affect the overall spermatogenic success in different mouse strains (Burgoyne and Mahadevaiah - personal communication). The paucity of spermatid stages and absence of sperm in the XOSxr^a mice of this study compared to those previously described (Cattanach *et al.*, 1971; Lyon *et al.*, 1981; Levy and Burgoyne, 1986a; Mahadevaiah *et al.*, 1988) could be explained by variation in genetic background. An interesting alternative explanation stems from recent investigations of sex chromosome aneuploid mice in our laboratory that suggests fertility of chromosomally anomalous mice may vary with age (Mahadevaiah and Burgoyne - personal communication). Some males in the aneuploid group that were assumed to be sterile following prolonged periods of non-productive matings, finally fathered their first litters after 7/8 months. Whilst the first meiotic prophase stages have been described for normal XY mice (Goetz *et al.*, 1984), no systematic study of the first meiotic wave in sex-reversed mice has previously been undertaken since all other XOSxr^a and XYSxr^a studies have been conducted on adult animals. If an age effect is applicable to XOSxr^a mice in this study, then adult XOSxr^a individuals would be expected to have an increased number of spermatids and occasional

sperm. In a recent study by Krzanowska (1989), a higher level of X-Y chromosome non-association was seen in pubertal males compared to that seen in the adult, and this was ascribed to an age effect. The XY control littermates of my study showed a proportion of diploid spermatids, and it is suggested that this probably arose from X-Y non-association in these pubertal mice. One possible explanation for an age effect could be a stricter cell selection against faulty cells in young adults with the screening system becoming less efficient with age (akin to the rationale of ageing oocytes in older females and the increased incidence of Down's Syndrome). If the rationale of age increasing spermatogenic success applies to XOSxr^a mice, this would conflict with the claim made by Goetz *et al.* (1984) that the first meiotic wave was entirely representative of that found in the adult.

In conclusion, the results of this developmental germ cell study in XOSxr^a and XYSxr^a mice are consistent with the pairing hypothesis predicted by Miklos (1974). As a further test of this model, Burgoyne (1987a) proposed that the XSxr^a chromosome be provided with a pairing partner, without additional Y chromosome material, and this experiment forms the basis of the following chapter.

CHAPTER 4

**EVIDENCE THAT X-Y PAIRING AND A Y-LINKED SPERM
MORPHOLOGY GENE ARE REQUIRED FOR NORMAL
SPERMATOGENESIS**

This experiment was undertaken as a joint laboratory project, assigned as follows:- (1) Breeding, PGK enzyme assay, bone marrow preparations, histology and quantitation was undertaken by the writer, (2) Synaptonemal complexes and sperm counts by Dr S K Mahadevaiah, (3) Partial splenectomies, vas smears, air dried testis preparations and MI analysis by Dr P S Burgoyne and Mr S J Palmer.

INTRODUCTION

A complex rearranged Y chromosome in the mouse has been described by Eicher (1982) and designated Y^* (Eicher *et al.*, 1983). The possible origin of Y^* was proposed by Eicher and Washburn in 1986, but recent findings that *Tdy* is located on the Y short arm rather than the proximal region of the long arm (McLaren *et al.*, 1988; Roberts *et al.*, 1988), precludes the Y^* rearrangement originating by a single event. Details of the Y^* rearrangement have therefore still to be determined, but we do know that Y^* lacks a short arm, the pairing and exchange region is located proximally on the long arm, and the testis-determining region is located distal to the pairing and exchange region. The chromosome products resulting from the cross over between the Y^* and X chromosomes at meiosis are shown in the schematic Fig. 4a. One of the recombinant chromosomal products, X^Y , comprises most of the X chromosome with most of the Y chromosome attached distally. The reciprocal product Y^X , referred to as Y^{del} (Hunt and Eicher, 1989 - unpublished), is a tiny Y fragment comprising little more than a centromere and the X-Y pairing and exchange region. Y^{del} is only just visible cytogenetically and, since it lacks the testis-determining region, XY^{del} mice are female.

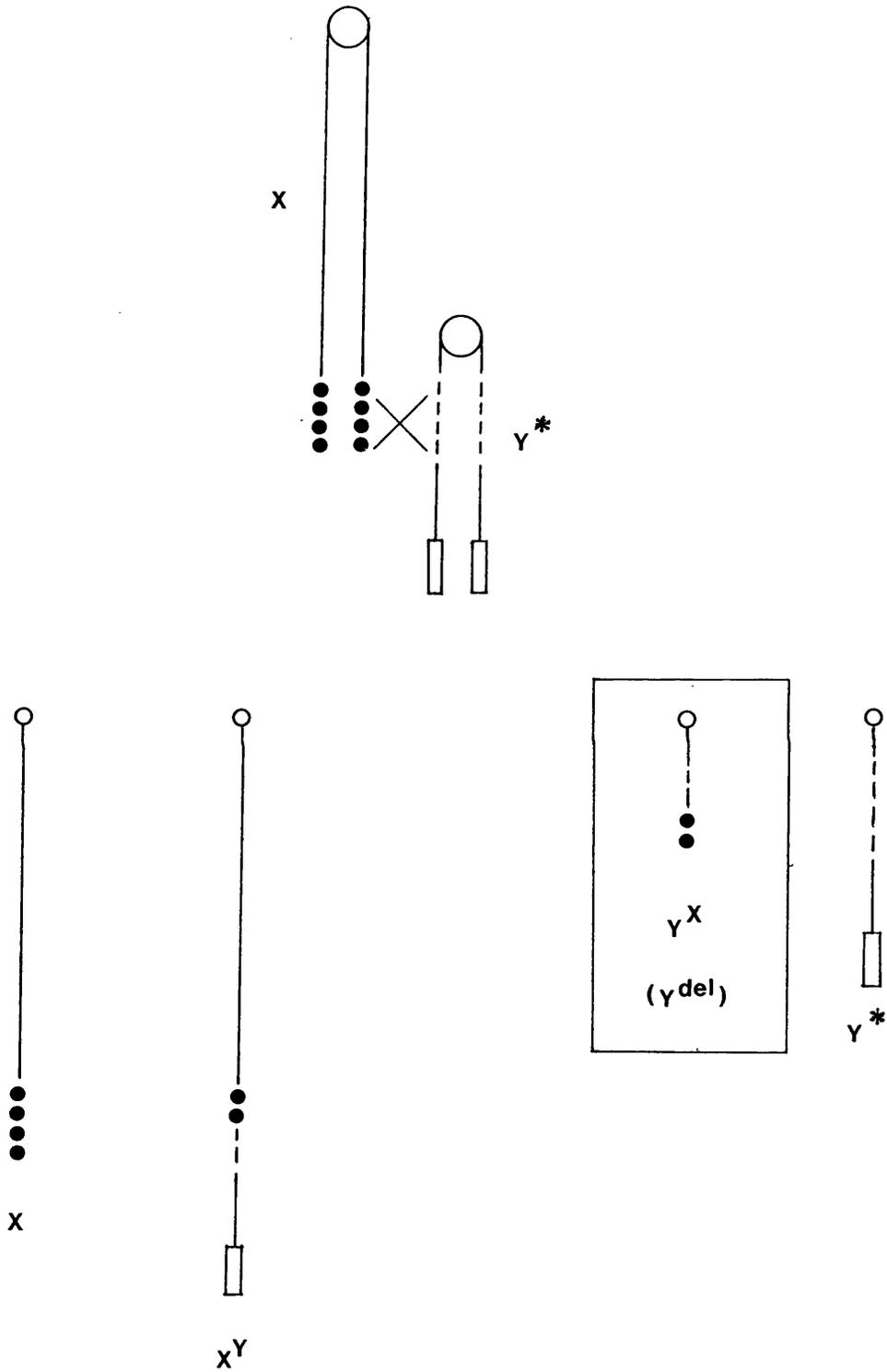


Fig. 4a. Schematic showing the chromosomal products following pairing and exchange between the X and Y* chromosomes at meiosis.

As described in the previous experiment, XOSxr^a male mice have normal spermatogenesis until meiosis and then varying degrees of impairment with greatly reduced numbers of spermatids (Cattanach *et al.*, 1971), most of which are diploid (Levy and Burgoyne, 1986a). If sperm are produced - and this seems to vary with genetic background - the sperm are few in number and have abnormal heads, but are motile (Burgoyne, 1987a).

The spermatogenic impairment seen in XOSxr^a mice may be a consequence of the lack of a pairing partner for the XSxr^a chromosome during meiosis or due to the absence of the long arm of the Y chromosome. These two postulates are not necessarily mutually exclusive.

Despite the pairing phenomenon of XOSxr^a mice being the subject of a number of studies and reviews (Cattanach, 1975; Chandley and Fletcher, 1980; Burgoyne and Baker, 1984; Levy and Burgoyne, 1986a; Burgoyne 1987b; Mahadevaiah *et al.* 1988), it has remained difficult to experimentally separate the two points raised in Cattanach *et al.*'s (1971) original paper. The experiment described in this chapter attempts to separate these two possibilities by providing the Y^{del} from the XY* system, as a pairing partner for the XSxr^a chromosome, without the addition of any known long arm Y chromosome material.

MATERIALS AND METHODS

Mice

The $X^{Sxr^a}Y^{del}$ mice used in this study were derived from a multi-generation breeding programme. This programme is detailed in Appendices 4a - g and is summarised below.

Part 1: The aim of Part 1 of the breeding programme was to produce $T16H^B/X^A Sxr^a$ females and $X^A Sxr^a Y^{Sxr^a}$ males.

Females heterozygous for Searle's translocation $T(X;16)16H$ and homozygous for the X-linked $PGK-1^b$ allele ($T16H^B/X^B$ females) were mated to males carrying the sex reversal factor Sxr^a and the $PGK-1^a$ allele ($X^A Y^{Sxr^a}$ males). (The original $T16H^B/X^B$ females were a gift from Dr C Beechey at the MRC Radiobiology Unit, Didcot, Oxon). The progeny of this cross include $T16H^B/X^A Sxr^a$ mice, approximately 60% of which were expected to develop as females (McLaren, 1986) (Appendix 4a). Initially the $X^A Y^{Sxr^a}$ males used in this cross derived their X^A chromosome from mothers who had a random-bred MFI background (Appendix 4b). These males consistently failed to father $T16H^B/X^A Sxr^a$ daughters. Only one out of the twenty-five agouti $PGK-1^B$ daughters was thought to carry Sxr^a , but became infertile during test matings before this was confirmed. The problem was resolved by using $X^A Y^{Sxr^a}$ males in which the X^A chromosome was derived from mothers with a C3H background, as was the case in the original study of McLaren and Monk (1982). This anomaly is currently under investigation, but falls outside the scope of this thesis.

The $T16H^B/X^A Sxr^a$ females eventually produced were identified as such by their PGK-1B phenotype (only the translocation X is expressed) and by their ability to produce striped $Ta/+$ male offspring (i.e. $X^{Ta}/X^+ Sxr^a$) when mated to $X^{Ta}Y$ males. These $T16H^B/X^A Sxr^a$ females were then backcrossed to their $X^A Y Sxr^a$ fathers (Appendix 4c). Adult PGK-1A male progeny with large testes were test mated to see if they produced all male offspring (McLaren and Burgoyne, 1983) (Appendix 4d) as is the case with $X^A Sxr^a Y Sxr^a$ males. One such $X^A Sxr^a Y Sxr^a$ male was identified.

Part 2: The aim of part 2 of the breeding programme was to produce $X^A O Sxr^a$ and $X^A Sxr^a Y^{del}$ males on the same genetic background.

The XY^* males were a gift from Dr E Eicher of the Jackson Laboratory, Maine, USA. X^{BY^*} males were crossed with homozygous inversion $In(X)1H$ females from our laboratory (Appendix 4e). Two types of progeny from this cross, heterozygous $In(X)^B/X^B$ females and $In(X)^B Y^*$ males were used for the next generation breeding.

$In(X)^B/X^B$ females crossed to the homozygous $X^A Sxr^a Y Sxr^a$ male from Part 1 (Appendix 4f), produced $X^A O Sxr^a$ males amongst their offspring. $In(X)^B Y^*$ males mated to $T16H^B/X^A Sxr^a$ females from Part 1 (Appendix 4g), produced the $X^A Sxr^a Y^{del}$ males.

Three adult $X^A O Sxr^a$ and four adult $X^A Sxr^a Y^{del}$ males were used in this study.

PGK Enzyme Assay

The biochemical microassay for the X-chromosome-linked phosphoglycerate kinase enzyme, PGK-1, was carried out using blood samples taken from the tail vein. The assay distinguishes between the electrophoretic variant isozyme bands PGK-1A and PGK-1B and is described in detail in Appendix 4h. Briefly, tissue extracts are diluted in sample buffer and loaded onto the cathodal side of a cellulose acetate strip soaked in running buffer. Electrophoresis is carried out using a water cooled Whatman electrophoresis tank run at 200 volts for one and half hours. Staining is achieved by the addition of a number of reagents that activate fructose 1,6 diphosphate in a forward PGK-1 reaction. When this reaction is coupled to an auxiliary enzyme system, fluorescent NADPH is produced which can be visualised under ultra violet light (Monk, 1987).

Karyotyping

Partial splenectomies were performed for the purpose of preparing mitotic spreads without sacrificing the animal. Mice were anaesthetised by injecting Avertin at a concentration of 1.25% into the peritoneal cavity at a dosage dependent upon body weight (e.g. 0.75ml/30g). Approximately one tenth of the spleen was removed, dissociated in a test tube by pipetting vigorously with a Pasteur pipette in 0.004% colcemid in Hepes-buffered Eagle's minimal essential medium and incubated for 60 minutes at 32°C. Preparations were then treated as for bone marrow cells, described in Materials and Methods, Chapter 2.

At autopsy, mitotic spreads were prepared from bone marrow cells as described in Materials and Methods, Chapter 2, as confirmation of the splenectomy results. G banding of a bone marrow preparation was undertaken by Dr E P Evans (Sir William Dunn School of Pathology, Oxford), which confirmed a T16HSx^{aYdel} karyotype, that was excluded from the subsequent quantitative analysis.

Air Dried Testis Preparations

Both testes of adult males were removed at autopsy and weighed using a Mettler balance. One testis was fixed in Bouins for histology (see Histology section). The other testis was divided into two halves. Tubules from one half of the testis were freed from the tunica and dissociated in 2.2% isotonic sodium citrate for one minute. The pellet was then treated with 1% sodium citrate for 13 minutes, followed by 3:1 ethanol:glacial acetic acid fixative carefully added drop-by-drop, with the pellet flicked into the suspension after each drop. A further five rapid changes of fixative were followed by a final resuspension of the pellet in 2 drops of 3:1 methanol:glacial acetic acid. The cells were dropped onto slides, air dried and stained in 2% Giemsa in pH 6.8 phosphate buffer for 25 minutes (Evans, Breckon and Ford, 1964).

Synaptonemal Complexes

From the remaining half testis, tubules were dissociated in a few drops of RPMI 1640 medium onto slides, incubated in 5% carbon dioxide at 37°C and used for synaptonemal complex preparations (Guitart et al., 1985; Mahadevaiah, 1987). Briefly, the cells are subjected to 0.4% sodium

chloride hypotonic solution, fixed in 0.03% sodium dodecyl sulphate in 4% formaldehyde and dipped in 0.4% photoflo at pH 8.0. Staining was carried out at 57°C with gel-developer and 50% silver nitrate. Plastic coated slides were used since all material was required for analysis under the electron microscope.

Histology

The contralateral testes from X^AOSxr^a and X^ASxr^aY^{del} males were then processed for histology, as described in Materials and Methods, Chapter 2.

Quantitative Analysis

Quantitative analysis of histological serial sections was carried out as described in Materials and Methods, Chapter 2, with the following modifications. The sampling was one tubule every 40th section, such that 20 tubule cross-sections were analysed per testis. A low power x4 objective was used to focus the grid underlying the slide. The high power objective used was x50 under oil immersion.

Sperm Count and Morphology

Vas smears for sperm dimension measurements and morphology, were prepared according to the technique of Burgoyne (1973). The vasa were held by forceps at one end and the sperm squeezed out and mixed into one drop of phosphate-buffered saline for one minute. Two drops of nigrosine/eosin were then added, mixed and left for a further two minutes. One drop was then smeared onto a clean slide and allowed to air dry.

Sperm counts were made from both capita epididymides which were dissected free from fat, weighed, placed in a petri dish with 0.2ml of 1% sodium citrate and chopped up using a scalpel and forceps. A further 1.8ml of sodium citrate was added, and the suspension mixed well with a Pasteur pipette. A small drop was placed on a haemocytometer and a count made of four squares, each square representing a chamber containing 0.0001 ml of cell suspension. The number of spermatozoa per ml was then calculated by dividing the total square count by 4×0.0001 .

RESULTS

For ease of description, the PGK-1A and B classification will be omitted from the genotype descriptions for the remainder of this thesis.

An example of the $XSxr^aY^{del}$ karyotype is shown in Plate 4a. This illustrates the extremely small size of the Y^{del} fragment and its unambiguous identification compared to the normal Y chromosome which approximates in size to the smallest autosome, chromosome 19.

The data on testis weights and sperm counts are shown in Table 4a for the three $XOSxr^a$ and four $XSxr^aY^{del}$ adult mice analysed in this study. The testis weights for the $XOSxr^a$ mice concur with previous findings and those seen in Chapter 3. Although sperm are occasionally found in $XOSxr^a$ mice, none were observed in these three individuals. By contrast, the testis weights of all four $XSxr^aY^{del}$ mice were approximately double those of $XOSxr^a$. The sperm count ranged from 3/4 to more than 3 million, the higher values within the range of normal XY mice from other genetic

backgrounds. Unfortunately, since all males in this cross have abnormal genotypes, no testis weight or sperm count data for normal XY mice on this background could be recorded, although data from a project involving males on a similar outbred background had testis weights ranging from 135.0 - 150.0 mg and sperm counts around 4 million (Mahadeviah - personal communication).

Plates 4b - d show electron micrographs of the synaptonemal complex association in XY, XOSxr^a and XSxr^aY^{del} pachytene spreads. The univalent XOSxr^a chromosome sometimes self pairs by forming a ring or hairpin loop. The Y^{del} fragment is seen to pair with the XSxr^a chromosome along the X-Y pairing and exchange region, confirming synapsis in XSxr^aY^{del} mice (Plate 4d).

Air dried preparations were used to score the percentage of XSxr^a-Y^{del} dissociation at metaphase I and the results, shown in Table 4b, average around 40%. A larger sample would have to be scored to determine whether there is a positive correlation between the amount of dissociation and sperm number.

The histological picture is in agreement with the testis weight and sperm count findings. Plate 4e. (i) - (ii) show that spermatogenesis breaks down at meiosis in XOSxr^a testes and most metaphase I cells are seen to be degenerating, Plate 4e. (i). A few metaphase I cells do survive, however, giving rise to a limited number of round spermatids although no sperm. (The number of spermatids and the occurrence of sperm is influenced by genetic background - Burgoyne - personal communication). XSxr^aY^{del}

mice, by comparison, have active spermatogenesis with both round and condensed spermatids and large numbers of spermatozoa, Plate 4e. (ii).

A quantitative analysis of germ cell types was undertaken from these histological serial sections. Since Sertoli cells cease dividing around 14 days *post partum*, adults have a stable Sertoli cell population and germ cell numbers were therefore expressed per 100 Sertoli cells (Russell and Clermont, 1977). The number for each meiotic germ cell stage is shown as a histogram for three $XOSxr^a$ and three of the four $XSxr^aY^{del}$ adult mice (Fig. 4b). There is clearly no difference in the total number of prophase cells between $XOSxr^a$ and $XSxr^aY^{del}$, but the high number of degenerating cells at metaphase I in $XOSxr^a$ explains the comparative paucity of round spermatids. There is also some evidence of degenerating metaphase I cells in $XSxr^aY^{del}$ but proportionally much less so than in $XOSxr^a$. Surviving $XOSxr^a$ spermatid cells are mainly diploid and a small proportion of $XSxr^aY^{del}$ spermatids are also diploid. The addition of the Y^{del} fragment to $XSxr^a$ dramatically alters the number of spermatids and sperm. A regression analysis of testis weights and total germ cell number for three $XOSxr^a$ and three $XSxr^aY^{del}$ adult mice demonstrates a strong positive correlation (Fig 4c), with the correlation coefficient for $XOSxr^a = 0.9970$ and for $XSxr^aY^{del} = 0.9557$.

Despite the high sperm number, the sperm in $XSxr^aY^{del}$ mice were all found to be abnormal. A typical example of the abnormal sperm head shape, compared to the normal for XY mice, is shown in Plate 4h (i) and (ii).

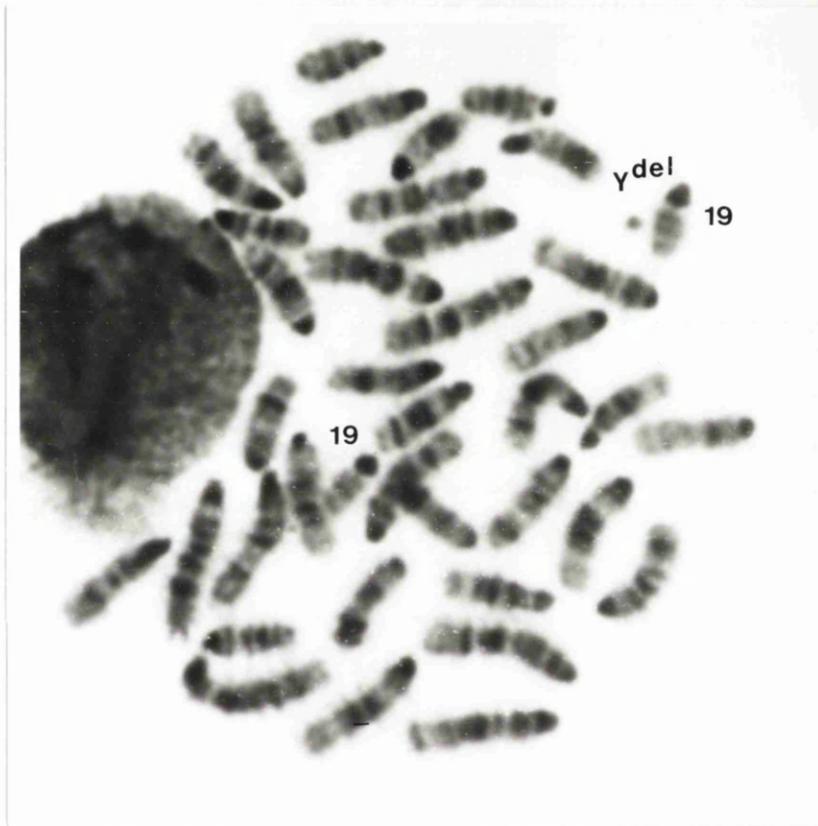
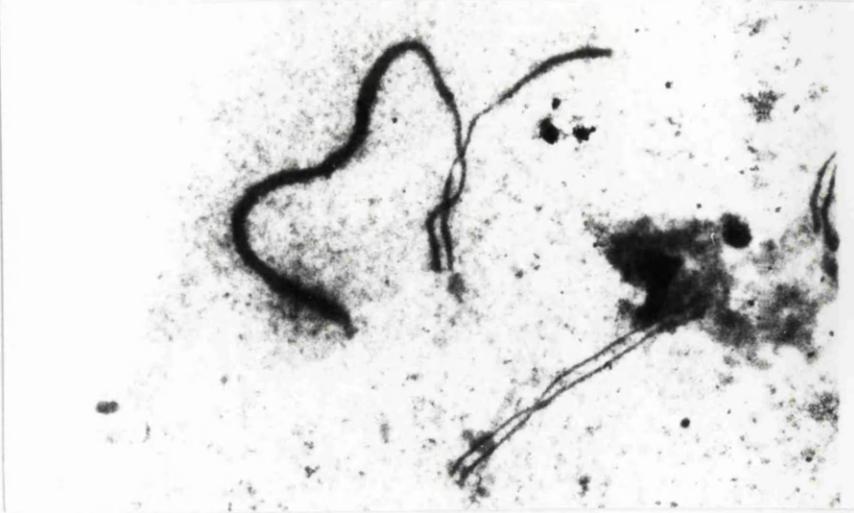


Plate 4a. Mitotic bone marrow spread showing the karyotype of $XSxr^aY^{del}$ mice. (courtesy of Dr E P Evans). The Y^{del} fragment can be seen lying alongside chromosome number 19 (the smallest autosome), which is approximately the same size as a normal Y chromosome.

<u>Genotype</u>	<u>No.</u>	<u>Testis weight (mg)</u>		<u>Sperm/caput</u>
		<u>Left</u>	<u>Right</u>	
XOSxr ^a	H25	52	53	0
	G23	43	49	0
	H67	46	43	0
XSxr ^{aYdel}	E12	136	136	3,095,000
	E11	112	119	1,242,500
	D 8	98	98	745,000
	A55	103	102	1,527,000

Table 4a. Table comparing the testis weights and sperm counts for three XOSxr^a and four XSxr^{aYdel} adult mice (courtesy Dr S K Mahadevaiah and Dr P S Burgoyne).

(b)



(c)



(d)

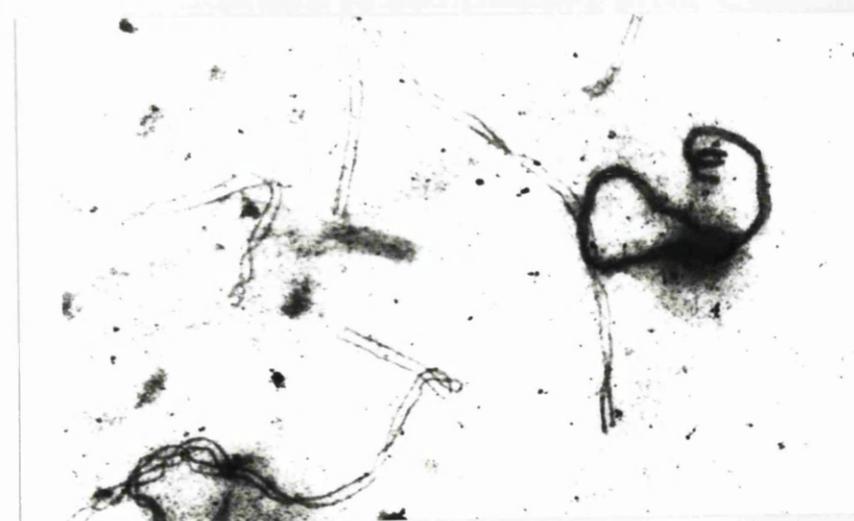


Plate 4b - d. Electron micrographs of synaptonemal complex preparations in (b) XY, (c) XOSxr^a and (d) XSxr^aY^{del} pachytene spreads (courtesy of Dr S K Mahadevaiah).

<u>Genotype</u>	<u>No.</u>	<u>% X-Y Pairing</u>	<u>% X-Y Dissociation*</u>
XSxr ^a Y ^{del}	E12	72.7	27.3
	E11	48.0	52.0
	D 8	70.7	29.3
	A55	49.0	51.0

* Allowances for non-appearance of Y^{del} (assumed to be present but sometimes difficult to visualise because of small size) made in the dissociation percentages.

Table 4b. Percentage X-Y dissociation scored from metaphase I testis preparations in four XSxr^aY^{del} mice (courtesy Dr P S Burgoyne).

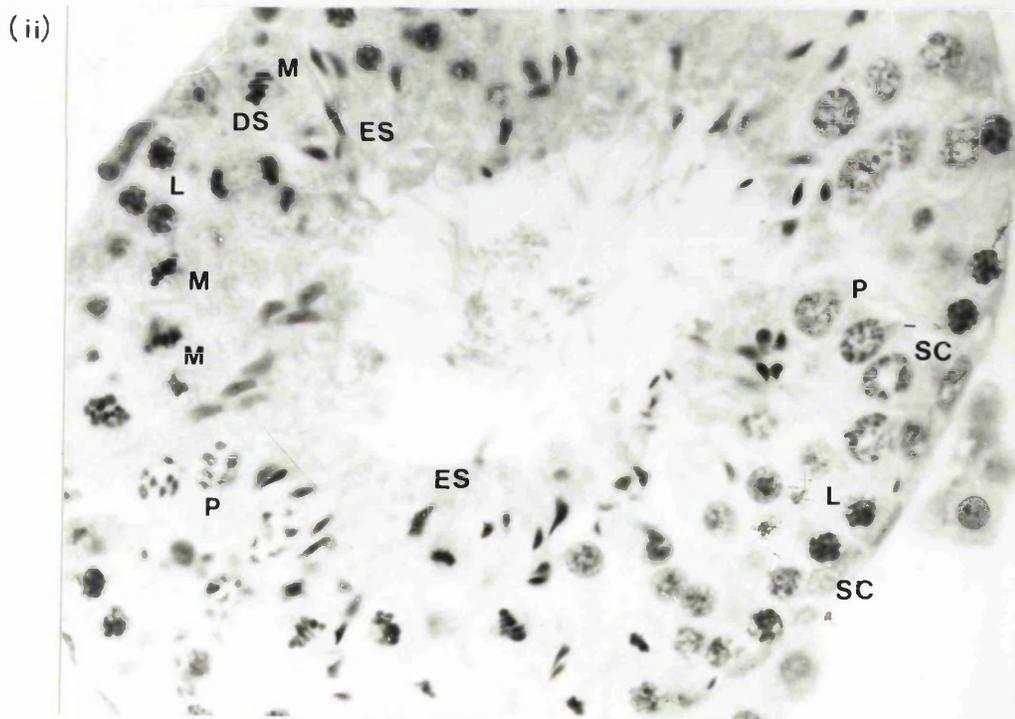
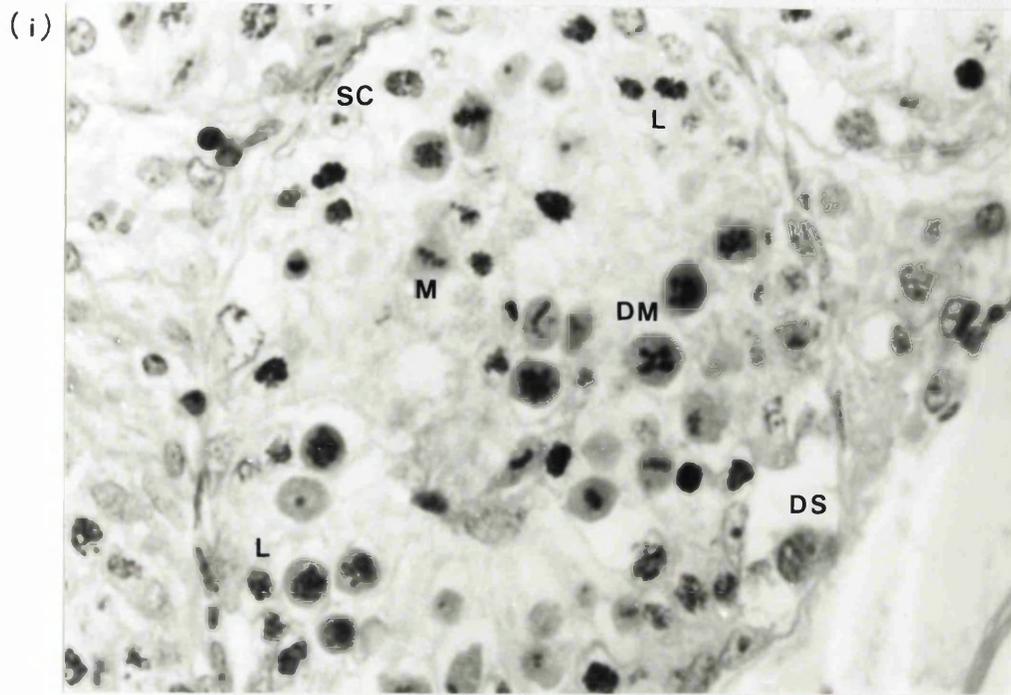


Plate 4e. Histological sections showing the condensed spermatids/spermatozoa stages in (i) $XOSxr^a$, and (ii) $XSxr^aY^{del}$ testes. SC = Sertoli cell; DS = differentiating spermatogonia; L = leptotene; Z = zygotene; P = pachytene; M = metaphase I; DM = degenerating metaphase I; RS = round spermatid; ES = elongated spermatid. Magnification X1500.

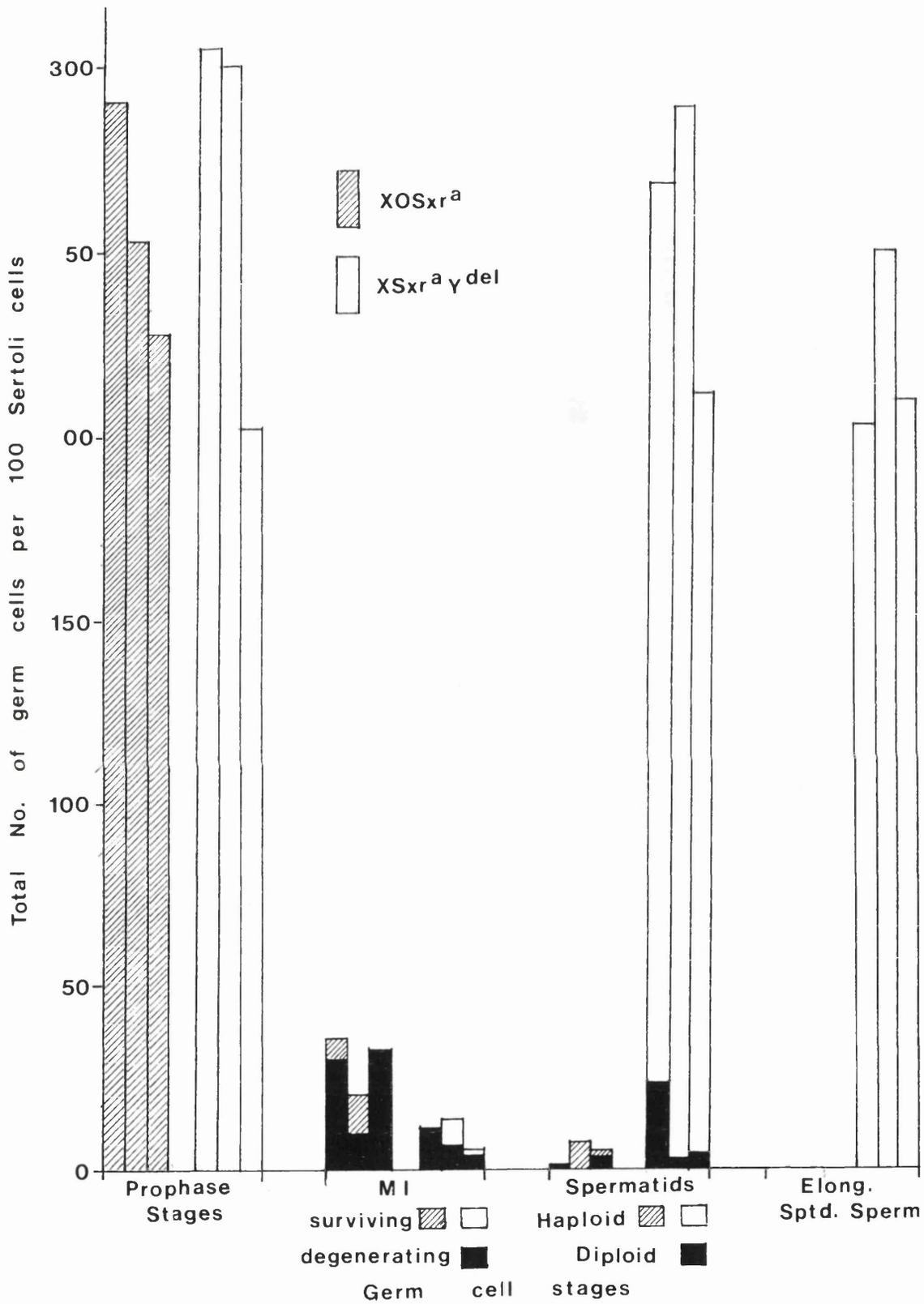


Fig 4b. Histogram showing the total number of germ cells per 100 Sertoli cells in three $XOSxr^a$ and three $XSxr^aY^{del}$ mice according to germ cell stage. The blocked areas show the proportion of MI degenerating spermatocytes and diploid spermatids in both genotypes.

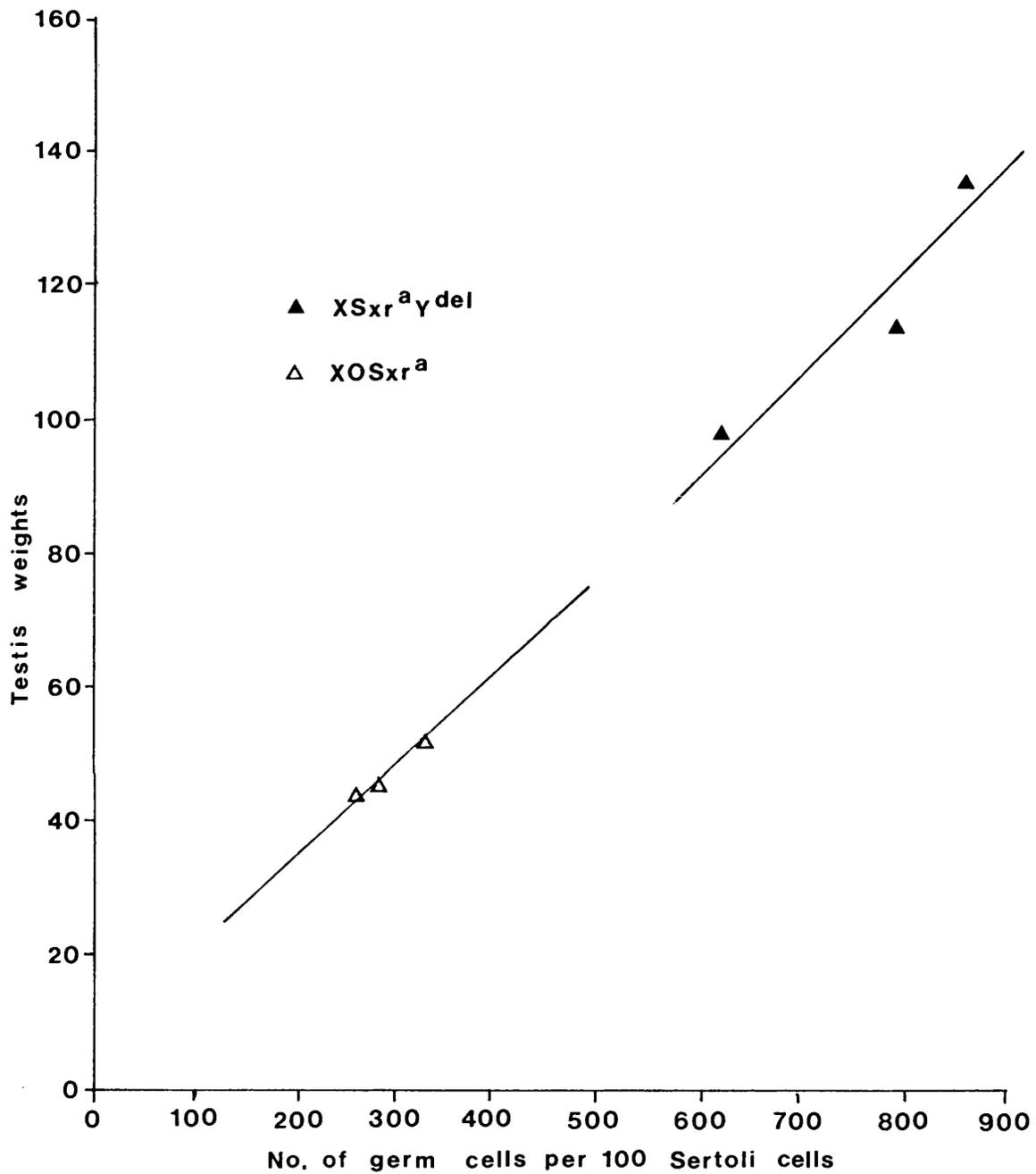
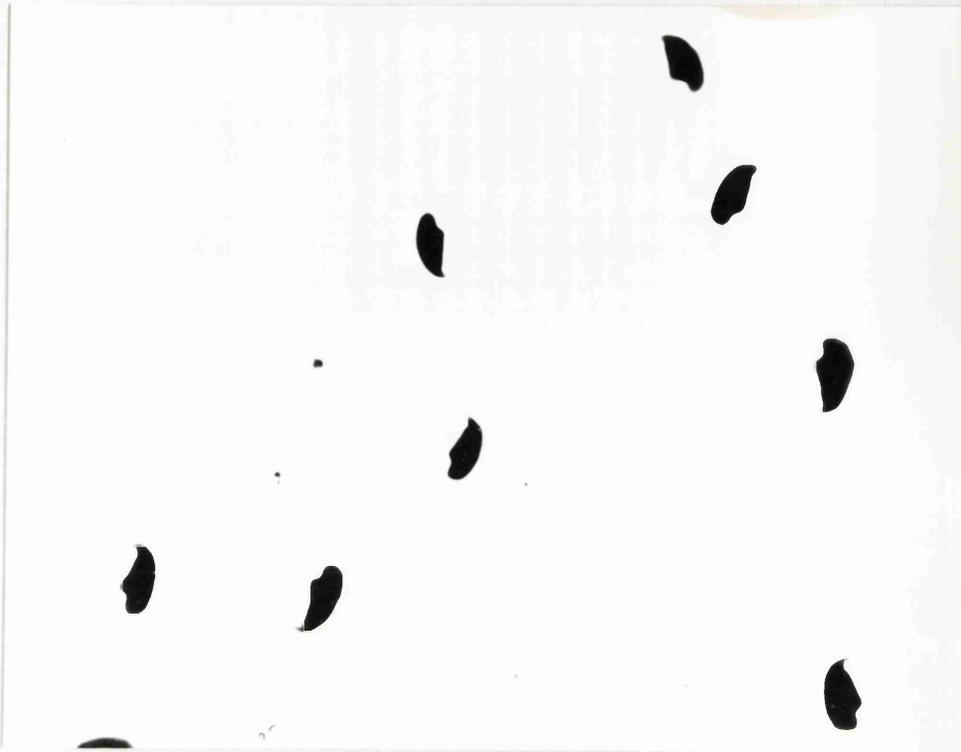


Fig 4c. Regression analysis of testis weights plotted against total germ cell number in XOSxr^a and XSxr^aY^{del} mice. Correlation coefficient : XOSxr^a = 0.9970; XSxr^aY^{del} = 0.9557.

(i)



(ii)



Plate 4f. Examples of sperm head morphology in (i) normal XY, and (ii) abnormal $XSxr^{aY^{del}}$ mice.

DISCUSSION

The breeding programme was set up to add the tiny Y fragment, Y^{del} (derived from Y^*) to $XSxr^a$. The second cross was set up to produce $XOSxr^a$ mice as controls on the same genetic background. Providing the Y^{del} pairing partner to $XSxr^a$ overcame the severe spermatogenic impairment seen at meiosis in $XOSxr^a$ mice and resulted in a dramatic increase in the number of spermatids and spermatozoa. However, in spite of the large number of sperm produced, all sperm had misshapen heads and the mice proved to be infertile.

When $XOSxr^a$ males were first described (Cattanach *et al.*, 1971) it was hypothesised that spermatogenesis might fail in these individuals because of lack of a pairing partner for the X-univalent or because of the loss of a gene required for ongoing development. The results of this study provide strong evidence in support of the former hypothesis.

It could be argued that the interpretation of this data need not relate to the provision of a pairing partner, but merely the provision of a second pseudoautosomal region, irrespective of actual pairing and exchange. Normal XY males with correct pseudoautosomal dosage show a percentage of X-Y dissociation, varying according to strain and genetic background, but the effect of this pairing failure is not apparent because the dissociation levels are usually too low to affect fertility (Chandley and Speed, 1987; Krzanowska, 1989). However, studies of $XYsXr^a$ males have demonstrated that, despite two pseudoautosomal regions, the presence of Sxr^a disrupts pairing with as much as 70 - 90% dissociation (Cattanach, 1975; Evans *et al.*, 1980; Chandley and Speed, 1987). Although these

XY Sxr^a mice have normal dosage for the pseudoautosomal region, they nonetheless often have profound pairing problems and are sometimes infertile. It is therefore unlikely that reinstatement of pseudoautosomal dosage alone, explains the spermatogonial success in X Sxr^aY^{del} mice.

Although the Y^{del} pairing fragment leads to successful spermatogenesis in X Sxr^aY^{del} mice, the absence of additional Y chromosomal material results in abnormal sperm. Since Sxr^a comprises only the short arm, the Y chromosomal long arm must be implicated in the sperm abnormality. Yq-deletions in humans have been associated with abnormal spermatogenesis and azoospermia (Tiepolo and Zuffardi, 1976; Davis, 1981; Fryns *et al.*, 1985). Despite deletions of the Y long arm in mice being postulated to control sperm motility (Eicher *et al.*, 1983), observations of motile sperm in X $O\mathit{Sxr}^a$ individuals negates this suggestion (Short and Aitken - quoted by Lyon *et al.*, 1981; Burgoyne, 1987a). However, the production of congenic mice with different Y chromosomes on the same genetic background, has demonstrated a Y effect, not on motility, but on the percentage of sperm head abnormality (Krzanowska, 1971; Krzanowska, 1976). Mice, originating in our laboratory, carrying a small Y chromosome have approximately two thirds of the long arm deleted. These mice are fertile, but have varying proportions of abnormal sperm (Burgoyne - unpublished). In addition, recent evidence by Moriwaki *et al.* (1988), has shown that deletions of the mid-region of the Y long arm in mice were correlated with more than 70% sperm head abnormality. In the case of X Sxr^aY^{del} mice the complete absence of the Y chromosome long arm leads to the entire sperm population being abnormal. Since the size of the deletion is correlated with the proportion of abnormality, it implies that a

gene, needed for normal sperm head development, is present in multiple copies along the length of the Y long arm.

A Y-specific 1.5 kb genomic sequence, Y353B, has recently been cloned (Bishop and Hatat, 1987). The pY353B insert is specifically transcribed in mouse testes and is present in multiple copies along the entire length of the Y long arm (Bishop and Hatat, 1987). Not surprisingly, the pY353B insert does not hybridise to the tiny Y^{del} fragment (Bishop - personal communication). It is suggested that Y353B is an ideal candidate for this multiple copy, sperm morphology gene (dubbed *Smy*), located on the mouse Y long arm.

One of the chromosomal products of Y^* is X^Y . X^YO males (XX^Y males would be infertile due to the two X chromosomes) are reported to have normal sperm shape and are apparently sometimes fertile (Eicher and Washburn, 1986), but unfortunately, there is no pachytene or metaphase I data available for these mice. How could X^YO males overcome their univalent pairing problem and undergo successful spermiogenesis? Certainly the length of the X^Y chromosome would enhance the chance of self-pairing (de Boer *et al.*, 1986; Mahadevaiah - personal communication) and this may contribute to the fertile sperm found in these males. A detailed analysis of synaptonemal complexes, testis histology and sperm morphology of X^YO mice would obviously provide interesting answers.

In conclusion, and to answer the original dichotomy, it appears that satisfying the pairing requirement alone without additional genetic information is sufficient to allow spermatogenesis to actively proceed.

However, to avoid sterility, additional genetic information, present on the Y chromosome, must act further down the line to produce fertile sperm.

CHAPTER 5

GENERAL DISCUSSION

The Y chromosome's role is not just that of testis determination, with the remainder comprising 'junk' DNA as was previously held to be the case up until the early 60's. Despite slow progress in genetic analysis (Goodfellow *et al.*, 1985), it is now accepted that the Y chromosome is multi-functional. Genes have been described for male-specific characteristics and various aspects of fertility in both man and mouse, furthermore the Y has an important role as a pairing partner for the X chromosome during meiosis. The purpose of this thesis was to investigate aspects of the Y chromosome's role in spermatogenesis during development from birth to puberty, using the sex reversed mouse as a model.

In the first project, a quantitative analysis was undertaken of the germ cell block in XOSxr^b testes. Sxr^b is a fragment of the Y short arm, now known to be the result of a deletion from Sxra (McLaren *et al.*, 1988; Roberts *et al.*, 1988; Bishop *et al.*, 1988; Mardon *et al.*, 1989). At birth, XOSxr^b mice are phenotypically normal males with testes that were shown in this project to have normal numbers of Sertoli cells and germ cells. The testis-determining gene, *Tdy*, must therefore be present in the Sxr^b fragment. By five days after birth, spermatogonial proliferation is impaired at the early differentiating A stages and, coupled with germ cell degeneration, few cells reach the A₃/A₄ differentiating stages. XOSxr^a mice of the same age show no such germ cell block. This evidence demonstrates that the gene required for ongoing spermatogenesis, which is present in Sxr^a but missing in XOSxr^b, is expressed during the first wave of differentiating A₁/A₂ spermatogonia.

The second project extended the quantitative analysis to XOSxr^a (and XYSxr^a) testes, covering the period throughout puberty. This study

demonstrated that in XOSxr^a testes, spermatogenesis proceeded normally through the meiotic prophase stages, culminating in a drastic germ cell block and degeneration at MI. Only 15% of germ cells reached the spermatid stages in these mice (on this genetic background). The Sxr^a fragment has been described as the Y short arm (Bishop *et al.*, 1988; Mardon *et al.*, 1989), so germ cell degeneration at MI could be due to the absence of essential sequences from the Y long arm. However, cell breakdown coincides with the stage in meiosis, MI, following bivalent pairing at pachytene and a hypothesis proposed by Miklos (1974) links pairing disruption with spermatid failure. XOSxr^a mice have 'unsaturated' pairing sites due to the univalent sex chromosome in every cell. It has remained difficult to distinguish between these two possible causative factors. The quantitative analysis in the XYSxr^a littermates showed degeneration commencing earlier at pachytene, as well as at MI, but no drastic germ cell block as in XOSxr^a mice. This finding has led to the suggestion that, since the Sxr^a fragment in XYSxr^a mice has already been shown to disrupt pairing (Winsor *et al.*, 1978; Hannapel and Drews, 1979; Chandley and Fletcher, 1980; Evans *et al.*, 1980; Chandley and Speed, 1987; Mahadevaiah *et al.*, 1988; Tease and Cattanach, 1989), some cells have two univalent sex chromosomes, and these could cause earlier impairment than that seen in XOSxr^a. This explanation is supportive of the Miklos theory, since there would be double the number of 'unsaturated' pairing sites in spermatocytes with X-Y separation in XYSxr^a testes.

Despite these findings, the XOSxr^a and XYSxr^a analysis only conclusively proved disruption to be post-pairing, but could not prove the Miklos model. In a further attempt to distinguish the causative factor of the XOSxr^a germ cell block and a further test of the Miklos hypothesis,

Burgoyne (1987a, 1987b) proposed that the XSxr^a univalent chromosome be provided with a pairing partner, but without additional Y chromosome material. This experiment has recently become feasible through a multi-step breeding programme using XY^{*} male mice (Eicher, 1982; Eicher *et al.*, 1983). One of the gametic products, Y^{del}, comprises little more than a centromere and a pairing and exchange region. This project formed the final part of this study. The provision of this tiny Y^{del} fragment to XSxr^a, overcame the germ cell block seen in XOSxr^a testes, resulting in all stages of spermatids and motile sperm in high numbers. It could be argued that successful spermatogenesis resulted from pseudoautosomal dosage alone, i.e. two pairing and exchange regions, irrespective of whether pairing occurred or not. However, in XYSxr^a mice, two normal pairing and exchange regions are unable to prevent spermatogenic disruption, implying that pairing and exchange is an important feature in spermatogenesis. The results of this study provide persuasive evidence to support the Miklos theory as well as demonstrating that the long arm of the Y chromosome is not required for spermatogenesis.

Although XSxr^aY^{del} mice had high numbers of motile sperm, the mice were found to be infertile. Sperm head morphology was found to be abnormal in all spermatozoa. This evidence supports recent findings that the Y long arm has a gene required for sperm morphology, and, since increased deletions of the long arm are correlated with increased percentage of sperm abnormality, this gene is probably present in multiple copies (Moriwaki *et al.*, 1988; Burgoyne - personal communication). It is tempting to suggest that a Y-specific genomic sequence, Y353B, present in multiple copies along the entire length of the Y long arm (Bishop and Hatat, 1987), may be the sperm morphology gene, *Smy*.

APPENDICES

ACKNOWLEDGEMENTS

APPENDIX 2a

BREEDING CROSS TO PRODUCE XOSxr^b MALE MICE

In(X)/X ♀ x X/YSxr^b ♂

Progeny Sex

In(X)/X ♀
 X/X ♀
 O/X ♀

In(X)/XSxr^b ♂
 X/XSxr^b ♂

In(X)/Y ♂
 X/Y ♂

In(X)/YSxr^b ♂
 X/YSxr^b ♂

(aborted) O/YSxr^b ♂

O/XSxr ^b ♂ (1 in 19 progeny)

APPENDIX 2b

SPERMATOGONIA

Training in the identification of prospermatogonia and spermatogonia was given by Professor Werner and Dr Barbara Hilscher, University of Dusseldorf, Federal Republic of Germany.

In the past, spermatogonial stages in the mouse and rat were identified by reference to the adjacent meiotic stages (Oakberg, 1956; Roosen-Runge, 1962; Huckins, 1971; Oakberg, 1971; Huckins and Oakberg, 1978) since there is a hierarchal order from the basement membrane towards the lumen in normal XY testis tubules. All six differentiating spermatogonial stages, A₁-A₄, In and B were confirmed in the adult mouse by Monesi (1962) who studied DNA synthesis and the cell cycles using tritiated thymidine.

'Early' spermatogenesis, as defined by Hilscher (1988), describes the formation of the stem cell stock and the first wave of differentiating cells, and many of the studies directed towards this period have investigated the undifferentiated stem cell stock and its mode of renewal (Hilscher, 1981b; Oakberg, 1981). The first wave of differentiating spermatogonia and the number of generations in the immature mouse is, however, less well understood (Kluin *et al.*, 1984; Sung *et al.*, 1986), but the inference has been that it is the same as in the adult.

Although a large number of studies have been undertaken in prepubertal mice and rats (Clermont and Perey, 1957; Huckins, 1965; Huckins, 1973; Bellve *et al.*, 1977; Kluin and de Rooij, 1981; Kluin *et al.*, 1982; Kluin *et al.*, 1984), there has been no confirmation of all six differentiating

spermatogonial stages in the first wave. There is a consensus of opinion that differentiating A, In and B spermatogonial stages are present but the A₁-A₄ and In-B stages have not been separately identified from each other during the early post-natal period. Discrepancies exist between authors with respect to the age when meiotic cells first appear (Clermont and Perey, 1957; Sapsford, 1962; Hilscher and Hilscher, 1976; Oakberg, 1981; Kluin and de Rooij, 1981), which could be accounted for by strain differences, but there seems to be general agreement that preleptotene cells are observed within three to four days of the first sighting of undifferentiated A spermatogonia. In the adult mouse and rat, the cell cycle time has been evaluated between 27 1/2 - 30 hours (Monesi, 1962; Hilscher, 1981b), whereas labelling experiments in immature mice have suggested this period is shorter (Oakberg, 1981; Kluin *et al.*, 1982), although this remains in dispute (Sung *et al.*, 1986). Certainly, the interval between the onset of dividing undifferentiated A cells and the appearance of the first preleptotene cells seems too rapid to accommodate a minimum of six successive generations of differentiating spermatogonia, unless they occur much more rapidly than in the adult (Hilscher, 1981b).

Whilst an A₁ cell is clearly distinguishable from an A₄, there is some overlap between consecutive stages, from a morphological point of view, particularly since scoring was undertaken at 3 μ m histology sections. The plane of the nuclear section could be misleading in terms of the number and amount of heterochromatic regions, which is a diagnostic morphological feature. For the purposes of scoring in the first project which was attempting to pin point the particular stage of breakdown in the first spermatogonial wave, therefore, germ cells A₁ and A₂ were pooled, as were A₃ and A₄, and also In and B. An attempt is underway to determine whether all six differentiating

spermatogonial stages are present in the first wave in prepubertal mice, but this is outside the scope of this thesis.

There is also some controversy surrounding the two neonatal prospermatogonial stages and the appearance of the undifferentiated A spermatogonia (the stem cell stock). There is agreement that the large round gonocytes seen at birth (named T₁-prospermatogonia by Hilscher *et al.*, 1974), divide mitotically within the first two post-natal days in the mouse or four days in the rat (the mouse is two days ahead of the rat - Nebel *et al.*, 1961). The dispute arises from the identification of the daughter cells of this T₁ division. Beaumont and Mandl (1963) and Oakberg (1981) state that these are the stem cells whereas Clermont and Perey (1957) and Kluin and de Rooij (1981) agree with Hilscher *et al.*, (1974) and Hilscher and Hilscher (1976) that there is another prospermatogonial stage (named T₂-prospermatogonia by Hilscher *et al.*, 1974). From this study it is evident that an additional stage does exist between T₁ and the undifferentiated A spermatogonia, and this has been described as T₂-prospermatogonia in accordance with Hilscher *et al.* (1974) for the purposes of this thesis.

APPENDIX 3a

Southern Blot Analysis

All males were karyotyped; XOSxr^a mice were identified as having 39 chromosomes and no Y chromosome. To distinguish XY and XYSxr^a littermates, genomic DNAs were analysed by Southern blots. Following a high stringency wash, the SX1 probe hybridises to three bands in XY mice, 7.0, 2.8 and 1.8 kb. However, in Sxr^a carriers, an additional 5.0 kb band is seen. The extraction of genomic DNA, Southern blotting and hybridisation followed the protocols described by Maniatis *et al.* (1982) and Hogan *et al.* (1986), with minor modifications. The steps are described below:-

1. Isolation of genomic DNA from tail tips

Approximately 1.5 cm of tail tip was removed, placed in an Eppendorf, snap frozen in liquid nitrogen and stored at -20°C. The tail tissue was subsequently chopped up in 700 µl of proteinase K buffer and incubated overnight in a water bath at 50°C (modification of Hogan *et al.*, 1986). The lysate was then either stored at 4°C or immediately extracted with phenol. An equal volume of buffered phenol was added to each Eppendorf, rotated on a vertical rotator for 10 minutes, then microfuged for 10 minutes. The aqueous phase plus interface were transferred to a fresh Eppendorf and an equal volume of phenol added and the above rotation and microfuging repeated. Prior to the third phenol addition, only the aqueous phase was removed, the interface being discarded. Phenol extraction is a standard method for the removal of proteins from the nucleic acid solution.

After the third phenol extraction, the aqueous phase was removed to a fresh Eppendorf and an equal volume of phenol:chloroform/isoamyl alcohol added to each tube. The tubes were then rotated and microfuged as above. The addition of chloroform facilitates the removal of phenol.

The aqueous phase was transferred to a polypropylene test tube, and three volumes of ethanol added. The fibrous precipitate was hooked out with a flamed glass pipette, washed twice in 70% ethanol to remove traces of phenol, allowed to dry at room temperature, and resuspended, by overnight rotation on a vertical rotor, in 150 μ l of TE buffer. The purified DNA was then stored at 4°C.

Optical density readings were recorded on a spectrophotometer (Pye Unicam SP8-150 UV/VIS) at a dilution of 1/100 (5 μ l DNA in 495 μ l H₂O; blank 5 μ l TE in 495 μ l H₂O) at 260 and 280 nm. Sample ratios ranged from 1.6 - 1.8 (1.8 - 2.0 optimum). Since 1 OD unit = 50 μ g/ml = 0.05 μ g/ μ l and dilution was 1 in 100, the DNA concentration was calculated in μ g/ μ l, by multiplying the OD reading x 0.05 x 100.

3. Restriction enzyme digestion of genomic DNA

Ten micrograms of tail DNA were digested with the restriction enzyme, *Eco* R1 using the *Eco* R1 restriction buffer supplied by the manufacturer (NBL). A 4-fold excess of enzyme was used per microgram of genomic DNA. An example of a typical enzyme digest is as follows:

10 μ g tail DNA	1 x <i>Eco</i> R1 buffer
40 Units <i>Eco</i> R1 enzyme	dd H ₂ O to a final volume of 40 μ l

The digest was incubated overnight at 37°C. The following morning a further 40 Units of enzyme were added and the incubation allowed to continue for three more hours.

Care was taken to avoid *Eco* R1 star activity. *Eco* R1 has a recognition sequence of GAATTC, but when a high excess of enzyme (25 Units *Eco* R1/ng) or glycerol concentrations of 5% are present, star activity (reduced specificity) is observed. The enzyme then cleaves at the sequence AATT. Following digestion, samples were stored at -20°C.

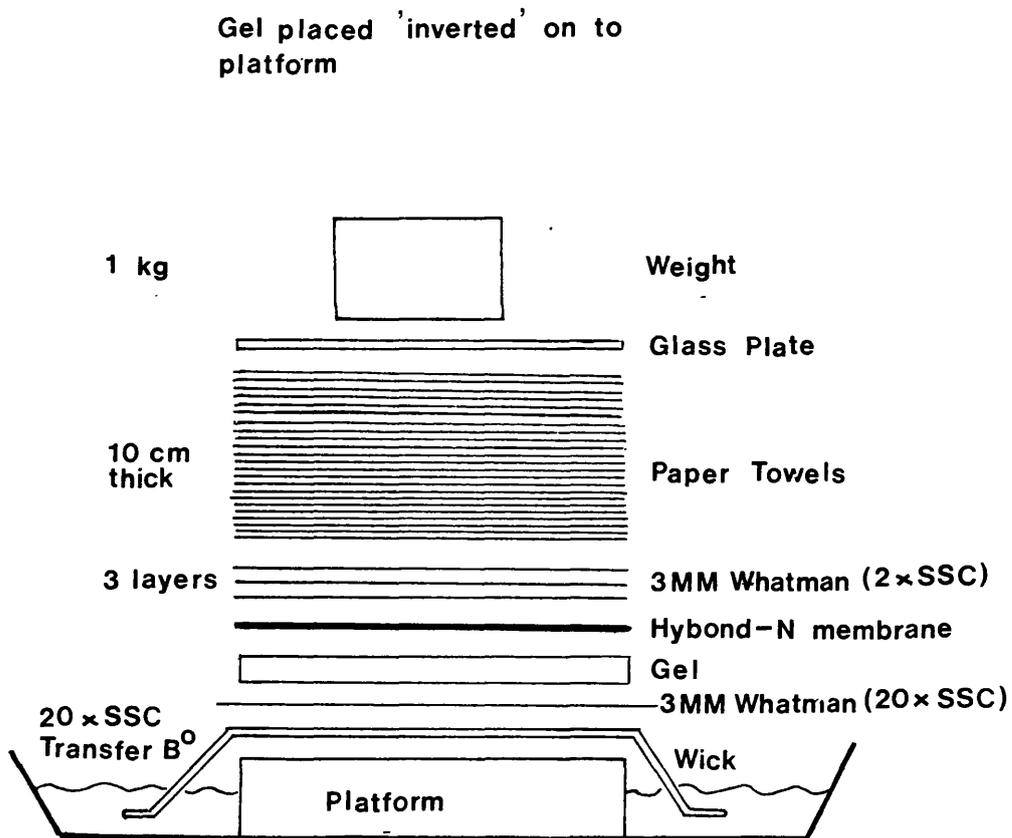
4. Electrophoresis of restricted DNA fragments

A 0.8 % agarose gel was prepared by boiling 2.4 g agarose in 300 ml of a 1 x TBE solution. After cooling the agarose to a temperature of approximately 60°C, ethidium bromide was added to a final concentration of 0.5 ug/ml, and the agarose poured onto a gel plate with a comb at one end. Once the gel had solidified, the comb was removed and the gel was placed in an electrophoresis tank. 1 x TBE buffer was poured into the tank to a level just covering the gel. 6µl of 5 x loading buffer was added to each sample, such that the total volume of DNA/enzyme/buffer was 50 µl, and the DNA loaded onto the gel. The gel was electrophoresed for 16 - 20 hours at 30 - 40 V, the DNA moving from the cathode to the anode, and then visualised and photographed under ultra violet light. An example of the digested DNA run on an ethidium bromide gel is shown in Appendix Plate 3a.

4. Transferring of DNA onto filter membrane by Southern blotting

The capillary blot was set up according to Southern's (1975) protocol, using 20 X SSC as the transfer buffer. The gel was depurinated (2 X 20 minutes),

denatured (2 X 15 minutes) and neutralised (1 X 30 minutes) in a glass dish on a rocking platform. The gel was then placed on the capillary blot. The schematic Appendix Fig. 3a, illustrates the main steps:



Appendix Fig.3a. Schematic illustrating the main features of the capillary blot (after Southern, 1975).

Saran wrap was used to surround the gel to prevent transfer buffer from flowing directly from the reservoir to the paper towels on top of the gel. The Hybond-N membrane (Amersham) was cut to the size of the gel and placed dry on top of the gel.

Care was taken to ensure that no air bubbles remained trapped between the filter papers, gel and membrane. Three layers of Whatman 3MM soaked in 2 X SSC were placed on top of the membrane followed by a 10 cm pile of paper towels, a glass plate and a 1 kg weight on top. The transfer was allowed to proceed overnight.

The following day, the Hybond-N membrane was removed, washed briefly in 2 X SSC, placed in dry Whatman paper and baked in a vacuum oven for 20 minutes at 80°C. The DNA was cross-linked onto the membrane under UV irradiation for five minutes, rebaked for 20 minutes at 80°C and finally bagged up in plastic.

5. Hybridisation of radioactive labelled probe to DNA

To radioactively label the probe, 100 ng pSX1 insert was added to 33 µl dd H₂O in an Eppendorf and boiled for 5 minutes to denature the DNA double helix (Wetmur and Davidson, 1968). An example of a typical labelling reaction is shown below:

3µl DNA	2 µl BSA
33 µl dd H ₂ O	2 µl Klenow
10 µl OLB	5 µl ³² P dCTP

The reaction was allowed to proceed for a minimum of five hours at room temperature. To separate the labelled probe away from unincorporated radionucleotides, a 1 ml syringe, plugged at the neck with siliconised glass wool, was used as a column to which Sephadex G50 was repeatedly added until the buffer stopped dripping through freely. The column was centrifuged for exactly two minutes, ensuring that the packed column height reached a minimum of 0.8 ml. 50 μ l of TES buffer was added to the probe solution, then the contents pipetted onto the top of the column. The column was then centrifuged for exactly two minutes. Probe with incorporated label forms the eluate, whilst unincorporated isotope adheres to the Sephadex column. 1 μ l of eluate was counted on a scintillation counter (LKB Wallac 1211 Minibeta liquid scintillation counter) to a specific activity not less than 1.0×10^8 cpm

Meanwhile, the membrane was pre-wet with 2 X SSC and re-bagged in plastic to which pre-warmed prehybridisation solution was added. Air bubbles were squeezed out of the bag before sealing, and the bag with enclosed membrane was placed in a water bath for two hours at 68°C.

The probe with incorporated isotope was added to the pre-warmed hybridisation solution. The prehybridisation fluid was tipped out of the bag and replaced by the hybridisation mix and the bag re-sealed (once again ensuring that air bubbles were removed). The bag was then placed in a plastic box in a shaking water bath and hybridised overnight at 68°C.

The following morning, the hybridisation fluid was tipped out of the bag, the membrane removed, washed briefly in 2 X SSC with 0.1% SDS, then placed in a plastic box with 2 X SSC plus 0.1 % SDS for 15 minutes at 68°C in a shaking water bath. This procedure was repeated twice more, with a final

high stringency wash at 0.1 X SSC plus 0.1% SDS salt solution for 12 minutes at 68°C.

Autoradiography was performed by exposing the membrane to Fuji RX100 X-ray film at -70°C with an intensifying screen (Laskey and Mills, 1977). The time of exposure was between 24 and 72 hours. A typical autoradiogram is shown in Appendix Plate 3b.

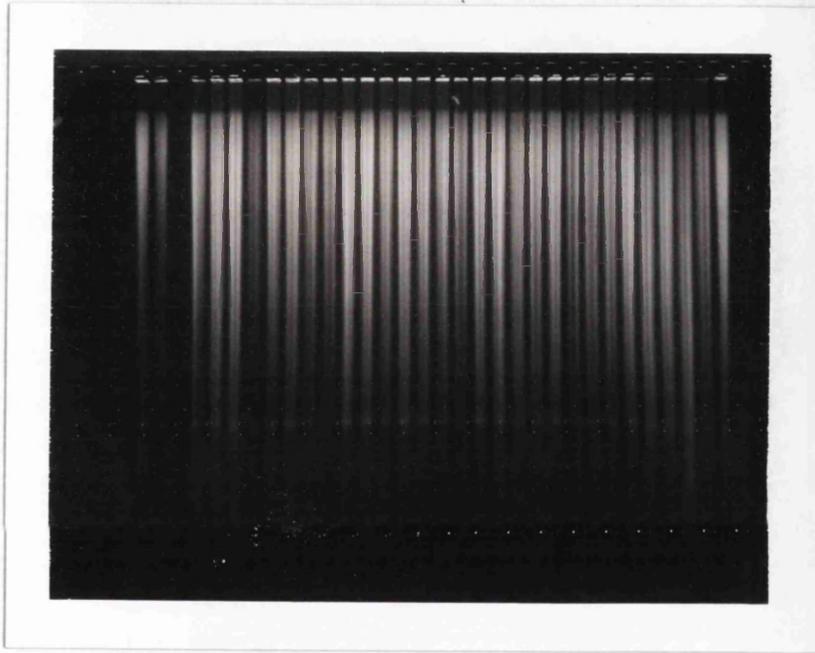
Troubleshooting

Although this protocol originally produced good results, problems were later encountered with poor resolution of the higher molecular weight 7.0 and single copy 5.0 kb bands. Test runs were undertaken varying the depurination times and concentration, and with no depurination (since it is not an essential step for DNA below 10.0 kb) but no differences were detected. Neither were differences found when the time of the digests was shortened to 3 hours or to a single digest. Varying the cross-linkage time did show that optimal timing was 3-4 minutes (rather than 5), but despite changing the timing, overall little improvement was found. Varying batches of *Eco* R1 enzyme, enzyme buffer and Hybond membrane had little overall effect.

Hybond-N positive membrane has recently been introduced by Amersham, which utilises an alkali transfer buffer such that denaturation, baking and UV cross linking are all unnecessary steps. Some improvement was detected when Hybond-N was used, but a number of samples were re-run several times with limited success. Unfortunately, the problem remained largely unresolved and it was concluded that the DNA quality must have been poor.

Control
Control
Blank

S A M P L E S



Appendix Plate 3a. Example of an electrophoresed agarose gel with *Eco*R1 digested tail DNA from XY and XYSxr^a mice.

SOLUTIONS AND BUFFERS

Proteinase K buffer

50mM Tris pH 8.0
100mM EDTA (Disodium ethylene diamine tetraacetate) pH 8.0
100mM NaCl (Sodium chloride)
1 % SDS 10 % (Sodium dodecyl sulfate)
0.5mg/ml Proteinase K

Phenol/Chloroform

Phenol with equal w/v buffered Tris 1M pH 8.0
0.1 % Hydroxyquinolone
2mM EDTA pH 8.0

Chloroform 24:1 v/v isoamyl alcohol

50 % Phenol)
50 % Chloroform) for final extraction

TE buffer

10mM Tris pH 7.6
1mM EDTA pH 8.0

Electrophoresis buffer

10 x TBE

890mM Tris pH 7.6
890mM Boric acid
20mM EDTA pH 8.0

Eco R1 Restriction Enzyme assay buffer (supplied by the manufacturer)

50mM Tris pH 7.5
100mM NaCl
10mM MgCl₂ (Magnesium chloride)
1mM DTT (Dithiothreitol)

Loading buffer (Hans Lehrach's)

5 x loading buffer

5 x TBE
50 % Glycerol
60mM EDTA pH 8.0
0.1 % BPB (Bromophenol blue)

Southern blot solutions using Hybond membrane

- (i) Depurination buffer:
0.25M HCl (Hydrochloric acid)

- (ii) Denaturation solution:
0.5M NaOH (Sodium hydroxide)
1.5M NaCl

- (iii) Neutralisation solution:
3.0M NaCl
0.5M Tris pH 7.0

Transfer solution for Hybond-N membrane

20 X SSC:

- 3.0M NaCl
- 0.3M Na₃Ct (Sodium citrate)

Transfer solution for Hybond-N membrane (positive)

Alkali:

- 0.4M NaOH

Prehybridisation solution

- 1 X SSC
- 5 X Denhardts
- 0.1 % Sodium pyrophosphate
- 100µg/ml Salmon/Herring sperm DNA (denatured by boiling 5 minutes)
- 0.1 % SDS

Hybridisation solution

- 3 X SSC
- 5 X Denhardts
- 0.1 % Sodium pyrophosphate
- 100µg/ml Salmon/Herring sperm DNA
- 0.1 % SDS

Oligonucleotide buffer (OLB)

Solution O:

- 1.25M Tris pH 8.0
- 0.125M MgCl₂

Solution A:

1 ml Solution O
18 μ l 2- mercaptoethanol (at a concentration of 0.1M).
5 μ l dATP)
5 μ l dTTP) in: 3mM Tris pH 7.0) at a concentration of 0.5mM
5 μ l dGTP) 0.2mM EDTA)

Solution B:

2M Hepes pH 6.6
4M NaOH

Solution C:

Hexanucleotide primers (Pharmacia) suspended in TE at 90 OD
Units/ml.

Solutions A:B:C are mixed in a ratio of 100:250:150 to make OLB.

Column buffer

TES-2 buffer

10mM Tris 1M pH 7.6
10mM EDTA 0.5M
0.1 % SDS 10 %

APPENDIX 4a

BREEDING CROSS TO PRODUCE T16^B/X^ASxr^a FEMALE MICE

T16^B/X^B ♀ x X^A/Y^{Sxr^a} ♂

<u>Progeny</u>	<u>Sex</u>	<u>PGK</u>
T16 ^B /X ^A Sxr ^a	♀ ♀ ♂	B
T16 ^B /X ^A	♀	B
X ^B /X ^A	♀	AB
X ^B /X ^{Sxr^a}	♂	AB
T16 ^B /Y	♂	B
T16 ^B /Y ^{Sxr^a}	♂	B
X ^B /Y	♂	B
X ^B /Y ^{Sxr^a}	♂	B

APPENDIX 4b

BREEDING CROSS TO PRODUCE $X^A Y S_{xr}^a$ MALE MICE

X^A/X^A ♀ x $X^B/Y S_{xr}^a$ ♂

Progeny Sex

X^A/X^B ♀

$X^A/X^B S_{xr}^a$ ♂

X^A/Y ♂

$X^A/Y S_{xr}^a$ ♂

APPENDIX 4c

BREEDING CROSS TO PRODUCE $X^{A_{Sxr^a}}/Y_{Sxr^a}$ MALE MICE

$T16^B/X^{A_{Sxr^a}}$ ♀ x X^A/Y_{Sxr^a} ♂

<u>Progeny</u>	<u>Sex</u>	<u>PGK</u>
$T16^B/X^A$	♀	B
$T16^B/X^{A_{Sxr^a}}$	♀	B
$T16^B_{Sxr^a}/X^A$	♂	B
$T16^B_{Sxr^a}/X^{A_{Sxr^a}}$	♂	B
X^A/X^A	♀	A
$X^A/X^{A_{Sxr^a}}$	♂	A
$X^{A_{Sxr^a}}/X^A$	♂	A
$X^{A_{Sxr^a}}/X^{A_{Sxr^a}}$	♂	A
$T16^B/Y$	♂	B
$T16^B/Y_{Sxr^a}$	♂	B
$T16^B_{Sxr^a}/Y$	♂	B
$T16^B_{Sxr^a}/Y_{Sxr^a}$	♂	B
X^A/Y	♂	A
X^A/Y_{Sxr^a}	♂	A
$X^{A_{Sxr^a}}/Y$	♂	A
$X^{A_{Sxr^a}}/Y_{Sxr^a}$	♂	A

Rare Double Cross-overs

<u>Progeny</u>	<u>Sex</u>	<u>PGK</u>
T16 ^A /X ^A	♀	A
T16 ^A /X ^A S _{xra}	♀ ♀ ♂	A
T16 ^A S _{xra} /X ^A	♂	A
T16 ^A S _{xra} /X ^A S _{xra}	♂	A
x ^B /X ^A	♀	AB
x ^B /X ^A S _{xra}	♂	AB
x ^B S _{xra} /X ^A	♂	AB
x ^B S _{xra} /X ^A S _{xra}	♂	AB
T16 ^A /Y	♂	A
T16 ^A /Y S _{xra}	♂	A
T16 ^A S _{xra} /Y	♂	A
T16 ^A S _{xra} /Y S _{xra}	♂	A
x ^B /Y	♂	B
x ^B /Y S _{xra}	♂	B
x ^B S _{xra} /Y	♂	B
x ^B S _{xra} /Y S _{xra}	♂	B

APPENDIX 4d

BREEDING CROSS TO VERIFY HOMOZYGOUS XSxr^aYSxr^a MALE MOUSE

x^B/x^B ♀	x	x^{ASxr^a}/Y_{Sxr^a} ♂
<u>Progeny</u>	<u>Sex</u>	<u>PGK</u>
x^B/x^{ASxr^a}	♂	AB
x^B/Y_{Sxr^a}	♂	B

Note: No females are possible from this cross, unless a null-O gamete is produced, i.e. XO ♀ (rare).

APPENDIX 4e

BREEDING CROSS TO PRODUCE $\text{In}(X)^B/\text{X}^B$ FEMALE AND $\text{In}(X)^B/\text{Y}^*$ MALE
MICE

$\text{In}(X)^B/\text{In}(X)^B$ ♀ x X^B/Y^* ♂

Progeny Sex

$\text{In}(X)^B/\text{X}^B$	♀
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$\text{In}(X)^B/\text{Y}^*$	♂
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$\text{In}(X)^B/\text{Y}^{\text{del}}$ ♀

$\text{In}(X)^B/\text{X}^{\text{Y}}$ ♂

APPENDIX 4f

BREEDING CROSS TO PRODUCE X^OS_{xra} MALE MICE

$\text{In}(X)^B/X^B$ ♀ x $X^{AS_{xra}}/Y^{S_{xra}}$ ♂

<u>Progeny</u>	<u>Sex</u>	<u>PGK</u>
$\text{In}(X)^B/X^{AS_{xra}}$	♂	AB
$X^B/X^{AS_{xra}}$	♂	AB
$\text{In}(X)^B/Y^{S_{xra}}$	♂	B
$X^B/Y^{S_{xra}}$	♂	B
$O/X^{AS_{xra}}$	♂	A

APPENDIX 4g

BREEDING CROSS TO PRODUCE X^{ASxra}/Y^{del} MALE MICE

$T16^B/X^{ASxra}$ ♀ x $In(X)^B/Y^*$ ♂

<u>Progeny</u>	<u>Sex</u>	<u>PGK</u>
$T16^B/In(X)^B$	♀	B
$T16^B/Y^{del}$	♀	B
$X^A/In(X)^B$	♀	AB
X^A/Y^{del}	♀	A
$T16^B/In(X)^B Y$	♂	B
$T16^B/Y^*$	♂	B
$X^A/In(X)^B Y$	♂	AB
X^A/Y^*	♂	A
$T16^B Sxra/In(X)^B Y$	♂	B
$T16^B Sxra/Y^*$	♂	B
$X^{ASxra}/In(X)^B Y$	♂	AB
X^{ASxra}/Y^*	♂	A
$T16^B Sxra/In(X)^B$	♂	A
$T16^B Sxra/Y^{del}$	♂	B
$X^{ASxra}/In(X)^B$	♂	AB
X^{ASxra}/Y^{del}	♂	A

Rare Double Cross-overs

<u>Progeny</u>	<u>Sex</u>	<u>PGK</u>
T16 ^A /In(X) ^B	♀	A
T16 ^A /Y ^{del}	♀	A
X ^B /In(X) ^B	♀	B
X ^B /Y ^{del}	♀	B
T16 ^A /In(X) ^B Y	♂	A
T16 ^A /Y [*]	♂	A
X ^B /In(X) ^B Y	♂	B
X ^B /Y [*]	♂	B
T16 ^A Sxra/In(X) ^B Y	♂	A
T16 ^A Sxra/Y [*]	♂	A
X ^B Sxra/In(X) ^B Y	♂	B
X ^B Sxra/Y [*]	♂	B
T16 ^A Sxra/In(X) ^B	♂	A
T16 ^A Sxra/Y ^{del}	♂	A
X ^B Sxra/In(X) ^B	♂	B
X ^B Sxra/Y ^{del}	♂	B

APPENDIX 4h

BIOCHEMICAL MICROASSAY FOR THE PGK-1 ENZYME (Monk, 1987)

X-chromosome-linked phosphoglycerate kinase (PGK-1) is one of the enzymes present in the glycolysis cycle. The glycolysis cycle acts by breaking down and oxidising carbohydrates (glycogen, glucose and fructose), by a sequential series of anaerobic reactions, and gives rise to pyruvic or lactic acid. The PGK-1 enzyme assay, originally developed for humans, was first described in the late 60's and utilized the backward PGK-1 reaction converting 3-phosphoglycerate into glycerol phosphate. This reaction couples conversion of NADH into non-flourescent NAD, and PGK-1 activity is visualised under ultra violet light by dark bands on a flourescent background. Improved, faster electrophoresis has now been developed and PGK-1 activity in a forward reaction can now be visualised by the production of flourescent NADPH. The biochemical steps are illustrated in Appendix Fig. 4h .

The constituents of the various buffers and stain reagents are listed in Appendix Table 4h.

A Whatman 'Cellogel' cellulose acetate strip, the 'gel', is hydrated in 150 ml of running buffer for 10 minutes, then 250ml, to which 25 mg dithioerythritol is added, for a further 10 minutes (dithioerythritol is a reducing agent used to stabilise the enzyme). This second running buffer is poured into both the cathodal and anodal sides of a Whatman electrophoresis tank. 45 mg of AMP is added to the cathodal side (AMP binds to the adenylate kinase, which would otherwise react with the ADP and ATP, and this accelerates the kinase

off the gel ahead of PGK-1). The gel is placed in the tank over a water cooled bridge, linked to a thermostatically controlled water bath and pump, which maintains the gel at 20°C during operation. The tissue samples, in this case venous blood, are diluted in sample buffer and PBS and loaded onto the cathodal side of the gel using a micropipette. A control sample is loaded at one end of the gel and the samples dotted along a straight line at least 3/4 cm apart. The gel is then electrophoresed for 1 1/2 hours at 200 volts.

Apart from the buffer, the staining solutions comprise reagents which are involved in various steps of the biochemical reaction described below and referred to in Appendix Fig. 4h.

Stain B: The continuous generation of 1,3-diphospho-glycerate, the unstable substrate of PGK-1, is maintained by adding fructose 1,6-diphosphate (Part 1),

Stain C: supplies glucose (Part 7) to initiate the auxillary system (Part 6),

Stain D: supplies Aldolase (Part 2); Glyceraldehyde 3 phosphate dehydrogenase (Part 3); Glycerol phosphate dehydrogenase (Part 4); Hexokinase (Part 8) and Glucose 6 phosphate dehydrogenase (Part 9).

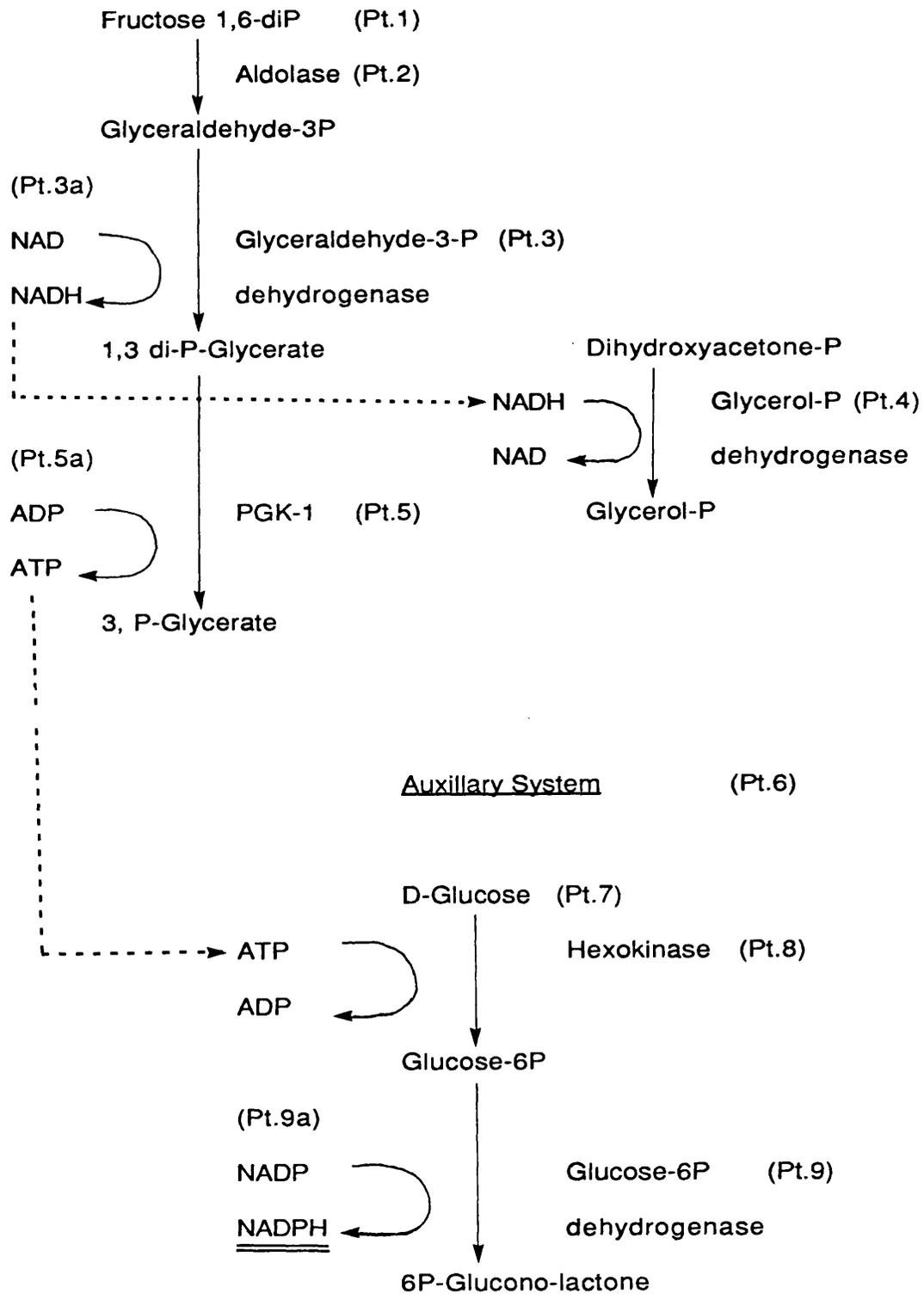
Stain B and C provide NAD (Part 3a) and ADP (Part 5a) for conversion into NADH and ATP, the former utilized in the reaction of dihydroxyacetone phosphate. The ATP generated is coupled to the auxillary sytem (Part 6). Stain C also supplies NADP which is reduced to flourescent NADPH. PGK-1 enzyme activity (Part 5) is measured by the position and intensity of these

flourescent NADPH bands which can be visualised under ultra violet light at 360 nm.

The component parts of the stain are mixed together 5 minutes prior to use and added to a clean support tray. A cellulose acetate strip (Cellogram) is soaked in the stain and the gel, which is cut from the bridge, is laid face downwards on the Cellogram. The tray is covered with a glass plate, placed in the dark, and allowed to develop (approximately 10 minutes). For the purposes of this experiment, it was sufficient to detect the presence or absence of the A, B or AB flourescent bands, but quantitative measurements can be obtained by calibration and the use of a spectrophotometer.

Appendix Fig.4h.

BIOCHEMICAL REACTION



Appendix Table 4h.

PREPARATION OF SOLUTIONS FOR PGK-1 CELLOGEL

ELECTROPHORESIS

Running Buffer

5,5-diethylbarbituric acid	4.12 g/l
Sodium citrate	2.94 g/l
MgSO ₄ .7H ₂ O	1.23 g/l
EDTA	0.74 g/l

1,4-dithioerythritol	25.00 mg
5' AMP	45.00 mg

Sample Buffer

Triethanolamine (50mM), pH 7.6	20.00 ml
1,4-dithioerythritol	6.00 mg
Bovine serum albumin	10.00 mg
Glycerol	20.00 ml

Stain Solution A

Triethanolamine (100mM), pH 7.5	14.92 g/l
MgSO ₄ .7H ₂ O	4.93 g/l

Stain Solution B

Triethanolamine (50mM), pH 7.6	10.00 ml
K ₂ HPO ₄ .3H ₂ O	91.00 mg
NAD	9.00 mg
Fructose diphosphate	220.00 mg

Stain Solution C

Triethanolamine (50mM), pH 7.6	5.00 mg
Glucose	135.00 mg
ADP	60.00 mg
NADP	165.00 mg
MgSO ₄ .7H ₂ O	160.00 mg

Stain Solution D

Aldolase	20.00 ul
Glucose-6-phosphate dehydrogenase	20.00 ul
Glycerol phosphate dehydrogenase	10.00 ul
Glyceraldehyde-3-phosphate dehydrogenase	10.00 ul
Hexokinase	10.00 ul

Stain solutions mixed 5 minutes before use in the following proportions:

800 µl	A
400 µl	B
100 µl	C
20 µl	D

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Analysis of the testes of H-Y negative XOSxr^b mice suggests that the spermatogenesis gene (*Spy*) acts during the differentiation of the A spermatogonia

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Summary

H-Y antigen negative XOSxr^b mice, like their H-Y positive XOSxr^a counterparts, have testes; but, in contrast to XOSxr^a males, XOSxr^b testes almost totally lack meiotic and postmeiotic stages of spermatogenesis. The quantitative analysis of the testes of XOSxr^b males and their XY±Sxr^b sibs, described in the present study, identified two distinct steps in this spermatogenic failure. First, there was a reduction in mitotic activity among T₁ prospermatogonia, so that approximately half the normal number of T₂ prospermatogonia were produced. Second, there was a dramatic decrease in the number of A₃ and A₄ spermatogonia and no Intermediate or B spermatogonia. These reductions were also largely due to decreased mitotic activity, there being a

shortage of A₁ and A₂ spermatogonial divisions and no divisions among A₃ or A₄ spermatogonia. Mitotic activity among the T₂ prospermatogonia and the undifferentiated A spermatogonia was normal. This means that the spermatogonial stem cells, which are a subset of the undifferentiated A spermatogonia, are unaffected in XOSxr^b mice. Sxr^b is now known to have arisen by deletion of DNA from Sxr^a. It is clear from the present findings that a gene (or genes) present in the deleted DNA plays a major role in the survival and proliferation of the differentiating A spermatogonia.

Key words: mice, spermatogenesis, sex reversal, H-Y antigen negative, *Spy*.

Introduction

Sex-reversed (Sxr) is a factor that causes an inherited form of sex-reversal, such that XX and XO mice carrying Sxr develop as phenotypic males (Cattanach *et al.* 1971). In 1982 evidence was obtained that Sxr was in fact an extra copy of the testis-determining region of the mouse Y chromosome which had become located distal to the pairing and exchange region of the Y, so that it regularly crossed over onto the X chromosome during male meiosis (Singh and Jones, 1982; Evans *et al.* 1982; Burgoyne, 1982; Eicher, 1982; Hansmann, 1982).

In addition to testis-determining information, the original Sxr (now termed Sxr^a – McLaren *et al.* 1988) included information required for H-Y antigen expression (Bennett *et al.* 1977). In 1984 McLaren *et al.* discovered a variant of Sxr^a (originally designated Sxr', but now Sxr^b) that retained the testis-determining information, but which had lost the Y-chromosomal gene required for transplantation H-Y antigen expression (Simpson *et al.* 1981, 1986). This finding, recently confirmed by the separation of TDF from H-Y loci in humans (Simpson *et al.* 1987), negated the hypothesis of Wachtel *et al.* (1975) that H-Y antigen was

the primary testis determinant (at least in so far as the transplantation H-Y antigen is concerned).

XXSxr males differ genetically from normal males not only in that they lack most of the Y chromosome, but also in having two X chromosomes. The presence of two X chromosomes is incompatible with male germ cell survival beyond the perinatal period (reviewed by McLaren, 1983) so that in order to investigate the effects of the Y-chromosomal deficiencies associated with Sxr^a and Sxr^b, it is necessary to produce Sxr males with single X chromosomes.

XOSxr^a mice were first described by Cattanach *et al.* (1971) and although all stages of spermatogenesis are represented in their testes, the later stages are severely depleted so that the testes are small and the mice are sterile. The majority of the spermatids are in fact diploid and the few sperm produced, whether haploid or diploid, are abnormal (Levy and Burgoyne, 1986a).

XOSxr^b mice have a more severe spermatogenic impairment with only a few germ cells reaching early meiotic prophase (Burgoyne *et al.* 1986). The XO germ cells in an XO/XY/XYY mosaic male described by Levy and Burgoyne (1986b) suffered a similar fate despite a normal XY Sertoli cell environment. These

findings led Burgoyne *et al.* (1986) to suggest that Sxr^a carries a spermatogenesis gene (*Spy*) that is lacking in Sxr^b , and that *Spy* is expressed cell-autonomously in the germ line. Recent studies have shown that the Sxr^b variant arose by deletion of DNA from Sxr^a (Bishop *et al.* 1988; Mardon *et al.* 1989).

The purpose of the present study was to define the spermatogenic block in $XOSxr^b$ mice by a quantitative analysis of germ cells in the two weeks following birth (when the block first becomes apparent) and from this deduce the function of *Spy*. During the course of the experiment, the finding of a significant body weight difference between $XOSxr^b$ and $XOSxr^a$ mice supported a hypothesis, under separate study, that a growth and development gene (dubbed *Gdy*) may also be deleted.

Materials and methods

Mice

$XYSxr^b$ males were mated with females heterozygous for the inversion $In(X)1H$. $In(X)/X$ females produce some nullo- X eggs following crossing-over within the inversion (Evans and Phillips, 1975), and approximately 1 in 19 of the progeny from this cross have the $XOSxr^b$ genotype. The $In(X)/X$ females were checked for vaginal plugs each morning, and coitus was presumed to have taken place at the midpoint of the previous dark cycle. Ages were calculated from conception, rather than birth, because it is known that the duration of pregnancy is affected by litter size. The majority of litters were born about $19\frac{1}{2}$ days *post coitum* (dpc), so in what follows this is equated with the day of birth. 157 litters were bred of which 59 included $XOSxr^b$ males. Litters were processed from $19\frac{1}{2}$ dpc (day of birth) through $30\frac{1}{2}$ dpc (11 days *post partum*), $32\frac{1}{2}$ dpc (13 dpp) and $59\frac{1}{2}$ dpc (40 dpp).

A similar breeding cross was set up to produce $XOSxr^a$ males as controls for a possible XO effect. Data from 35 litters are included in this study. The litters were processed at $19\frac{1}{2}$, $21\frac{1}{2}$ through $24\frac{1}{2}$, $27\frac{1}{2}$, $29\frac{1}{2}$, $31\frac{1}{2}$ and $33\frac{1}{2}$ dpc.

Body weights were recorded at autopsy. Following exclusion of 'runts' (Burgoyne *et al.* 1983b), litters were evaluated provided at least one $XOSxr$ and one $XY\pm Sxr$ male was present. Since a qualitative analysis suggests that XY and $XYSxr$ testes are not significantly different during the premeiotic stages (results not shown) XY and $XYSxr$ males were not separately identified. 52 Sxr^b and 35 Sxr^a litters finally provided data.

Karyotyping

Mitotic spreads were prepared either by dissociating liver fragments ($19\frac{1}{2}$ and $20\frac{1}{2}$ dpc) or by flushing out bone marrow cells ($21\frac{1}{2}$ dpc onwards) in 0.04% colcemid in HEPES-buffered Eagle's minimal essential medium, and incubating at 32°C for 60 min (liver) or 15 min (bone marrow). Cells were then treated with 0.56% KCl for 20 min followed by five changes of 3:1 methanol:glacial acetic acid. The cells were then air-dried on slides and stained for 15 min in 2% Giemsa in pH 6.8 buffer. $XOSxr$ males were identified by scoring at least 5 consecutive spreads with 39 chromosomes and no evidence of a Y chromosome. $XY\pm Sxr$ males were identified by 40 chromosomes, with a Y recognised by size and the presence of splayed short arms (Ford, 1966).

Histology

Both testes from each male were weighed using a Cahn electrobalance, and were then retained in Bouin's fixative awaiting the results of karyotyping. Testes from $XOSxr$ and $XY\pm Sxr$ littermates were dehydrated and cleared according to standard procedures, embedded in paraffin wax, serially sectioned at $3\ \mu\text{m}$ and stained with haematoxylin and eosin.

Quantitative analysis

This analysis was carried out 'blind' with respect to genotype of the mice from which the sections were taken. The sampling was one tubule cross-section from every 20th section, or every 10th section for smaller testes, such that between 25 and 35 tubule cross-sections were analysed per testis. The procedure for selecting tubules for analysis was as follows: (1) A 0.25 mm square grid (R-4 grid, Graticules Ltd, Tonbridge, Kent) was 'stuck' to the bottom of the microscope slide with a film of water and a Chalkley grid (G52, Graticules Ltd) was inserted in the eyepiece. (2) When a section was selected, the square grid was focused under low power with a $\times 10$ objective and a square chosen at random. The central cross of the Chalkley grid was centered over the square and the section was brought back into focus. (3) The tubule cross-section adjacent to the central cross was analysed under oil immersion, provided it could be encompassed within the field of view.

This selection procedure ensures that all regions of the gonad have an equal chance of being sampled. Once a tubule was selected, all cells within the tubule cross-section were classified as to cell type except dead or dying cells which could not be classified. Sertoli cells were scored as being in interphase or division. Gonial cells were scored as being in interphase or division, and were also classified as to stage (i.e. T_1 prospermatogonia, T_2 prospermatogonia, undifferentiated A spermatogonia, differentiating A_1 or A_2 spermatogonia, A_3 or A_4 spermatogonia, Intermediate or B spermatogonia) using the criteria described by Clermont and Perey (1957), Oakberg (1971), Hilscher *et al.* (1974), Hilscher and Hilscher (1976), Bellve *et al.* (1977), Huckins and Oakberg (1978) and Kluin and de Rooij (1981). It was often difficult to assign divisions to specific spermatogonial stages and in these cases they were classified according to the adjacent interphase stages in the same tubule. A category existed for cells that could not be classified. This group formed less than 0.5% of germ cells scored and have been omitted from the analysis. It should be pointed out that these cell counts are crude counts, uncorrected for cell sizes and thickness of the sections.

Results

The body weight data for the Sxr^a and Sxr^b litters are given in Table 1. The best estimates for the body weights of the four genotypes ($XOSxr^a$, $XY\pm Sxr^a$, $XOSxr^b$, $XY\pm Sxr^b$) at the various ages studied are provided by the means of litter means. In order to compare the two genotypes in each cross, mean weighted differences between these genotypes and the significance of these differences have been calculated from 'within litters' as described by Burgoyne *et al.* (1983b). From these mean weighted differences it is clear that $XOSxr^b$ mice are underweight when compared with $XY\pm Sxr^b$ mice. Despite the limited number of mice at each age, the difference is significant for 5/13 age groups, and pooling across age groups (the mean weighted differences are similar throughout the age

Table 1. Mean body weights for (A) *XOSxrb* and *XY±Sxrb*, and (B) *XOSxra±Sxra* mice and the estimated difference between them for the period 19½–33½ dpc

(A)

Days post coitum	No. of Mice		Mean±s.e.m. body weights (g)*		Mean±s.e.m. weighted <i>XOSxrb</i> – <i>XY±Sxrb</i> difference (g)	Significance of <i>XOSxrb</i> – <i>XY±Sxrb</i> difference (P)
	<i>XOSxrb</i>	<i>XY±Sxrb</i>	<i>XOSxrb</i>	<i>XY±Sxrb</i>		
19½	3	5	1.570±0.03	1.693±0.08	–0.120±0.046	NS
20½	3	9	1.917±0.10	2.200±0.10	–0.284±0.070	<0.005
21½	4	12	2.065±0.15	2.175±0.10	–0.120±0.180	NS
22½	7	16	2.436±0.12	2.862±0.11	–0.496±0.160	<0.005
23½	11	14	3.297±0.24	3.645±0.30	–0.237±0.135	NS
24½	6	19	3.876±0.16	4.240±0.13	–0.347±0.197	0.05–0.025
25½	5	19	4.094±0.19	4.932±0.26	–0.842±0.108	<0.005
26½	3	8	5.130±0.66	5.500±0.79	–0.330±0.266	NS
27½	4	8	5.290±0.25	5.525±0.16	–0.324±0.144	<0.005
28½	3	7	6.385±0.11	6.500±0.10	–0.115±0.194	NS
29½	6	12	6.618±0.66	6.675±0.47	–0.219±0.284	NS
30½	4	6	7.505±0.71	8.025±0.67	–0.498±0.332	NS
32½	4	6	8.060±0.50	8.563±0.53	–0.483±0.407	NS
Pooled mean weighted difference					–0.359±0.059	<0.005

(B)

Days post coitum	No. of Mice		Mean±s.e.m. body weights (g)*		Mean±s.e.m. weighted <i>XOSxra</i> – <i>XY±Sxra</i> difference (g)	Significance of <i>XOSxra</i> – <i>XY±Sxra</i> difference (P)
	<i>XOSxra</i>	<i>XY±Sxra</i>	<i>XOSxra</i>	<i>XY±Sxra</i>		
19½	3	6	1.467±0.04	1.450±0.01	+0.010±0.050	NS
21½	4	6	2.727±0.27	2.927±0.01	–0.241±0.103	0.05–0.025
22½	5	14	3.142±0.43	3.148±0.45	–0.003±0.102	NS
23½	4	12	3.448±0.14	3.465±0.25	+0.004±0.082	NS
24½	4	12	4.123±0.31	4.213±0.17	–0.130±0.219	NS
27½	4	10	5.868±0.40	6.273±0.54	–0.404±0.162	0.025–0.010
29½	6	17	6.658±0.45	6.305±0.32	+0.529±0.230	0.025–0.010
31½	4	15	7.918±0.38	8.285±0.47	–0.367±0.275	NS
33½	4	12	9.145±0.62	9.295±0.33	–0.092±0.157	NS
Pooled mean weighted difference					–0.044±0.069	NS

* Mean of litter means.

range studied) gives an overall estimated weight deficit of -0.359 ± 0.059 g ($P < 0.005$). *XOSxr^a* mice are not significantly underweight when compared with *XY±Sxr^a* mice (pooled mean weighted difference = -0.044 ± 0.069 g).

The testis weight data for the *Sxr^a* and *Sxr^b* litters are given in Table 2. *XOSxr^a* testes (Table 2B) are not underweight when compared with *XY±Sxr^a* litter mates, but *XOSxr^b* testes (Table 2A) are significantly underweight for 9/13 of the ages studied. Since *XOSxr^b* mice are underweight, this testis weight deficit could simply be a reflection of the overall reduction in body weight. The *XOSxr^b* testis weights were therefore corrected by dividing by individual body weight and multiplying by the mean *XY±Sxr^b* body weight for the relevant litters. The mean weighted *XOSxr^b*–*XY±Sxr^b* differences for these corrected testis weights are plotted in Fig. 1. *XOSxr^b* testes are significantly underweight by 23½ dpc and the weight deficit rapidly increases thereafter.

Fig. 1. Mean weighted differences in testis weights (corrected for body weights) for *XOSxr^b* and *XY±Sxr^b* mice for the period 19½–32½ dpc. Where error bars are shown the differences are significant (*t*-test, 1-tailed).

The reason for the reduced testis weight in *XOSxr^b* mice is apparent in Fig. 2, which gives the mean number of germ cells and Sertoli cells per tubule cross-section in *XOSxr^b* and *XY±Sxr^b* mice, throughout the period

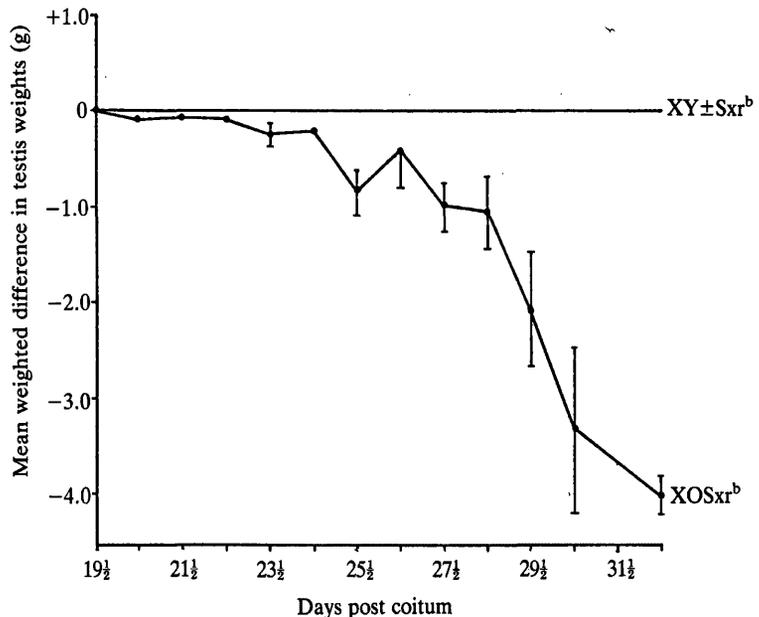


Table 2. Mean testis weights for (A) XOSxrb and XY±Sxrb, and (B) XOSxra±Sxra mice and the estimated difference between them for the period 19½–33½ dpc

(A)

Days post coitum	No. of mice		Mean±S.E.M. testis weights (mg)*		Mean±S.E.M. weighted XOSxrb–XY±Sxrb difference (mg)	Significance of XOSxrb–XY±Sxrb difference (P)
	XOSxrb	XY±Sxrb	XOSxrb	XY±Sxrb		
19½	3	5	0.883±0.01	0.960±0.26	–0.063±0.019	0.05–0.025
20½	3	9	0.980±0.17	1.167±0.18	–0.185±0.167	NS
21½	4	12	1.183±0.10	1.260±0.02	–0.091±0.163	NS
22½	7	16	1.440±0.10	1.782±0.17	–0.380±0.167	0.025–0.010
23½	11	14	2.190±0.14	2.622±0.20	–0.373±0.117	<0.005
24½	6	19	2.528±0.13	2.942±0.05	–0.402±0.268	NS
25½	5	19	2.734±0.18	4.148±0.22	–1.396±0.239	<0.005
26½	3	8	3.580±0.18	4.207±0.25	–0.606±0.405	NS
27½	4	8	3.708±0.27	5.003±0.23	–1.232±0.255	<0.005
28½	3	7	3.385±0.68	4.555±0.48	–1.114±0.383	0.025–0.010
29½	6	12	4.555±0.40	6.248±0.41	–1.974±0.613	<0.005
30½	4	6	4.615±0.16	7.685±1.80	–3.617±0.896	<0.005
32½	4	6	6.027±0.19	10.347±0.64	–4.356±0.293	<0.005

(B)

Days post coitum	No. of Mice		Mean±S.E.M. testis weights (mg)*		Mean±S.E.M. weighted XOSxra–XY±Sxra difference (mg)	Significance of XOSxra–XY±Sxra difference (P)
	XOSxra	XY±Sxra	XOSxra	XY±Sxra		
19½	3	6	0.837±0.02	0.757±0.08	+0.068±0.106	NS
21½	4	6	1.863±0.18	1.940±0.10	–0.097±0.091	NS
22½	5	14	2.108±0.33	2.104±0.30	–0.036±0.105	NS
23½	4	12	2.608±0.24	2.563±0.26	+0.078±0.188	NS
24½	4	12	2.818±0.22	3.030±0.27	–0.226±0.228	NS
27½	4	10	4.938±0.56	4.898±0.40	+0.059±0.156	NS
29½	6	17	6.358±0.69	5.655±0.38	+0.907±0.192	<0.005
31½	4	15	8.898±1.20	9.323±1.15	–0.435±0.620	NS
33½	4	12	12.688±2.87	12.343±1.05	+0.691±0.944	NS

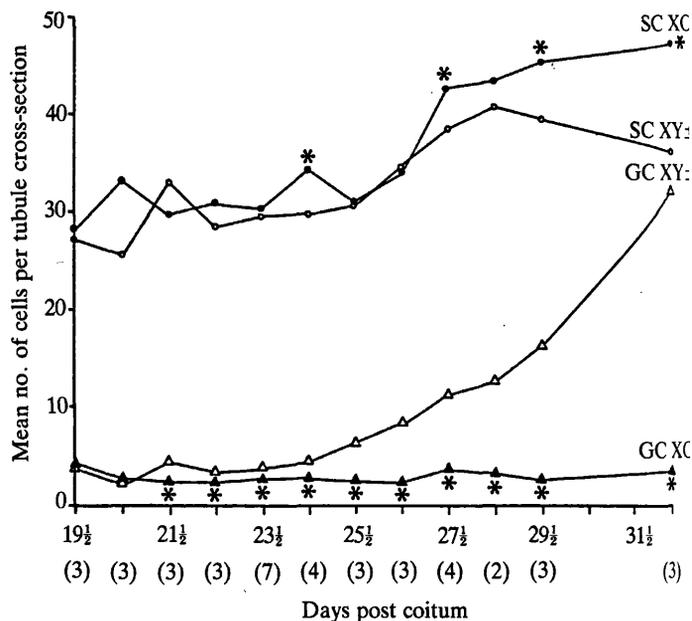
* Mean of litter means.

studied. As expected, there is a marked increase in the number of germ cells per tubule cross-section in XY±Sxrb mice, but by contrast there is no increase in XOSxrb mice. There is no deficiency of Sertoli cells in XOSxrb mice. Indeed the mitotic index for Sertoli cells during the period 19½–23½ days was found to be very similar in XOSxrb mice (0.85%) and XY±Sxrb mice (0.90%). The mitotic index for Sertoli cells drops to less than 0.3% after 24½ dpc in both genotypes. Clearly, the testis weight deficiency in XOSxrb mice is due to germinal failure.

In view of the normal numbers of Sertoli cells in XOSxrb mice, in the more detailed analysis of the germ cell deficiency that follows, germ cell numbers are expressed per 100 Sertoli cells, rather than per tubule cross-section.

Fig. 2. Mean number of Sertoli cells (SC) and germ cells (GC) per tubule cross-section in XOSxrb and XY±Sxrb mice for the period 19½–32½ dpc. The numbers in parentheses are the numbers of litters scored at each age. Asterisks indicate XOSxrb points which are significantly different from controls (*t*-test, 2-tailed). The significantly higher numbers of Sertoli cells in XOSxrb tubules at 29½ and 32½ dpc is a scoring artifact: at these ages some large tubule cross-sections from the controls had to be excluded because they would not fit in the field of view, resulting in an underestimate of the numbers of Sertoli cells and germ cells for controls at these ages.

In Fig. 3, germ cell numbers are plotted against age for the various classes of germ cells identified in the scoring procedure. The numbers of T₁ prospermatogonia are indistinguishable in XOSxrb and XY±Sxrb mice. However, XOSxrb mice clearly have fewer T₂ prospermatogonia than the controls and pooling over the period 20½–24½ dpc reveals that XOSxrb have only 39% of the control value. By contrast, XOSxra mice have 91% of the control value. Since T₂ prospermato-



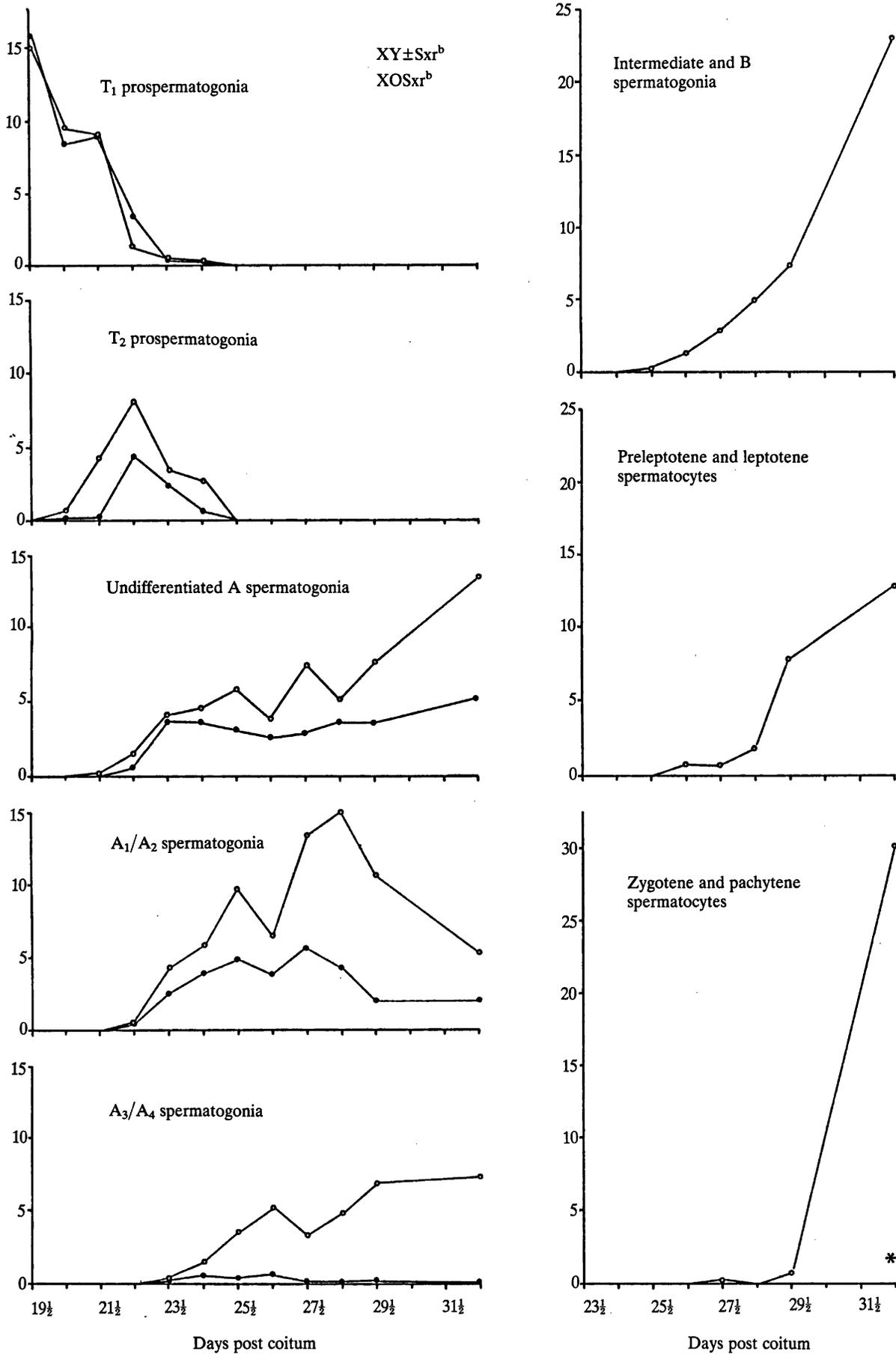


Fig. 3. Number of germ cells per 100 Sertoli cells for each germ cell stage during the period 19½–31½ dpc. The asterisk denotes occasional *XOSxr^b* zygotene or pachytene cells.

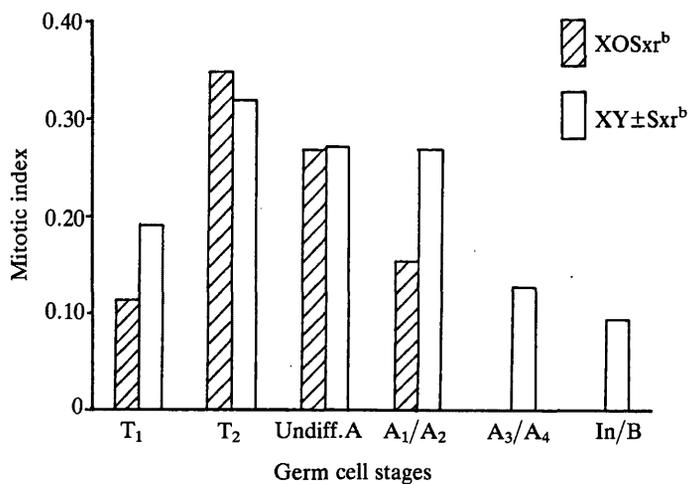


Fig. 4. Histogram showing the mitotic index according to germ cell stage of XOSxr^b and XY±Sxr^b mice.

gonia are assumed to be the progenitors of the undifferentiated A spermatogonia, a deficit of undifferentiated A spermatogonia is expected in XOSxr^b mice, and is indeed observed (XOSxr^b is 54% of XY±Sxr^b). Similarly, there is the expected deficit of differentiating A₁/A₂ spermatogonia (XOSxr^b is 42% of XY±Sxr^b). The number of A₃/A₄ spermatogonia, however, is reduced much more than expected (XOSxr^b is 7% of XY±Sxr^b) and there are no Intermediate or B spermatogonia.

This pattern of germ cell deficiency in XOSxr^b mice is largely accounted for by observations on mitotic index (Fig. 4). That is to say, there is a shortage of dividing T₁ prospermatogonia, accounting for the drop in the number of T₂ prospermatogonia; a reduced frequency of divisions among A₁/A₂ spermatogonia accounting for the much more severe shortage of A₃/A₄ spermatogonia; and no dividing A₃/A₄ spermatogonia accounting for the absence of In/B spermatogonia.

During the scoring procedure the gonidia with the morphological characteristics of A₁ and A₂ spermatogonia were pooled, although it is assumed that they are distinct generations of spermatogonia, as in the adult. When the mitotic index of the A₁/A₂ spermatogonia is plotted against age (Fig. 5), there is no marked shortage of divisions in XOSxr^b mice until 25½ dpc, raising the possibility that it is the A₂ rather than the A₁ spermatogonia that are affected.

If A₁/A₂ spermatogonia rarely divide to give A₃ or A₄, but the undifferentiated A spermatogonia continue to divide, one might expect a 'piling up' of A₁/A₂ stages. This is not observed, implying that the cells that fail to divide are degenerating. This is supported by observations on the germ cell degeneration index (Fig. 6), which has been calculated on the assumption that all the dying cells observed were germ cells. The degeneration index is very low in XOSxr^b and XY±Sxr^b mice. Nevertheless, from 26½ days onwards XOSxr^b mice clearly have more degenerating cells than controls, which is consistent with the increased degeneration of A₁/A₂ spermatogonia. It is tempting to suggest that the increased degeneration index in

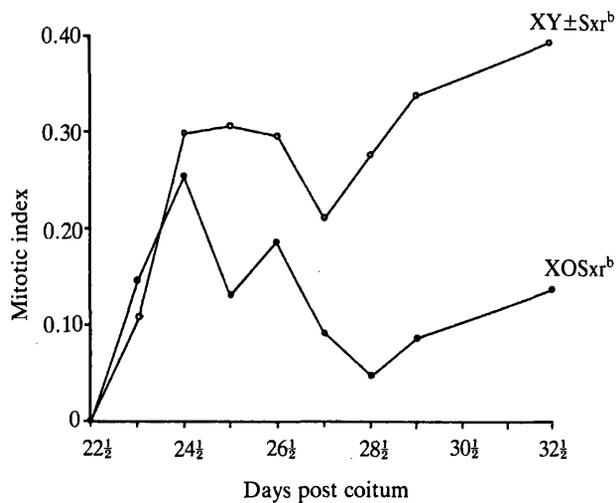


Fig. 5. Mitotic index of A₁/A₂ spermatogonia in XOSxr^b and XY±Sxr^b mice during the period 19½–32½ dpc.

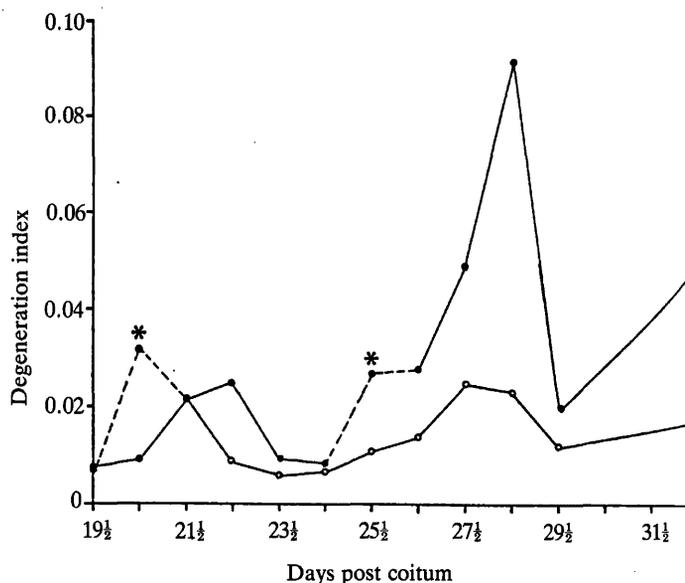


Fig. 6. The germ cell degeneration index for the period 19½–32½ dpc was calculated on the assumption that all dying cells were germ cells. The two points marked with an asterisk are artificially high, in that only one of the males at each of these points showed an elevated degeneration index.

XOSxr^b mice at 22½ days is similarly due to the death of T₁ prospermatogonia that failed to divide.

Although no Intermediate or B spermatogonia were scored during the quantification, very rare patches of these spermatogonia, and also of early meiotic stages, can be found in 32½ dpc and adult (59½ dpc) XOSxr^b testes. They occur without the normal hierarchy of stages, and in small patches, as if an occasional A₃/A₄ spermatogonium divides and the products proceed *via* the usual stages up to early pachytene.

Discussion

The present results show that XOSxr^b testes have normal numbers of germ cells at birth, but become

severely deficient in germ cells in the ensuing two weeks. During the same period the numbers of Sertoli cells remain normal. These findings are consistent with the view of Burgoyne *et al.* (1986) and Levy and Burgoyne (1986b) that the spermatogenic failure in XOSxr^b mice is due to the loss of a gene (*Spy*) that acts cell autonomously in the germ line.

The quantitative analysis of the germ cell deficiency in XOSxr^b mice revealed a reduction in mitotic activity among T₁ prospermatogonia, which resulted in a shortage of T₂ prospermatogonia, and consequently a reduced pool of undifferentiated A spermatogonia. However, mitotic activity among the undifferentiated A spermatogonia, which include the spermatogonial stem cells, was found to be normal. It was during the early differentiating spermatogonial stages that the spermatogenic block occurred, with mitotic failure leading to an almost complete absence of Intermediate and B spermatogonia and subsequent meiotic stages.

XO female mice are developmentally retarded in early pregnancy (Burgoyne *et al.* 1983b) and are significantly underweight postnatally (Burgoyne *et al.* 1983a). It was anticipated that XOSxr^b mice would also be underweight from birth, and this proved to be the case. Unexpectedly, however, the XOSxr^a mice originally included as controls for this 'XO effect' showed little, if any, postnatal weight deficit. Coincidentally, the genetic basis for the early developmental advantage of XY over XX embryos (Tsunoda *et al.* 1985; Seller and Perkins-Cole, 1987) was being investigated in this laboratory, concurrently with the present study of XOSxr^b mice, and the findings may provide an explanation for this difference in postnatal weight between XOSxr^b and XOSxr^a mice. Briefly, it was shown that the Y chromosome carries a factor that accelerates the early growth and development of XY embryos, and it appears that this factor (*Gdy*) may be present in Sxr^a (P. S. Burgoyne, S. Kalmus, E. P. Evans, K. Holland and M. J. Sutcliffe, unpublished) but deleted from Sxr^b (P. S. Burgoyne and C. E. Bishop, unpublished). Thus it may be that the 'XO effect' is ameliorated by the presence of *Gdy* in XOSxr^a but not XOSxr^b mice.

The deletion of Y-chromosomal material involved in the generation of Sxr^b has thus removed genetic information required for H-Y antigen expression (McLaren *et al.* 1984), for spermatogenesis (Burgoyne *et al.* 1986) and for an early acceleration of growth and development (P. S. Burgoyne *et al.* unpublished). Burgoyne *et al.* (1986) pointed out that the spermatogenesis gene (*Spy*) and the gene controlling H-Y expression (*Hya*) might be one and the same, and this possibility still holds. Similarly, *Gdy* may not be a separate gene from *Hya* and/or *Spy*. At the molecular level, it has been shown that *Zfy-2*, one of the Y-chromosomal copies of a gene encoding a zinc finger protein, present along with *Zfy-1* in Sxr^a, has been deleted from Sxr^b (Roberts *et al.* 1988; Mardon *et al.* 1989; Nagamine *et al.* 1989a). Because it is strongly expressed in testes, probably in germ cells (Mardon and Page, 1989; Nagamine *et al.* 1989b), it is an obvious candidate for *Spy*.

As to the function of the 'spermatogenesis gene' *Spy*,

we have clearly shown that the spermatogenic failure seen in XOSxr^b mice is due to a failure of proliferation during the differentiating A spermatogonial stages, and so by definition *Spy* is important for the survival/proliferation of these spermatogonial stages. Whether the deficiency of T₁ or prospermatogonial divisions in XOSxr^b mice is also a consequence of the deletion of *Spy*, or whether it is due to the deletion of a gene separate from *Spy*, remains to be determined.

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