THE PHYSIOLOGY AND AETIOLOGY OF INTERSEXUALITY IN PIGS

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DECLARATION

I declare that this thesis is my own composition and that the work described herein was carried out by me, except where acknowledgment is made to the contribution of others (see below).

Surgery was performed by Dr RHF Hunter, with the assistance of Dr AA Macdonald and Mr R Nichol (University of Edinburgh). Dr G Foxcroft (University of Nottingham) helped with jugular catheterisation and developed and supervised the assay for luteinizing hormone (Chapter 10). Steroid assays were carried out at Glasgow Royal Infirmary under the direction of Dr B Cook (Chapters 10 and 11). Dr U Wiberg (University of Freiburg) was responsible for the H-Y antigen assay (Chapter 12).

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ABSTRACT

The term "intersex" covers a range of conditions in which sexual development is ambivalent. Individuals may possess ovarian and testicular tissue, possibly in the form of ovotestes, or the gonadal sex may be contrary to what is expected from the appearance of the external genitalia. Intersex pigs are usually regarded, clinically, as female, but further investigation reveals testicular tissue, with masculine characteristics in the genitalia and behaviour.

Fifteen intersex pigs were included in this study. Karyotypes were assessed in 8 animals and all were found to have a normal 38,XX chromosome constitution. Histological examination of gonadal tissue revealed fertile ovarian and sterile testicular tissue, whether from abdominal or scrotal, newborn or mature testes.

Behavioural observations indicated varying degrees of male sexual behaviour, including mounting of oestrous gilts and frothing at the mouth in the presence of a boar. The "brain sex" of selected animals was assessed by measuring blood levels of luteinizing hormone secretion following an injection of oestradiol benzoate. Normal, sexually mature female pigs would respond to such a challenge with a "surge-like release" of luteinizing hormone (as seen prior to ovulation) 37 to 55 hours after the injection, a response not seen in male pigs. Of the five intersex pigs used in this experiment, none showed a female response in the pattern of secretion of luteinizing hormone.

The male specific antigen, H-Y antigen, has previously been implicated in testicular development. The transplantation antigen status of two intersex pigs was therefore assessed; both animals were found to be H-Y antigen negative, indicating that testicular development can occur in the absence of the antigen.

Aberrant adrenal development may also cause masculinization of the genitalia in female mammals. To investigate the possible role of the adrenal cortex in the differentiation of testicular tissue, the response in gonadal steroid secretion following an ACTH challenge was measured in several intersex pigs. Although the results indicate the presence of gonadal ACTH receptors, this was not thought to be a cause of transformation from ovarian to testicular tissue.

The origin of the intersex condition in the pig is unknown. A systemic factor, hormonal or genetic, would not account for the asymmetry in gonadal development. The germ cells may play a more important role in gonadal differentiation than previously thought, and a proposal is made for their involvement in the aetiology of intersexuality.

CONTENTS

PART B Experimental

Abbreviations used:-

ACTH	adrenocorticotrophic hormone
CAH	congenital adrenal hyperplasia
CRF	corticotrophin releasing factor
DHA	dehydroepiandrosterone
DHT	dihydrotestosterone
FSH	follicle stimulating hormone
HCG	human chorionic gonadotrophin
LH	luteinizing hormone

INTRODUCTION

According to Greek mythology, Hermaphroditus, the son of Hermes and Aphrodite, merged with the nymph Salmacis to form one body, with male and female characteristics. The term "hermaphrodite" is thus used to describe an organism possessing the reproductive organs of both sexes.

Some plant and animal species (invertebrates) are true hermaphrodites in that they are able to self-fertilise; those which are not able to do so are often called intersexes. Amongst mammals, however, sexual dimorphism is the norm and classification as male or female on the basis of phenotype is, in most cases, simple. Indeed, recognition of gender is an important factor in determining how we treat our domestic animals, and, in our own species, the manner in which we behave towards each other. The occurrence of mammals presenting characteristics of both sexes is therefore intriguing, and challenges the assumptions made about sexual determination and differentiation. These assumptions (based on evidence from many sources) are 1) that the undifferentiated gonad will develop as a testis in the presence of a Y chromosome, and as an ovary in its absence (sexual determination) and 2), if a testis develops, gonadal secretions result in male differentiation of phenotype and behaviour (sexual differentiation). If no testis develops, a phenotypic female results.

Amongst the domestic farm species, pigs show one of the highest incidences of intersexuality. Unlike freemartinism in cattle, which is caused by placental fusion between a male and a female fetus, the condition in pigs does not, as yet, have an explanation and therefore cannot be predicted. Affected animals are apparently genetic females in which testicular tissue develops, with associated consequences on genital morphology and on sexual behaviour.

This thesis is divided into two parts; part A reviews the literature on determination and differentiation in mammals, and on intersexuality in mammals including man, mouse and the pig. Separate chapters on adrenal/gonadal interactions and on H-Y antigen are included as background to the experimental work described in Chapters 11 and 12.

Part B describes the experimental work conducted to investigate the physiology and aetiology of intersexuality in pigs. On the basis of results obtained, no firm conclusion can be drawn on the aeiology of the condition, but in the discussion suggestions are made as to the possible causes of the development of testicular tissue in what appear to be female mammals.

PART A SEXUAL DETERMINATION, DIFFERENTIATION AND INTERSEXUALITY

CHAPTER 1 SEX DETERMINATION AND DIFFERENTIATION IN MAMMALS

1.1 INTRODUCTION

Sexual or asexual reproduction

Sexual reproduction is not the only option available to an individual to produce offspring; asexual reproduction might be seen as a faster, more efficient and, for the individual (since it would result in all the individual's genes being passed on), more beneficial method. Coelenterates (*eg. Hydra*) reproduce by budding, asexual fission occurs in bacteria and protozoa, and vegetative propagation is common amongst higher plants. Parthenogenesis occasionally occurs in chickens and turkeys (Ohno, 1976) but has not been reported in mammals. The reasons for adopting sexual as opposed to asexual reproduction are complex (Maynard Smith, 1978), but are generally to do with maintaining genetic diversity through "reshuffling" of the chromosomes and crossing over at meiosis to provide for recombination of genes. Since natural selection acts on variability of phenotype and behaviour, and animals, because they are mobile, are likely to be exposed to different environments, the ability to produce genetic diversity helps to ensure the survival of a population, even if at the expense of the individual.

If we assume that sexual reproduction is beneficial to a population, there has to be some form of separation of strains, or sexes, so that like does not attempt to reproduce with like, and the simplest mechanism of achieving a division is to have two sexes, male and female. Differentiation of these two sexes is determined by one of a variety of mechanisms.

Genetic versus environmental sexual determination

It is generally accepted that mammals share a common mechanism of sexual determination, the XX/XY dichotomy in which the male is heterogametic, with the Y chromosome determining "maleness" and initiating testicular development. Differentiation of the reproductive tract follows from gonadal sex, and whilst environmental influences may alter the gender of the tract, gonadal sex in thought to be immutable. This is not so in birds, which share a sex chromosome determination mechanism, but hormonal imbalance is able to change the sex of a gonad even in an adult bird. Unlike the situation in mammals, female birds are the heterogametic sex, the W chromosome causing "femaleness"; male birds have the sex chromosomes ZZ, females ZW.

Genetic sex determination is not the only method employed to achieve differentiation of the sexes; environmental factors are, in some species, all-important. Some reptiles hatch as males or females depending upon the egg incubation temperature. In map turtles, for example, males are obtained at incubation temperatures of 23°-28°C, both sexes in the range 28°-30°C and only females are obtained above 30°C. In lizards and alligators, warm temperatures produce males, cool temperatures females and in some species *eg.* snapping turtles, females develop at low and high temperatures with males in the middle around 30°C (Bull, 1980).

Other sex determining mechanisms may be genetic, but involving many genes in addition to environmental effects, such as in the swordtail fish, *Xiphophorus helleri* (Kosswig, 1964). Species of *Hymenoptera* (ants, bees and wasps) utilise a sex determining mechanism known as arrhenotoky, in which males arise from unfertilised eggs and females from fertilised eggs (Bull, 1983).

The evolution of a genetic sex determining mechanism

Most reptiles *eg.* turtles, tortoises, lizards, crocodiles and alligators, have no heteromorphic pair of sex chromosomes. However, amongst the snakes and in whiptail lizards and mud turtles, heteromorphism of the sex chromosomes is apparent. The "primitive" *Boidae* (pythons and boas) show no heteromorphism; the *Colubridae* (non-venomous garden snakes) show some difference in size between the two sex chromosomes, and the *Viperidae* (rattlesnakes and vipers) and *Elapidae* (cobras and mambas) show distinct chromosome heteromorphism in the female (Ohno, 1967). There appears to be a process of sex chromosome evolution in which the X and Y originate from autosomes, but undergo a gradual accumulation of differences until they become heteromorphic. How this might have happened is discussed by Bull (1983). He suggests that crossing over between the X and Y chromosome had to be suppressed to avoid distribution of genetic material to both chromosomes, followed by a loss of gene function from the Y chromosome, possibly as a result of the lack of recombination and hence an increase in lethal genes on the Y chromosome. The result is heteromorphism between the X and Y chromosome, the X being larger than the Y and carrying many functional genes absent from the Y chromosome. Most of the long arm of the human Y chromosome consists of repetitive DNA sequences of doubtful functional significance which are common to the autosomes (Kunkel and Smith 1982). These authors suggest that the human Y chromosome has evolved in part by the incorporation or loss of specific fragments, rather than by the gradual modification of DNA already present on the Y chromosome.

Lyon (1974) has proposed a mechanism whereby the X chromosome could accumulate genetic material at the expense of the Y. A differential segment of genes for determining testis formation came to be present on the Y but not the X chromosome. Crossing over between this new segment and the X chromosome was prevented due to lack of homology, whereas between the homologous portions of the chromosomes, crossing over continued. At some point, a transfer of Y material to the X chromosome resulted in the heteromorphism between the two chromosomes, see Figure 1.1.

Since today's mammals and birds are thought to have evolved from a common reptilian-like ancestor, Short (1982) argues that sex was originally determined by environmental temperature, as occurs in many reptiles today. In animals that incubated their eggs, such a mechanism became pointless, and genetic sex determination became important, with the consequent development of heteromorphic sex chromosomes.

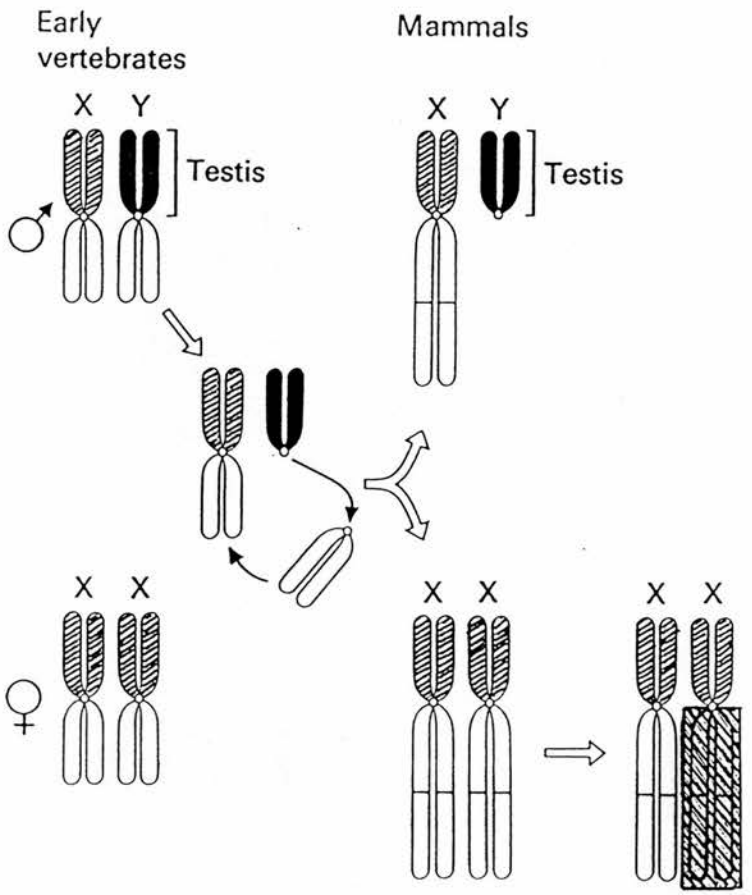


Figure 1.1. Lyon's proposal for the origin of mammalian XY heteromorphism. At the pre-mammalian ancestral stage, the X and Y Chromosomes were morphologically identical (top left). A differential segment on the Y which contains testis-determining genes (solid black) avoids genetic crossing-over with its counterpart on the X (hatched). Free cross-overs were permitted between the remaining homologous segment from the Y and promoted its subsequent transfer to the X. Thus, the Y became small, and the X has effectively doubled. This doubling of X in mammals (bottom right) necessitated the subsequent invention of a peculiar dosage compensation mechanism which inactivates one of the X chromosomes in all somatic cells.
(After Lyon, 1974).

The sex ratio

The evolution of a heteromorphic sex determining mechanism results in a 1:1 sex ratio since the genes segregate in a 1:1 ratio at meiosis. In fact this does not necessarily result in a 1:1 sex ratio in the population since the sex determining genes may alter meiosis, or affect the survival of the gamete or of the offspring. Bar-Anan and Robertson (1975) found that in domestic cattle there was a significant difference between bulls in the ratio of male:female offspring sired, and a correlation between sire and son in the sex ratio of their progeny, indicating a genetic difference between the bulls, which influenced sex determination.

A strong argument for the evolution of a 1:1 sex ratio is that of the evolutionary stable strategy (Fisher, 1958). If the majority of offspring being born are female, then an individual who has only male offspring will have more grand-sons and grand-daughters than one who has only female or a mixture of female and male offspring. Therefore, in populations of excess females, genes tending to restore the ratio to unity will spread.

In our own species, a 1:1 ratio of males to females is accepted as normal and animal populations where this ratio varies are seen as slightly bizarre. However, there are many advantages in reducing the proportion of non-productive males in a population. In effect this is what happens in animal production systems - resources are shared amongst the breeding females and castrated males, one male siring many offspring from more than one female. As Short (1982) points out, in the case of animals with environmentally determined sex, if the population is declining, altering the incubation temperature of the eggs (by choosing a specific nesting site) could result in an increase in the number of females born and hence a rapid population increase the following year. Unfortunately, aspects of maternal choice of nest site among reptiles remain unknown.

Hermaphroditism

Species of animal with separate males and females are gonochoristic, for plants the term is dioecious. Hermaphrodite animals produce both sperm and eggs, either at the same time (synchronous hermaphrodites) or at different stages of their life cycle (asynchronous). Hermaphrodite plants produce ovules and pollen on the same flower, if male and female flowers occur on the same individual the plant is monoecious. Hermaphroditism is known amongst several species of fish as well as lower animals such as Platyhelminthes, Annelids and Molluscs. If hermaphroditism were of evolutionary advantage, we might expect it to be widespread, but in fact hermaphrodite animals and plants often have fairly complex methods of avoiding self-fertilisation, implying that to cross-fertilise is advantageous. Self-fertilisation amongst some helminth parasites is recorded, and presumably in these cases the advantage of colonising a host outweighs the disadvantage of loss of increased fitness obtained through outcrossing.

Maynard Smith (1978) describes situations in which hermaphroditism in animals may be selected for, *eg.* in situations of low population density or amongst sessile individuals, where finding a mate may be difficult. In a hermaphrodite population, each individual a hermaphrodite meets is a potential mate, whereas only half the population is so for a gonochoristic species.

Amongst mammals, however, gonochorism is the reproductive strategy, and before describing the various anomalous sexual conditions found, we should first look at the "normal" process of events leading to development of a male or a female individual.

1.2 MECHANISMS OF SEXUAL DETERMINATION

Gonadal development

The mammalian embryo possesses the potential to develop as a male or a female, since the gonad is undifferentiated and both male (Wolffian) and female (Müllerian) ducts are present. Genetic sex is first reflected in gonadal morphology, and once the sex of the gonads is decided, that of the adjoining tract is determined by gonadal secretions. In mammals the male is termed the "dominant" sex, since the presence of a Y chromosome determines testicular development. In the absence of the Y, female differentiation occurs "by default". Testicular secretions are required for Wolffian duct development and Müllerian regression and, in the absence of these secretions, the Wolffian duct regresses and Müllerian duct remains.

From the 6th week of fetal life in the human (Hamilton and Mossman, 1972), and from day 10 in mice, (Taketo *et al.*, 1984) a swelling on the ventral side of the mesonephros can be identified. This is the anlage which will form the gonad. It consists of several cell types, mesenchymal cells, ingrowing mesonephric cells, coelomic epithelial cells and germ cells which have migrated from an extra-gonadal site. How exactly this mixture of cells goes on to form an ovary or testis has been the subject of much speculation; the review by Jost *et al.* (1973) covers the sequence of embryological events. The testis differentiates first and is recognised by seminiferous cords which develop from the centre of the gonad to the periphery, enclosing the germ cells. These cords connect with ingrowing mesonephric tissue and remain in the adult animal, constituting the rete testis. In the fetal pig, testicular cords are apparent in XY gonads by day 26 whereas the XX gonad remains undifferentiated (Pelliniemi, 1975). In humans, the genital ridge does not appear until the embryo is about 5 weeks old, and gonadal differentiation is not apparent for a further week. The male mouse gonad can be identified 12 days after fertilisation (Taketo *et al.*, 1984)

Ovarian differentiation is characterised by the clustering of germ cells in the cortex of the gonadal swelling and by the absence of development of a connective tissue sheath, the tunica albuginea, around the gonad. In the females of some species *eg.* sheep and pigs, the germ cells become enclosed in cords called medullary or sex cords (Jost *et al.*, 1973; Byskov, 1982). In these species, meiosis does not occur immediately, as it does in species such as man and the mouse where the germ cells are not confined to sex cords.

The ingrowing mesonephric tissue which forms the rete testis in XY gonads is also the precursor for Sertoli cells, at least in sheep (Zamboni and Upadhyay, 1982). The female equivalent to the rete testis is the rete ovarii, a network of tubules lined with cuboidal cells, found in all mammalian species examined (Opend'hal *et al.*, 1986). The close resemblance between the structure of rete cells and granulosa cells indicates a common origin (Byskov and Lintern-Moore, 1973; Byskov, 1978) and Zamboni *et al.* (1979) claim that the mesonephros is indeed the source of follicular cells in sheep, just as it is the Sertoli cell forerunner in the male. The rete ovarii apparently play a role in the initiation of meiosis in female germ cells, since ovaries separated from their extraovarian rete contained only oogonia, the diploid precursors of oocytes (Byskov, 1974). Recently, Opend'hal *et al.* (1986), have found that, rather than being a closed system, the rete ovarii in cattle and deer open directly into the infundibula, and are secretory. These authors suggest that the structure may play more of a role in reproductive physiology than previously thought.

In the male gonad, the formation of seminiferous tubules is one of the earliest signs of differentiation. These tubules are lined by Sertoli cells, apparently the first cells to differentiate in the testis (Magre and Jost, 1980). Recent work by Burgoyne *et al.*, (1988), has shown that, in XX/XY mouse chimaeras which develop testes, whilst XX cells may contribute to the Leydig and peritubular cells and the tunica albuginea, Sertoli cells are always XY. This suggests that Sertoli cell differentiation is triggered by cell-autonomous activity in Y-bearing cells. Sertoli cells secrete Müllerian inhibiting substance or anti-Müllerian hormone (AMH), which causes regression of the female duct system. It has been shown that AMH can also induce formation of

"tubular-like structures" resembling seminiferous tubules in cultured fetal rat ovaries (Vigier *et al.*, 1987); Sertoli cells may thus play a role in testicular differentiation as well as in masculinization of the tract.

The gonadal sex of an individual is effectively the key to somatic sex since gonadal secretions have an effect on almost every tissue in the body (Short, 1982), a matter discussed in more detail later on. Gonadal differentiation is determined by the sex chromosomes, the presence of a Y chromosome determining a male offspring in mammals. Exactly how gonadal differentiation occurs has been explained using several different models.

Mechanisms of gonadal sex determination

a) Cortical versus Medullary inducers

Since testicular development is characterised by growth of the medulla of the indifferent gonad, and ovarian development involves concentration and growth of follicles in the gonadal cortex, Witschi (1939) devised the cortical versus medullary inductor theory. This held that a cortical inducing system led to ovarian development, whereas medullary induction formed a testis. Isolated cortex from the gonads of duck embryos aged between 5 and 9 days apparently was capable of forming a complete gonad according to the genetic sex of the donor. Cortex from female gonads of 5-10 days of incubation developed into ovarian tissue whereas cortex from male gonads would only form testicular tissue after 6.5 days of incubation with the medulla. The fact that cortex could develop into testis at all was put down to the origin of testicular cords, *i.e.* they are mesonephric and invade from the epithelium (McCarrey and Abbott, 1979). Since testicular tissue would only form from the isolated cortex after exposure to some factor in the medulla, it was assumed that a diffusible substance, named "medullarin", was instrumental in testicular differentiation.

b) Hormonal influences

The original interpretation of the freemartin condition in cattle (where male and female twins share a common placental circulation) put forward by Lillie (1917), was that hormones from the male calf caused masculinization of the female reproductive tract and sex reversal of the gonads. Attempts to reproduce these results in mammals using administered sex hormones have never resulted in gonadal masculinization in the female fetus. Testicular differentiation has been induced in fetal mouse ovaries by transplanting them into adult male mice (Taketo *et al.*, 1984) and testicular differentiation has been inhibited by neonatal oestrogen treatment in a marsupial (Fadem and Tesoriero, 1986). Early androgen treatment of female mammals can masculinize the genitalia and disrupt ovarian development (Turner, 1939; Angelova, 1984), but testicular or ovarian differentiation has not been induced in fetal mammals using hormone treatment alone. It may be that administration of exogenous hormones to the pregnant mother does not mimic endogenous and local production by gonadal tissue. However, administration of testosterone directly to pig fetuses through the uterine wall at days 30 or 40 of gestation, does not cause gonadal sex reversal (Elsaesser *et al.*, 1978a).

The situation in birds is different. Removal of the left ovary from 6 week old leghorn chicks results in hypertrophy of the right gonad and its transformation into a functional testis (Domm, 1939). Application of oestradiol to genetically male embryos *in vivo* apparently causes gonadal sex inversion, although this is not maintained in the adult animal and the gonad reverts to being a testis (Jost, 1960; Narbaitz and Teitelman, 1965; Narbaitz and DeRobertis, 1970). In birds the female is the heterogametic and "dominant" sex, castration of the male does not lead to feminization as would happen in mammals. On the contrary, ovariectomy causes masculinization, in for example the plumage (Domm, 1927).

Gonadal sex reversal can also be achieved in fish, amphibians and invertebrates by treatment with hormones (Ohno, 1976). In mammals, the role of hormones is seen as a consequence of gonadal differentiation and not as causative. During gestation, the fetus is well protected from the high concentrations of maternal oestrogen by the presence of α-fetoprotein, a plasma protein in the fetal circulation which binds and

effectively sequesters much of the oestrogen present in the fetal and neonatal circulations. However, the fetus is not protected in the same way against raised testosterone concentrations (MacLusky and Naftolin, 1981).

c) Differential growth rates

Mittwoch proposed that a difference in mitotic rate between male and female gonads results in variation in the rate of growth, and that this determines testicular or ovarian development (Mittwoch *et al.*, 1969; Mittwoch, 1971).

There is evidence that gonadal volumes do vary between males and females even at very early stages of development (Mittwoch and Mahadevaiah, 1980). In humans, the heterogametic male fetus has heavier gonads with a higher content of protein and DNA than female counterparts. In chick embryos it is the heterogametic female which has a greater gonad:body weight ratio than the male, although only after day 7 of embryonic life (Gasc, 1978). There is also evidence from the rat that these differences in size occur before histological differentiation has begun (Mittwoch *et al.*, 1969). Figure 1.2 expresses these theories.

Mittwoch and Mahadevaiah (1980), also propose a possible link between hermaphroditism and the expression of H-Y antigen through differential growth rates of the undifferentiated gonad. It is known that not only does testicular size exceed that of the ovary, but, in humans the right gonad is larger than the left. In hermaphrodite humans, the right gonad is more likely to develop as a testis than the left, supporting the proposal that an increased growth rate leads to testicular development. Whilst it may be difficult to show a causative relationship between growth rate and sexual differentiation, the recent interest in growth factors and their early expression in embryos may be enlightening (Mercola and Stiles, 1988).

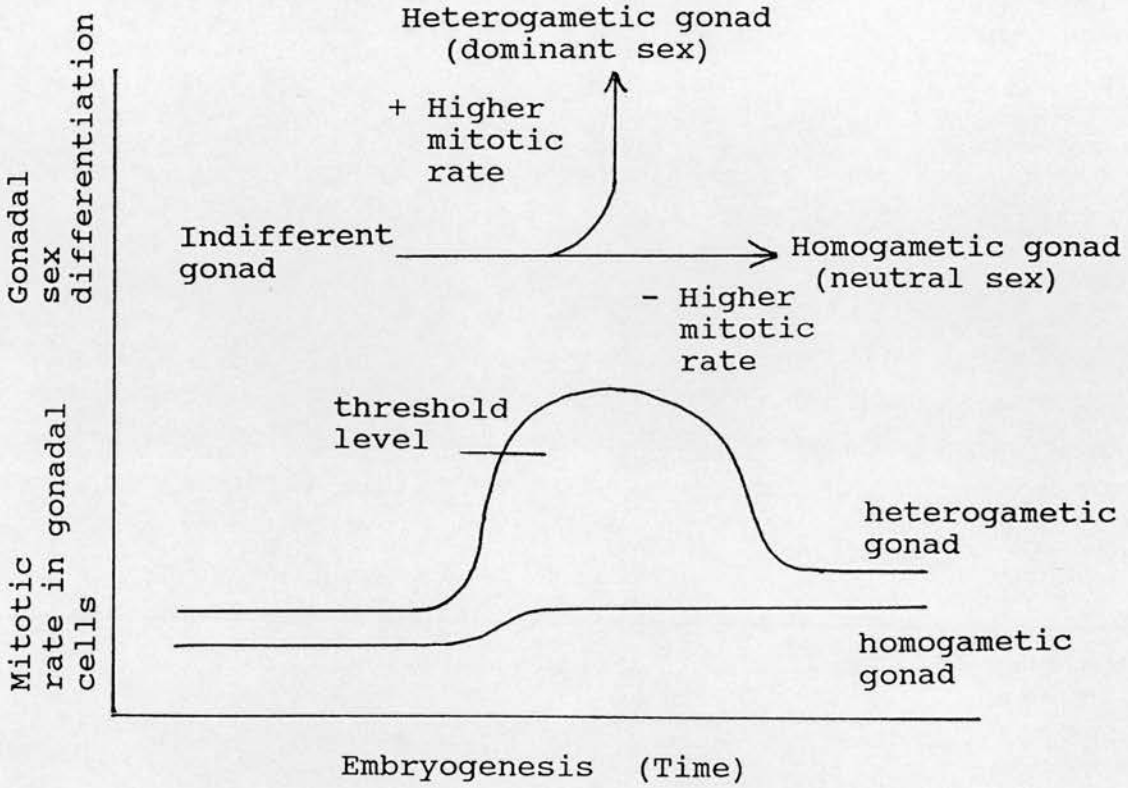


Figure 1.2. Differential growth theory of sexual differentiation. Development of the dominant gonadal phenotype (testis in mammals) is correlated with the surge in mitotic rate in the gonadal cells, above a threshold level. The neutral gonadal phenotype results from the failure of mitotic rate in the gonadal cells to surpass the threshold level. From McCarrey and Abbott (1979).

d) H-Y antigen

The finding that, within an inbred strain of mice, which are taken to be genetically identical, male skin grafts are rejected by females, led to the belief that the Y chromosome codes for a male specific antigen (Billingham and Silvers, 1960). There followed a search for what many considered to be the male determining antigen (Wachtel Koo *et al.*, 1975), sometimes called the heterogametic sex determinant, since female birds are H-Y positive (Wiberg, 1987). The possible genetic control and mode of action in sexual development is described in detail in Chapter 5.

The method of programming from a Y chromosomal gene or genes to testicular development is not known. At present, there seem to be three possibilities. Firstly there is the idea of a diffusible substance which induces tubule formation in the male gonad; H-Y antigen would be one candidate. Second, Burgoyne *et al* proposal (1988) is that Sertoli cell differentiation is the key and that this occurs in the absence of external influence. Once these cells have differentiated, tubule formation results from the secretion of AMH. Thirdly there is the "growth rate" theory supported by Mittwoch (1971) which goes some way towards explaining anomalous gonadal development in hermaphrodite individuals, but at present, lacks conclusive evidence of a causal relationship.

1.3 THE GENETICS OF SEXUAL DETERMINATION

X and Y chromosomes

Whatever the mechanism involved in the process of testicular or ovarian growth from an undifferentiated gonadal anlage, the evidence suggests that a Y chromosome must be present for the transformation to a testis to occur. If a Y chromosome is present, a male phenotype will ensue even if there are, in addition, two, three or four X chromosomes (Jacobs and Strong, 1959), whereas XO or XX individuals develop as females (Welshons and Russell, 1959). It was Henking (1891) who first recognized the sex chromosome; he noticed a densely staining body at meiosis and, examining a pair of secondary spermatocytes, he noticed that the body was always incorporated into one of the two spermatocytes. Rejecting his original idea that the body was a nucleolus, Henking labelled it "X" for unknown (Ohno, 1976). Eleven years later, McClung (1902) realized that the sex of the zygote was determined by whichever of the two kinds of spermatozoa fertilized the egg in the grasshopper species he was studying. Perhaps it was fortunate that he wasn't studying the fruitfly, *Drosophila melanogaster*, since in this species the Y chromosome is not required for the development of the male phenotype but to ensure motility of the sperm; male and female development depends on the ratio of the number of X chromosomes to the number of autosomes. An XX or XXY constitution *i.e.* two X chromosomes, results in a female whereas with one X (XY or XO), males develop (Bridges, 1916). Because of the work done by Bridges, when an X and Y chromosome were identified cytologically in humans (Painter, 1923) it was assumed that sex was decided by the number of X chromosomes. Studies of humans and mice with abnormal sex chromosome constitutions led to the belief that it was in fact the Y chromosome that was male determining.

The X chromosome is not composed of genes involved only with sex determination. Only one of the mammalian X-linked genes is directly concerned with sexual development, that is the Testicular Feminization locus (*Tfm*). In individuals suffering from testicular feminization, target tissues are unable to respond to testosterone and normal sexual

differentiation of the tract and other target organs does not occur. *Tfm* is also known to be X-linked in the mouse (Lyon and Hawkes, 1970) and many gene products or diseases known to be X-linked are also so in other species *eg.* glucose-6-phosphate dehydrogenase production (Ohno, 1976). There are also similarities in banding patterns between the X chromosome of species as diverse as chimpanzees and pigs (Pathak and Stock, 1976), which points to evolutionary conservation of the X chromosome. This is supported further by the finding that sex associated DNA sequences from the Indian banded krait (termed Bkm sequences) are present in a wide spectrum of eukaryotes (Singh *et al.*, 1980; Singh and Jones, 1986).

Despite the relatively small mammalian Y chromosome, its strong male determining role in all species implies the presence of major sex determining genes. Since the first step in male differentiation is the gonadal decision to follow a testicular pathway, it follows that the Y chromosome should encode for testis determination, and a putative testis determining gene is designated *TDF* in man and *Tdy* in the mouse. On the human Y chromosome, functional genes seem to lie within the region from the tip of the short arm (p) to the centromeric, proximal part of the long arm (q) (Ferguson-Smith *et al.*, 1987). The remaining portion of the long arm consists of heterochromatin which fluoresces brightly with a quinacrine mustard dye (Caspersson *et al.*,1970). Deletions of this portion of the chromosome do not interfere with testicular differentiation, although meiosis may be affected (Müller *et al.*, 1986).

The notion that one, or a few genes can code for some factor which determines testicular differentiation and hence sexual determination, is an attractive one. H-Y antigen was put forward as one possible candidate and it was suggested that this male specific antigen was coded for by the Y-linked *TDF/Tdy* gene (Wachtel Ohno *et al.*, 1975). The repetitive DNA sequences comprising Bkm, whilst apparently being sex specific in the mouse and concentrated at the paracentric region of the Y chromosome (Jones and Singh, 1981), are not convincingly sex specific in humans (Singh and Jones, 1986).

The study of human XX males and XY females has led to the pinpointing of the exact location of *TDF*. DNA hybridization studies (using Y-specific probes) of individuals with only parts of the normal Y chromosome, have

yielded an 8 interval deletion map of the Y chromosome. From studies of XY females (who are presumed to have lost the necessary DNA) and XX males (who have gained *TDF* in their genomes), it is shown that *TDF* resides in interval 1, representing a portion of Yp (Simpson *et al.*, 1987). XX male individuals do indeed carry Y derived DNA in their genome (Magenis *et al.* 1982; Guellaen *et al.*, 1984; Andersson *et al.*, 1986; Müller *et al.*, 1986), conversely, XY females are shown to lack small portions of the Y short arm (Disteche *et al.*, 1986).

Page *et al.*(1987) have recently cloned a part of the Y chromosome believed to contain some or all of *TDF*, identified using DNA probes on the DNA of sex-reversed humans. The portion believed to be responsible for testis determination maps to the short arm of the Y chromosome, close to the centromere. A DNA probe was constructed which included all of the particular portion of the Y chromosome involved, designated interval 1A2. The probe hybridized with a sequence on the Y chromosome in a variety of species including gorillas, dogs and goats, inferring evolutionary conservation. The probe also hybridized to the *Sxr* region in sex-reversed mice (see Chapter 2). Page *et al.* (1987) found that the gene included within the probe pDP1007, encodes a protein with a nucleic acid domain, implying that the protein binds directly to RNA or DNA. This infers that it only affects those cells in which it is expressed and is not released from the cell as a cell surface protein, although the authors add that, whilst the initial steps in sexual differentiation may be cell-autonomous, subsequent steps need not be so.

Interestingly, this cloned region of DNA not only detected a Y specific fragment in many different species tested, but also a fragment present in males and females. Page *et al.* (1987) offer four models for sexual differentiation taking into account this homologous gene on the X chromosome:

1) the Y encoded protein is sex determining and the X homologue does not play a role in testis determination.

2) The X and Y loci act antagonistically.

3) The X and Y loci act together, encoding different proteins in concert to those encoded by two X loci (or rather one active locus), as in an XX individual.

4) The X and Y loci are both testis determining and sex is determined by the number of expressed loci. Due to X inactivation in females, a single gene dose would result in ovarian development, whereas XY individuals, with two active loci, develop testes. In this model sex determination thus becomes a function of X inactivation. Chandra (1985, 1986) has also proposed that X inactivation may be more than a "dosage compensation" mechanism in homomorphic individuals, and may play a role in sex determination, a view which is challenged by Lyon (1986) and Newton (1986) on evolutionary grounds. Newton claims that inactivation occurs because the chromosomes are heteromorphic, and that heteromorphism could only evolve if fixation of genes onto one chromosome occurred *i.e.* heteromorphism came before inactivation.

The identification of Y specific DNA in XX human males is evidence that, during meiosis, transfer of genetic material from the Y to the X chromosome has occurred. The concept of a crossing over between these chromosomes was put forward to explain the unusual inheritance of the sex-reversed factor (*Sxr*) in the mouse (discussed later). Burgoyne (1982) concluded that a single "obligatory" cross-over occurred in the X-Y pairing segment. The idea of crossing over between the X and Y had, prior to 1959 (Ohno *et al.*, 1959) been rejected on cytological evidence since the X and Y were found to be associated end to end at diakinesis, with no visible chiasma.

Burgoyne (1986) presented a model for crossing over between the human X and Y chromosomes. He proposed that the distal portions of the X and Y short arm are homologous and hence crossing over occurs, possibly with one "obligatory" cross-over point. This homologous portion of the X and Y chromosome is termed the pseudoautosomal segment, since genes located here will not show sex linkage; the segment does not undergo inactivation in females since dosage compensation is unnecessary when both males and females carry double doses of the genes.

Proximal to the X-Y homologous pairing segment is a non-homologous segment including *TDF* on the Y chromosome and *Xg* (a gene controlling

expression of a red blood cell antigen) and *STS* (a gene responsible for steroid sulphatase deficiency) which also escape X inactivation (Burgoyne, 1982 and 1986; Müller *et al.*, 1980). Crossing over between the X and Y chromosomes over this segment can occur (Polani, 1982) as shown by the occurrence of XX *Xg(a-)* males whose fathers are *Xg(a+)* *i.e.* at meiosis the paternal chromosome has gained *TDF* and lost *Xg(a+)* (de la Chapelle *et al.*, 1984).

Whilst the Y chromosome seems to be essential for normal male development, there are autosomal and X-linked genes which are also functional in sexual determination. A dominant gene mapped to chromosome 17 has been identified in mice, which causes ovarian or ovotesticular development in C57BL/6J mice (Washburn and Eicher, 1983). The gene has been named *T-associated sex reversal* or *T-as*. Similarly, if a Y chromosome (Y^{DOM}) from stocks of certain wild mice *Mus musculus* is placed on the C57BL/6J background, the gonads develop as ovaries or ovotestes (Nagamine *et al.*, 1987 a and b). It is thought that the animals must be homozygous at an autosomal locus in order to develop as hermaphrodites in the presence of this Y chromosome. The gene, not yet mapped to any particular autosome, is called *Tda-1 (Testis-determining autosomal-1)* (Erickson *et al.*, 1987). In humans, de la Chapelle (1987) suggests an autosomal gene, *TDFA*, is responsible for hermaphroditism in the absence of any Y specific DNA (Waibel *et al.*, 1987).

X-INACTIVATION

The XX/XY sex determining mechanism in mammals poses certain problems in the female (XX) since, with two X chromosomes, she effectively carries double the dose of X genes when compared with the XY male. This problem of a double dosage is overcome by X-inactivation, a system first proposed by Lyon (1961) see Figure 1.1, and since found to occur in all eutherian mammals and in marsupials. X-inactivation occurs at an early embryonic stage , and involves switching off almost all of the genes on one X chromosome in all somatic tissue. Lyon (1974) gives examples of mammals in which the time of X-inactivation is known, in many cases by observing the appearance of the Barr body (sex chromatin), a dense mass of intranuclear heterochromatin representing the inactivated X chromosome. The inactivated X can also be identified in dividing cells since the chromosome replicates later than its homologue and consequently stains differently with fluorescent dye (Kondra and Ray, 1978). More accurate methods have been employed to assess the time of inactivation (Lyon, 1974) and for the mouse this seems to occur between 3½ and 4 days after fertilization. In the pig, sex chromatin is first apparent at the 50 cell stage (Lyon, 1972).

The tortoiseshell cat is often used as an illustration of random X-inactivation, apparently the norm in eutherian mammals. The cat's coat is a mosaic of black and yellow hairs, black hairs are determined by the dominant X-linked gene *B*, yellow by the recessive allele *b*. A heterozygote female *Bb*, will be a tortoiseshell if random inactivation occurs, producing patches of yellow and black hairs. Male tortoiseshell cats are rare and are chimaeras of XX and XY cells, or two populations of XY cells, presumably formed by the fusion of two XY zygotes in a mating between a heterozygote female (*Bb*) and a black or ginger male. If more than two X chromosomes are present, all but one is inactivated, and in humans, defective X chromosomes are preferentially inactivated.

There are exceptions to the rule of random X-inactivation, notably in mules and kangaroos, animals in which the paternal X is inactivated (Hamerton, Giannelli *et al.*, 1969; Cooper *et al.*, 1971). As mentioned earlier, there are also genes on the X chromosome which are not

inactivated. The mechanism of X-inactivation appears to involve travel of some signal along the chromosome from an inactivation centre (Rastan, 1983), and in humans this process does not extend to the genes *Xg*, *STS* and *MIC 2x* (Buckle *et al.*, 1985) although there may be partial inactivation of *STS* (Lyon, 1986). Similarly, in the mouse, the *STS* gene escapes inactivation (Keitges *et al.*, 1985)

Whilst dosage compensation is apparently vital in somatic XX cells, in the germ cells both chromosomes must be active as evidenced by sterility in XO humans (Ferguson-Smith, 1965) and reduced fertility in XO mice (Lyon and Hawkes, 1973). However, in male germ cells, the X chromosome is only active in the early spermatogenic stages, both the X and Y chromosomes become condensed and inactive later on (Lyon, 1972). Male individuals with more than one X chromosome are sterile apparently because of the excess X chromosome activity.

EXCEPTIONS TO THE RULES

a) Sex determination in the wood lemming

Mention should be made of the wood lemming *Myopus schisticolor*, a Scandinavian creature in which it was found that wild populations had very high proportions of females. Further studies showed that the females could have the karyotype XX or XY and that XY females were fertile, producing only female offspring (Fredga *et al.*, 1976). Two types of X chromosome are identified in the wood lemming, the normal X and X*, distinguishable cytogenetically. The X* chromosome carries a suppressor gene/s which results in female development in X*Y animals. Furthermore, at meiosis, only X* gametes result since the Y chromosome is lost from most of the germ cells. Since X* determines femaleness, the offspring of an X*Y female x XY male are all female, an X*X x XY produces a ratio of 3 females : 1 male. Arctic varying lemmings (*Dicrostonyx torquatus*) have a similar X* chromosome in their population, but at meiosis in X*Y females, the Y chromosome survives and X* and Y bearing oocytes result.

Sexual differentiation in marsupials

Amongst mammals, there seems to be an exception to the rule that gonadal sex precedes and hence determines sexual differentiation of the rest of the animal. It was mentioned earlier that treatment of gray opossums (South American marsupials) with oestrogens prevents testicular development (Fadem and Tesoriero, 1986), and that this goes against the general finding in placental mammals that, whilst ovarian development may be disrupted by exogenous testosterone treatment, oestrogen treatment of testes is without effect. In either case gonadal development is not completely inhibited and is never reversed.

Recent work by O *et al.* (1988) on tammar wallabies has shown that sexual dimorphism is noticeable in newborn young before gonadal differentiation

is perceptible. Scrotal bulges, a gubernaculum and processus vaginalis were all recognizable in pouch-young karyotyped as males; females of the same age were identified by the presence of mammary anlagen and a short gubernaculum and processus vaginalis. It seems that in this animal, there must be sex-linked genes affecting dimorphism in sexual development even before gonadal differentiation begins.

1.4 THE GERM CELLS

The origin of the germ cells

The germinal epithelium covering the ovary is so-called because it was at one time thought that the germ cells originated from this tissue layer, (Young, 1961), which is now known to be a continuation of the visceral peritoneum. Experiments by Everett (1943) showed that genital ridges transplanted from 9½-10 day old mouse embryos developed into ovaries or testes when transplanted into kidney capsules, but were sterile. This indicated that the germ cells were not present in the undifferentiated gonad, and the use of alkaline phosphatase to stain preferentially primordial germ cells (McKay *et al.*, 1953) helped pinpoint their position.

Chiquoine (1954) claimed that primordial germ cells were first identifiable in 8 day old mouse embryos, when they were seen in the yolk sac endoderm; in 9 day old embryos, some germ cells were seen in the embryonic hindgut. The yolk sac endoderm was also described by Clark and Eddy (1975) as the site of germ cell origin, although there was a suggestion that the germ cells may have come from the mesoderm before that (Spiegelman and Bennett, 1973). Although pinpointing the exact origin of primordial germ cells using alkaline phosphatase is not accurate (Eddy *et al.*, 1981), the yolk sac endoderm is generally taken to be the site from which germ cells migrate, via the hind gut of the embryo, to the gonadal ridge. (Witschi, 1948; Clark and Eddy, 1975). How the germ cells find their destination is not known, but locomotion seems to be amoeboid *i.e.* by means of pseudopodia, recognizable *in situ* (Everett, 1943; Witschi, 1948). See Figure 1.3.

The number of germ cells reaching the gonadal ridge is, in females, several times that leaving the site of origin since vigorous mitotic activity increases numbers, reaching approximately 7 million in humans. Mitosis also occurs in male germ cells, at this stage, apparently not to the same extent. In the female, diploid oogonia enter meiosis before birth (in the majority of species) but are arrested at the diplotene stage of prophase 1 as primary oocytes, meiosis being resumed again by the

pre-ovulatory gonadotrophin surge in females at puberty. Once the oogonium has entered meiosis, further mitotic divisions do not occur and a mammal is born with a lifetime supply of eggs. Obviously several million could last a long time, but the process of meiosis is apparently hazardous and a huge loss in numbers of oocytes occurs during the second half of fetal life (see Figure 1.4.). The process of transformation of oogonia to oocytes, the maturation of the oocyte, and the possible reasons behind the dramatic atresia are subjects covered by Baker (1982).

In contrast to the prenatal initiation of meiosis seen in female germ cells, in the male, gonocytes remain inactive until shortly before puberty. Mitotic division results in spermatogonia which then undergo meiosis to form primary then secondary spermatocytes. The production of spermatids and finally spermatozoa from spermatocytes is reviewed by Setchell (1982). Unlike oocytes, numbers of which are fixed at the time of birth, continuous mitotic activity in male germ cells (from puberty) ensures an almost indefinite supply.

The position of male germ cells within the developing testis seems to be of importance in regulating the timing of meiosis. Germ cells within testicular cords may enter meiosis at the same time as those in the ovary, but only reach the leptotene stage of prophase 1. Germ cells which are not enclosed within testicular cords may however reach zygotene or pachytene, but then degenerate and disappear (Byskov, 1982). Meiosis can be induced in germ cells if an undifferentiated testis (*i.e.* lacking seminiferous tubules) is cultured with a differentiated ovary (separated by means of a filter) co-incubation with a differentiated testis however, cannot induce meiosis in the germ cells within the seminiferous tubules (Byskov and Saxén, 1976). These observations led the authors to conclude that meiosis is prevented in testicular tubules by a diffusible substance, termed meiosis-preventing substance (MPS) and that ovaries secrete meiosis inducing substance (MIS). In pubertal and adult testes, meiosis begins under the influence of MIS. Meiosis has been observed in XY germ cells in the ovaries of XX/XY mouse chimaeras (Evans *et al.*, 1977) supporting the notion of meiosis-inducing substance in the ovary, although the presence of growing oocytes in testicular tissue of XXSxr mice is difficult to explain (Mclaren, 1980). In this case it is suggested that the germ cell carrying two X chromosomes is more

susceptible to MIS than XY germ cells. The location of these oocytes near the rete ovarii does lend support to Byskov's postulate that MIS is produced in this region (Byskov, 1974)

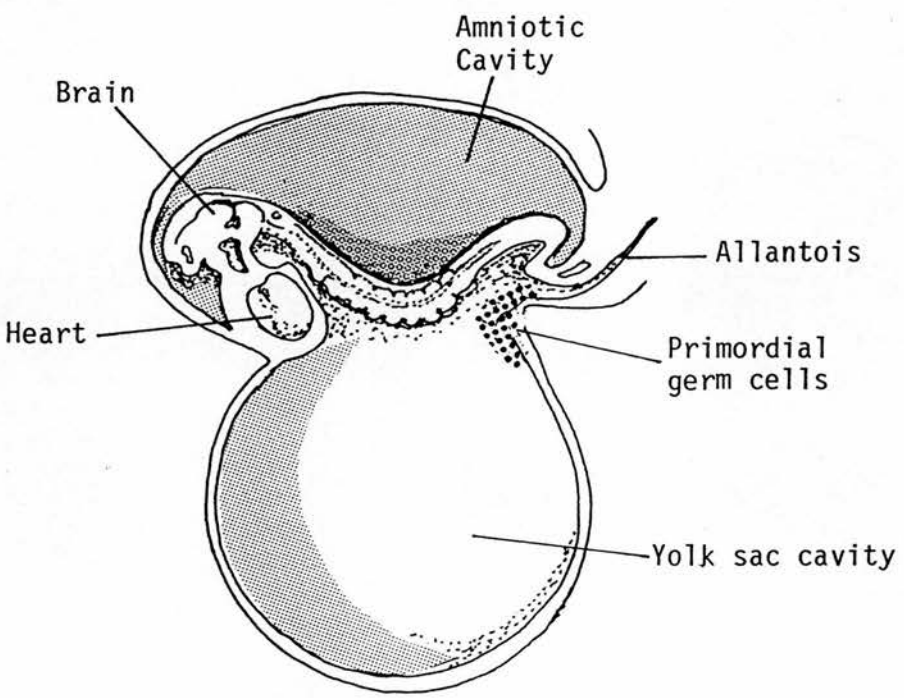


Figure 1.3. The apparent site of origin of primordial germ cells, as demonstrated in a human embryo at 24 days of gestation. From Byskov (1982).

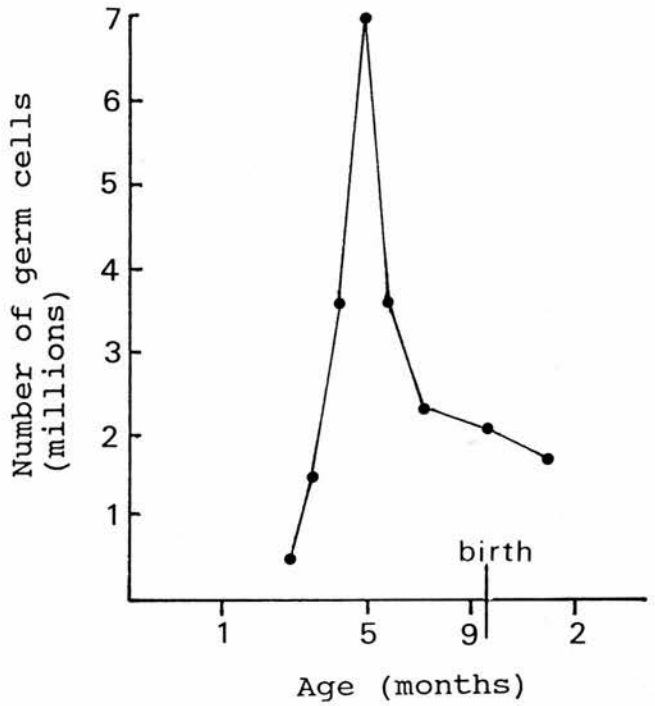


Figure 1.4. Fluctuations in the number of germ cells in the human embryonic and fetal ovary. From Baker (1982)

The role of germ cells in gonadal differentiation

Witschi (1951) observed that ovarian development was impaired if germ cells were prevented from reaching the gonadal ridge, and Dantchakoff (see McCarrey and Abbott, 1979) proposed that germ cells were a prerequisite for gonadal development. However, experiments on chick embryos have since shown that organogenesis can proceed even if germ cells are killed by irradiating the developing embryo (reviewed by McCarrey and Abbott, 1979). Merchant (1975) used busulfan to kill primordial germ cells in rat embryos and demonstrated that the ovary can, nevertheless, differentiate.

Despite the evidence suggesting that organogenesis does not require the presence of germ cells, Witschi's observations are not contradictory since the elimination of meiotic germ cells does result in ovarian regression (Macintyre *et al.*, 1960). Merchant (1975) also observed that follicular formation was impaired in the absence of germ cells, the gonad remaining in the embryonic "cord stage" since the presence of the oocytes is necessary for follicular formation. Hypophysectomy in rats results in the formation of testis-like tubules one year later, similar to the tubular structures seen in ageing rat ovaries following follicular atresia (Crumeyrolle-Arias and Aschheim, 1981). Vigier *et al.*(1987) showed that treatment of fetal rat ovaries with anti-Müllerian hormone causes loss of the germ cells, reduction in gonadal volume and reorganisation of the gonad.

The genetics of germ cells

It was previously mentioned that XX germ cells differ from somatic tissue in that X-inactivation does not occur; evidence from XO individuals shows that, in order to survive, female germ cells need two X chromosomes (Lyon, 1974). In XY individuals, by contrast, it seems that more than one X chromosome is lethal to the germ cell since animals with an XXY or XXXY karyotype are sterile (reviewed by Lyon, 1974), although X activity

is apparently confined to early spermatogenic stages and later both the X and Y chromosome are inactivated. There are exceptions to these rules, examples are given in Short (1982) of the creeping vole, *Microtus oregoni*, in which the X chromosome is lost from the male germ line, which nonetheless survives, producing Y and O sperm. In certain bandicoots (*Isodon obesulus*), the situation is reversed, in that male and female are XO, but germ cells are XY and XX.

There has been some discussion as to whether the fate of the germ cells is determined by their genetic constitution *i.e.* does the germ cell differentiate as an oocyte or a spermatocyte regardless of the karyotype of the gonad in which it finds itself. Chimaeric mice, formed by the fusion of XX and XY embryos can offer a model for the study of the fate of germ cells which find themselves in an environment of different genotype (McLaren *et al.*, 1972). The majority of XX⇔XY mouse chimaeras develop as fertile males with two testes (McLaren, 1983), although some develop ovotestes. A study of the gonads in fetal mouse chimaeras showed that meiotic germ cells sometimes occurred in the testis or testicular portions of an ovotestis (McLaren *et al.*, 1972), in normal males meiotic germ cells are not seen until puberty. McLaren *et al.* (1972) concluded that these germ cells were probably XX in constitution and were entering meiotic prophase under the influence of neighbouring XX somatic tissue. In sex-reversed male mice, oocytes have been observed in the testes of animals up to 15 days of age (McLaren, 1980). These animals are of an XX genotype, but carry the testis determining region (including *Tdy*) on an X chromosome, causing sex reversal (see Chapter 2). McLaren (1980) suggested that, since the germ cells possessed two X chromosomes, they were more susceptible to MIS than XY germ cells. However, due to random X-inactivation, XX*Sxr* males should be considered as mosaics with the Y(*Tdy*) sequence expressed in some cells but not others. Oocytes may then be observed, either because of their two X chromosomes, (no meiotic germ cells are seen before birth in XO*Sxr* mice), or because *Sxr* (or *Tdy*) normally prevents meiosis, but in some germ cells, entry into meiosis occurs before reactivation of the *Sxr* segment. There is also the possibility that the gonad surrounding the germ cells consists of cells in which X*Sxr* is inactivated and the germ cell is thus allowed to enter meiosis because it is in an XX rather than an XX*Sxr*

environment, although this implies testicular development in the absence of the testis determining region of the Y chromosome (McLaren, 1983). Using Searle's X-autosome translocation T(X;16)H, or T16H, which causes preferential activation of the translocated chromosome, it is possible to produce sex-reversed mice carrying an X chromosome with the sex-reversed segment *Sxr* (which will be inactivated), and an X chromosome X*T16H*, which is preferentially activated. Inactivation of the X*Sxr* chromosome is not always complete and the animal T16H/X*Sxr* may develop as a fertile female, hermaphrodite, or a sterile male depending on the extent of inactivation of the *Sxr* region (McLaren, 1983). Within the seminiferous tubules of these intersex mice, growing oocytes may be seen (Ward *et al.*, 1988). Since both X chromosomes will be activated in the germ cells, and hence *Sxr* is expressed, it would seem that this testis determining region does not prevent entry into meiosis. Also, if the oocytes are in a testicular region of the ovotestis, presumably *Sxr* is not inactivated in the gonadal cells. Such observations suggest that the presence of two X chromosomes is indeed a deciding factor as to whether germ cells enter meiosis or not. However, there is an account of an XY germ cell entering meiosis and developing into an oocyte, in an XX⇔XY chimaera (Evans *et al.*, 1977) which implies that an XX environment may induce meiosis in germ cells.

Zamboni and Upadhyay (1983) give a detailed account of germ cells which are found in the adrenal glands of embryonic and postnatal mice. Whatever the sex of the animals, the stray germ cells enter meiosis prenatally and develop as oocytes, disappearing in animals over 3 weeks old. These observations imply that germ cells will differentiate as oocytes whatever their genetic sex, unless they are enclosed within developing seminiferous tubules. The developing oocytes also showed meiotic arrest at the diplotene stage of prophase I, indicating that enclosure within a follicle is not a prerequisite for inhibition of meiosis, a finding which contradicts those of previous workers who showed that isolated oocytes undergo spontaneous maturation in culture (Tsafriri *et al.*, 1976).

1.5 DIFFERENTIATION OF THE REPRODUCTIVE TRACT

The experiments of Jost (1946-47), showed that the developing testis is required for Wolffian duct differentiation into the vas deferens, seminal vesicle and epididymis. If a male fetus is castrated before testicular differentiation is completed, the Wolffian duct regresses and the Müllerian duct differentiates into a female tract *i.e.* Fallopian tubes, uterus and vagina. A castrated female fetus also develops these structures, hence showing that the ovary is not required for Müllerian duct differentiation. Jost also showed that the masculinizing effects of a testis on the adjacent duct are localised, a testis transplanted next to the fetal right ovary of a rabbit causes development of the tract on that side, but does not induce masculinization of the left duct. Furthermore, a crystal of testosterone placed next to the undifferentiated ducts resulted in development of the Wolffian duct without regression of the Müllerian duct, whereas placement of a testis instead of a testosterone crystal caused Wolffian duct development and Müllerian duct regression. Jost therefore postulated that the testis produced two classes of hormone - testosterone and a Müllerian inhibiting substance.

Müllerian-inhibiting substance (MIS) or anti-Müllerian hormone (AMH) is now known to be a glycoprotein dimer secreted by Sertoli cells, and also granulosa cells (Vigier *et al.*, 1984). MIS activity is high during fetal life and disappears progressively in the perinatal period or shortly after birth, although weak activity has been found in rete testis fluid from the mature boar (Josso *et al.*, 1979) and in the testes of adult rats and 6 week old dogs (Donahoe *et al.*, 1976; Meyers-Wallen *et al.*, 1987)

Testosterone secretion by the Leydig cells begins in the early fetus and continues throughout life. In the human fetus, Leydig cell differentiation and testosterone production are apparent in the 8th week. Experiments in the pig fetus indicate that initial secretion is independent of pituitary stimulation since decapitation does not affect activity (van Vorstenbosch *et al.*, 1982). However, decapitation of 42-day old fetuses leads to regression of Leydig cells later in fetal life and van Vorstenbosch *et al.*, (1984) concluded from their experiments that, in

the pig, Leydig cell dependency upon LH begins between days 60 and 75 *post coitum*.

As Figures 1.5. and 1.6. show, the external genitalia and, in males,the prostate and bulbourethral glands, develop from a common anlage. The genital tubercle gives rise to the glans penis in the male and clitoris in the female. The paired urethral folds develop into the labia minora in the female and fuse to form the shaft of the penis in the male, displacing the urethral opening to the tip of the penis; the genital swellings form the female labia majora and fuse to form the scrotum in the male. In the human fetus, labio-scrotal fusion occurs around weeks 12-14, during which time the prostate and bulbourethral glands also differentiate, penile organogenesis is complete by week 14. These events are androgen dependent, administration of testosterone to female fetuses resulting in masculinization of the genitalia (Figure 1.7.). However, once the male genitalia have differentiated and, in the female, the vagina and urogenital sinus have separated, after week 14 even large doses of androgen no longer produce labial fusion in fetuses of either sex (Forest, 1983).

Within the cytoplasm, testosterone may be transformed, by means of a 5α-reductase enzyme, into dihydrotestosterone (DHT). This steroid binds to the nucleus and exerts its action on protein synthesis; it was at one time thought to be responsible for differentiation of the urogenital sinus. XY patients lacking the 5α-reductase enzyme show incomplete penile organogenesis at birth. In these individuals, however, Wolffian duct development is usually unaffected (Jaffe, 1978; Forest, 1983), suggesting that testosterone is responsible for differentiation of the Wolffian duct, but that DHT is necessary for normal development of the urogenital sinus and external genitalia (Peterson *et al.*, 1977). At puberty, these individuals undergo dramatic masculinization with deepening of the voice, an increase in muscle mass, enlargement of the phallus and descent of the testes.

It is now thought that both testosterone and dihydrotestosterone cause these changes in the male, but that high circulating concentrations of progesterone in the fetus compete with testosterone for binding sites in the cell; binding of DHT is not affected to the same extent. Conversion

of testosterone to DHT, and of progesterone to metabolites which do not compete with testosterone, are brought about by 5α-reductase. The enzyme is therefore seen as an adaptation to deal with competition from progesterone, in the fetus, for testosterone binding sites (Hodgins, 1982). Individuals lacking the enzyme therefore do not show the same extent of masculinization at birth as their normal counterparts. When puberty is reached, testosterone concentrations are elevated, and since there is no maternal progesterone to inhibit the steroid's activity, secondary sexual characteristics develop, *eg.* deepening of the voice. Such developments are, to some extent, reversible so that removal of the gonads after puberty will result in raising of the tone of the voice and reduction in facial hair growth (Jeffcoate, 1962).

In females, DHT does not appear to play any role in pubertal maturation as XX individuals with 5α-reductase deficiency have a normal phenotype and are fertile (Imperato-McGinley and Peterson, 1976). However, androgens do play a role in follicular development, being necessary for the growth and maintenance of pre-ovulatory follicles, and stimulation of pubic hair growth. Under gonadotrophin control, waves of follicular growth occur in the prepubertal female with a resultant rise in oestrogen and androgen production by the ovary. It is the combined effects of these hormones which causes the growth spurt seen in prepubertal girls. Epiphyseal closure *i.e.* attainment of maximum height is brought about by raised oestrogen levels in pubertal females, testosterone in males, so that castration may result in excessive long bone growth since critical concentrations of these steroids are not attained.

Secondary sexual characteristics in the female (notably breast development) are oestrogen dependent, and gynaecomastia can also occur in XY individuals treated with oestrogen (Short, 1982) even after puberty.

The development of secondary sexual characteristics is most closely documented for humans (Marshall, 1970), but the principles of hormonal influence on genital development also hold for other species *i.e.* androgen administration will cause masculinization, male castration results in a female habitus. The stage at which androgens are administered is of importance since there appears to be a "critical period" during which complete masculinization of the external genitalia can occur. In the

sheep, this critical period seems to be between days 40 and 50 of a 148 day gestation, since testosterone treatment after this time results in only partial masculinization of the genitalia (Clarke *et al.*, 1976). Testosterone treatment of female fetal pigs results in development of a penis-like structure if treated between days 29 and 35 but only enlargement of the clitoris if treated on days 39-45 of a 114 day gestation (Ford and Christenson., 1987). In humans, and possibly in other animals, the clitoris remains sensitive to testosterone throughout life (New *et al.*, 1983), but the genital tract (the Fallopian tubes, uterus and upper vagina) is only sensitive to locally raised testosterone concentrations (Forest, 1983) and cannot be masculinized by systemic administration of testosterone to the female fetus. In the male, stability of the differentiated Wolffian duct is apparent and, once the critical period of differentiation is over, castration, which may affect the external genitalia, will not cause regression of the Wolffian duct or growth of the Müllerian duct.

Sexual differentiation involves tissues other than those concerned with reproduction since the administration of testosterone *in vivo* induces a retention of nitrogen, promotes protein synthesis and increases the respiratory rate by stimulating the number of mitochondria and of mitochondrial membranes. Because of these effects, sexual dimorphism occurs in the kidney, liver and muscle, and also in the brain.

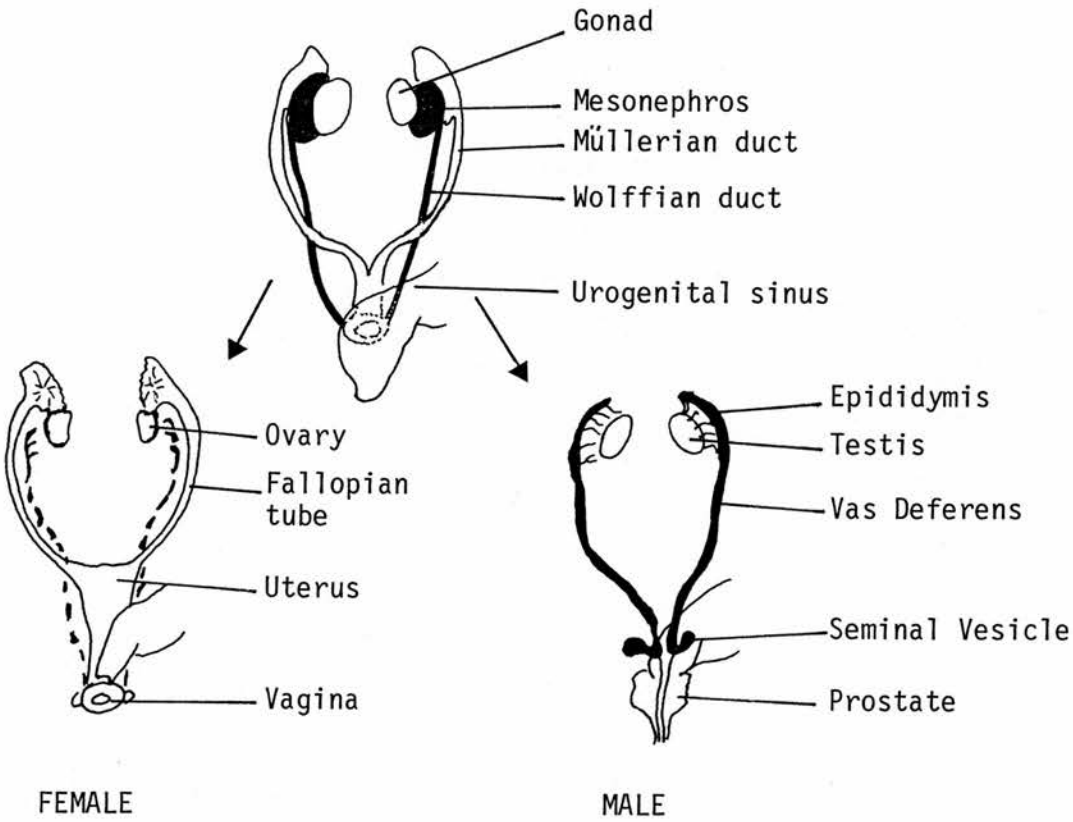


Figure 1.5. Internal genital development in the male and female.

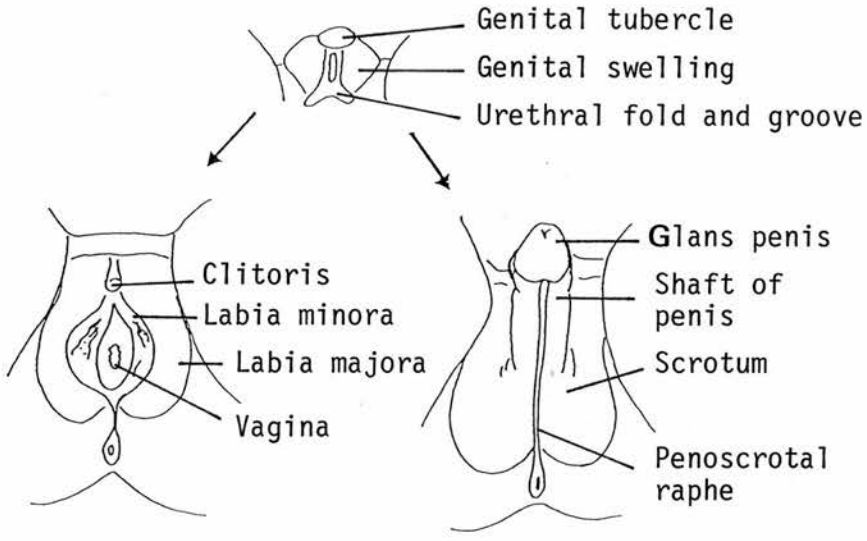


Figure 1.6. External genital development, demonstrating homologies and common anlage in the male and female.

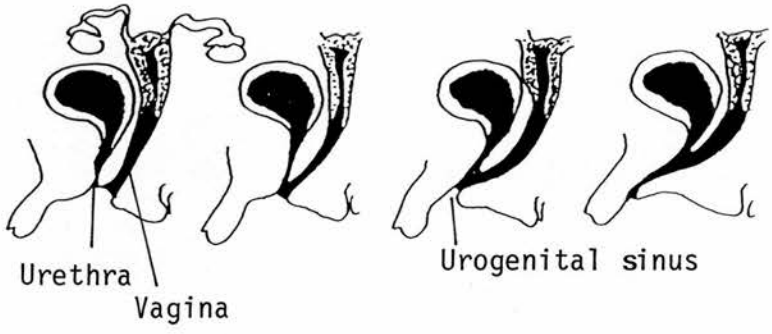


Figure 1.7. Female pseudohermaphroditism induced by prenatal exposure to androgens. Exposure after 12th week leads to clitoral hypertrophy (left). Exposure at progressively earlier stages (left to right) leads to retention of urogenital sinus.

1.6 BRAIN SEX AND BEHAVIOUR

Just as the liver functions differently in a male or female rat (Bardin and Catterall, 1981), so too the brain responds to stimuli according to its sex. The differences in brain anatomy, gonadotrophin secretion and behaviour seem to be induced by exposure to certain hormones during prenatal or neonatal life, the exact timing of differentiation depending on the species. In some cases, behaviour patterns are reversible by administration of hormones.

Morphological differences in the CNS can be seen, and MacLusky and Naftolin (1981) list five known differences between male and female brain morphology in the rat. For example, the medial preoptic nucleus is much larger in the male than in the female. An increase in the size and number of neurones within the nucleus is apparent in the male starting at around the time of birth and continuing throughout the first ten days of postnatal life. If male rats are castrated on the day of birth, this increase in size of the preoptic nucleus does not occur (Gorski *et al.*, 1978).

The pituitary release of gonadotrophins is sexually dimorphic, with females producing a pattern of gonadotrophin secretion allowing normal ovulatory cycles . Pfeiffer (1936) showed that castration of male rats at birth resulted in the development of characteristically feminine patterns of gonadotrophin release. A measure of hypothalamic/pituitary sex often used is the luteinizing hormone response to an exogenous oestradiol stimulus, since adult females will show a surge release of LH not seen in males. Early exposure of the brain to testosterone seems to be the determinant of brain gender as measured in this way. In the rat, exposure to testosterone soon after birth, but not prenatally, will abolish the LH response to oestradiol in the adult; in the guinea pig, the critical period is prenatally, around day 30 of gestation. In fact, the period at which the brain is sensitive to testosterone (in determining gonadotrophin secretion) seems to be related to the stage of development at birth. In species that are relatively immature at birth (*eg.* rat, hamster) the critical period extends into postnatal life, whereas in

species such as the guinea pig and rhesus monkey, the period is during prenatal life.

It seems that testosterone is not, as was first thought, the functional steroid in the masculinization of patterns of gonadotrophin secretion, since treatment of female rats with oestrogen in the neonatal period also results in anovulation in the adult (Wilson *et al.*, 1941). It was subsequently found that 5α-DHT (a non-aromatisable androgen) was much less effective than testosterone or oestrogen in masculinizing the brain, and the hypothesis was put forward that androgens first had to be aromatised to oestradiol-17β in the brain before they could exert their effect (Naftolin, *et al.*, 1975). Subsequent studies have shown that the developing brain is oestrogen-sensitive and that the minimum dose required to masculinize the neonatal rat brain is considerably lower for oestrogen than it is for testosterone (MacLusky and Naftolin, 1981).

Sexual behaviour is controlled by an area of the brain different from that controlling gonadotrophin secretion, but behaviour is also influenced by early exposure to steroid hormones. Testosterone given to pregnant guinea-pigs produces female offspring that, on reaching maturity, respond to exogenous androgen by showing mounting behaviour similar to that in normal males (Young, 1961). Prenatal treatment with oestrogen does not result in feminine behaviour, probably because of the presence of α-fetoprotein which protects the fetus against raised concentrations of oestrogen (MacLusky and Naftolin, 1981). Oestrogens given to castrated males do not induce female sexual behaviour, but may in fact restore male libido. Castrated ewes and red deer hinds may also have their oestrous behaviour restored by testosterone, whereas prolonged exposure to low levels of oestrogen can cause masculine behaviour, for example cows with cystic ovaries begin to show bull-like behaviour (Short, 1982). Spayed ewes exposed to testosterone prenatally show male behaviour patterns, including mounting of oestrous ewes, when injected with oestradiol-17β or testosterone, indicating that prenatal organization of the brain determines the response to a steroid in adult life, whether it be oestrogen or androgen (Clarke and Scaramuzzi, 1978). The time scale of exposure to steroids is important, as shown by administration of testosterone to female sheep; repeated injections induce masculine behaviour (Johnston *et al.*, 1956) whereas a single injection causes

oestrous behaviour (Lindsay and Robinson, 1961). Since animals which have been androgenized by prenatal administration of testosterone not only show male behaviour patterns but also fail to show characteristic female behaviour, several authors view the prenatal organization of the male brain as a double process involving defeminization and masculinization; *eg.* failure to show oestrus indicates defeminization, mounting behaviour indicates masculinization (Clarke and Scaramuzzi, 1978; Short, 1982). Prenatal treatment of females with testosterone may result in masculinization without defeminization. *eg.* some of the spayed ewes mentioned above (Clarke and Scaramuzzi, 1978) showed oestrous behaviour, but also urinated like rams.

CHAPTER 2 INTERSEXUALITY IN MAMMALS

2.1 INTRODUCTION

This section describes some cases in which the processes of gonadal determination and sexual differentiation do not proceed normally, resulting in an individual with characteristics of both sexes. Since there are many stages between the development of an undifferentiated gonad into an ovary or a testis, and the resultant functional adult male or female, there are also many steps at which things can go wrong. Before describing some of the situations in which normal mammalian sexual development has not occurred, it is worth clarifying the nomenclature used to describe affected individuals.

The term "hermaphrodite" should, strictly speaking, be applied only to those species in which sperm and eggs are produced within the same animal so that, theoretically, self-fertilisation is possible. However, the term "true hermaphrodite" is often applied to cases in which both testicular and ovarian tissues are present, even if only one tissue-type is fertile. If two testes are present but the external appearance is female, the term is male pseudohermaphrodite ; individuals with two ovaries but a male phenotype are female pseudohermaphrodites. The problems with these terms are that:- a) categorization can only be made once both gonads have been identified in their entirety *i.e.* after surgery or at autopsy, and b) the terms imply a difference in aetiology between hermaphroditism and pseudohermaphroditism. This may be so in some cases, *eg.* testicular feminization and sex-reversal in mice are caused by two different genetic anomalies and result in a male pseudohermaphrodite and, in certain circumstances, true hermaphroditism respectively, but the sex-reversed mouse may also develop as a sterile XX male or a fertile female. In the domestic farm species a wide spectrum of sexual "malformations" may occur *eg.* in the polled goat the range of phenotypes stretches from almost normal female to almost normal male in affected animals, but there is no evidence that different causes are responsible. For these reasons, the term intersex is often used since this can cover a range of conditions and simply means that characteristics of the male and female

sex are present, reflected in gonadal histology and/or morphology of the tract or external genitalia. In this thesis, the word "hermaphrodite" is used exclusively in those instances where ovarian and testicular tissue are known to be present in the same animal, but no implication of a specific aetiology is intended. Intersexuality is used to cover all forms of morphological sexual ambiguity, including true hermaphrodites.

The following sections are by no means exhaustive but have been included, in the case of man and the mouse, because extensive studies in these species have resulted in explanations (to some extent) for the causes of intersexuality, which may be of relevance to species less well studied. The section on domestic farm species covers those animals in which an intersex condition has been described in sufficient detail to merit inclusion for the purpose of comparison with the condition in pigs.

2.2 INTERSEXUALITY IN MAN

2.2 (1) GENETIC DISORDERS

Gonadal dysgenesis or Turner's Syndrome

Turner's syndrome patients have a 45X karyotype, rather than the normal 46XX constitution. These individuals usually fail to develop functional gonads. In the place of ovaries are found "streak" gonads consisting of whorls of connective tissue but containing no primary follicles. It has been estimated that 0.8% of zygotes have an XO karyotype, but less than 3% of these zygotes develop to term and a higher than normal mortality rate in infancy means that, amongst newborn females, 1 in 2700 may be suffering from Turner's syndrome (Jaffe, 1978). A sex chromosome may be lost during meiosis (by non-disjunction) resulting in an egg or sperm without a sex chromosome, or the loss may occur after fertilization. Since the embryo lacks a Y chromosome, female gonadal development should ensue, but since XO germ cells do not survive, follicular development in the embryonic gonad does not occur. Germ cells can be found in the ovaries of human XO fetuses, but by birth few oocytes remain (Carr *et al.*, 1968) presumably because two activated X chromosomes are necessary for oogenesis (Lyon, 1974). In the absence of follicular activity, the Fallopian tubes, uterus and vagina remain infantile and secondary sexual development (menstruation, breast development) does not occur. A variety of other symptoms are commonly associated with the syndrome, in particular, short stature, "webbing" of the neck, high-arched palate and low-set prominent ears, The absence of germ cells means that XO individuals are infertile, but there are rare reports of menstruation and/or pregnancy occurring, seemingly some XO germ cells do survive (King *et al.*, 1978). Grumbach and Conte (1985) also describe familial gonadal dysgenesis in XX and XY females who had streak gonads, but none of the other symptoms associated with Turner's syndrome.

Klinefelter's syndrome

This syndrome refers to individuals in whom an XXY (*i.e.* 47 chromosomes) constitution is found in some if not all of the cells. A frequency of 1 in 750 adult males are reportedly affected in a population (Chandley, 1984). The presence of a Y chromosome means that testicular development occurs but the testes are small, with hyalinized seminiferous tubules and Leydig cell hyperplasia. Spermatogenesis is impaired and testosterone concentrations are usually below normal, resulting in elevated gonadotrophin concentrations. In many patients there is excessive growth of the long bones, decreased intellectual function and personality disorder (Jaffe, 1978). There are other chromosomal abnormalities which result in symptoms similar to those of Klinefelter's syndrome although perhaps with greater effects on mental development, 48XXXY and 49XXXXY karyotypes are recorded (Chandley, 1984).

Mosaics and Chimaeras

Mosaicism results from a mitotic error in an individual (non-disjunction) giving rise to two or more cell types of different genotype *eg.* XO and XXX cells. The extent of abnormalities associated with an XO karyotype depends upon the proportion of XO cells present; in the case of XO/XY patients (non-disjunction followed by loss of a Y chromosome) a range of phenotypes is found from Turner's syndrome individuals to those with masculinized genitalia due to the presence of testicular tissue. If two zygotes (or blastocysts) fuse (or are artificially fused), a chimaera consisting of cells of different genetic constitution again results. In this case, an XX/XY chimaera may occur.

Sex chromosome deletions and X- and Y- autosome translocations

Deletions of portions of chromosomes may be identified cytologically *eg.* the normally metacentric X chromosome may appear acrocentric. XXp- patients (suffering a deletion of the short arm of the X chromosome) have somatic abnormalities similar to those seen in Turner's syndrome *i.e.* short stature and webbing of the neck. If part of the long arm of the X is lost (XXq-) the patients have a normal appearance, but with streak gonads and sexual infantilism. Studies of women with deletions of various portions of Xq indicate that the region of the X chromosome critical for normal ovarian function covers about 2/3 of the long arm (Chandley, 1984). These women (both Xp- and Xq-) were heterozygous for the condition, and since the abnormal X chromosome would usually be inactivated, this is evidence that the inactivated X chromosome does play a role in development.

In XY individuals, the Y chromosome may be dicentric (XYdic), the patients showing varying disorders of reproductive development (Robinson and Buckton, 1971).

X-autosome translocations are rare in humans (Chandley, 1984) apparently causing azoospermia in men. In *Drosophila* 80% of affected XY males with an X-autosome translocation are sterile (Lindsley, 1981). Y-autosome translocations are described in a variety of species including man, apparently also causing azoospermia (Chandley, 1984).

X-Y Translocations

In a number of species, including mouse and man, the X and Y chromosomes are seen to pair and form a synaptonemal complex at the zygotene stage of meiotic prophase. In man, pairing occurs between the short arms of the X and Y chromosomes, at its maximum, pairing extends almost to the Y centromere (Moses *et al.*, 1975). Normally any crossing over which occurs between the two chromosomes involves genes not concerned with testis

determination, but occasionally crossing over may extend into the Y-linked testis determining region, close to the centromere. Page *et al.* (1987) describe XX males who inherited different amounts of the X-pseudoautosomal region from their father's X and Y chromosomes, as well as *TDF* from their father. The authors conclude that the XX males were the result of unequal crossing over between the X and Y chromosomes (during meiosis in their father) so that the X chromosome, instead of simply receiving pseudoautosomal Y material, also received *TDF*, resulting in a testis determining X chromosome.

Using Y specific DNA probes (Andersson *et al.*, 1986; Müller *et al.* 1986), measuring the relative lengths of X chromosomes (Evans *et al.*, 1979) or using X-linked markers (de la Chapelle *et al.*, 1984), other workers have shown that XX human males do indeed carry Y-specific segments of DNA in their genomes (Andersson *et al.*, 1986; Müller *et al.* 1986). *In situ* hybridization has located Y specific DNA on the short arm of an X chromosome in XX males (Magenis *et al.*, 1987). Most work describes the occurrence of this DNA in XX males resulting from Xp-Yp translocation, but it is not certain how or exactly when the exchange occurs. That loss of *TDF* from the Y chromosome does occur is shown by the presence of XY females (Rosenfeld *et al.*, 1979; Magenis *et al.*, 1984).

True Hermaphroditism

As explained in the introduction, this term refers to those individuals possessing ovarian and testicular tissue, either as an ovotestis or in separate gonads. According to Van Niekerk and Retief (1981) out of 409 cases of human true hermaphroditism studied, 121 (29.6%) possessed an ovary and testis, and an ovotestis was the most common gonadal type (44.3%). An XX karyotype was found in 116/195 cases with various combinations of XX, XY or XXY cells seen in the remaining individuals. Spermatogenesis was observed in only 12% of cases, but ovulation could occur. Phenotypes ranged from normal male to normal female including phenotypic males with an XX constitution and XY phenotypic females. AMH

production seemed to be unaffected since of 169 cases, only 10% had a fully formed uterus, in 46% of the cases the uterus was hypoplastic. The study also revealed that an ovary was more likely to occur on the left side and testicular tissue, whether as an ovotestis or testis, occurred on the right.

As for the aetiology of the condition, the presence of XY cells in an XX/XY individual, or XX/XXp+ (carrying *TDF*) mosaics may explain the development of testicular tissue. However, Waibel *et al.* (1987) used different Y-specific probes to test true hermaphrodites with 46XX chromosome constitutions, but with no success. De la Chapelle (1972) offers three different theories which may account for the development of testicular tissue in XX humans:-

a) The Gene Theory

This theory involves an autosomal gene or genes causing testicular development in an XX individual. In *Drosophila melanogaster* an autosomal gene *tra* transforms XX zygotes into sterile males, and the *polled* gene *p* in goats is believed to be linked with intersexuality in these animals. However, if an autosomal gene were responsible in human hermaphrodites there should be evidence of consanguinity between parents of XX males and this does not seem to be so. The incidence of XX males would also be expected to be high amongst sibs and cousins of the patient, but the condition is extremely rare *eg.* one male in 15363 live male births (Evans *et al.*, 1979). There are some exceptions, *eg.* where several XX males occur within one family (Armendares *et al.*, 1975), although Evans *et al.* (1979) suggest, in these cases, a Y-autosomal translocation with expression of the Y determined genes dependent on other autosomal genes.

b) The Interchange Theory

This involves translocation of part of the Y chromosome onto the X or an autosome. This is indeed the case in many XX males, but as Waibel *et al.* (1987) found, XX hermaphrodites do not seem to carry Y genetic material.

c) The Mosaicism or eliminated Y chromosome Theory

XX/XY mosaicism does account for some cases of intersexuality and de la Chapelle (1972) suggests that XX males or true hermaphrodites are the result of the triggering of male differentiation by a line of cells containing a Y chromosome, with this cell line becoming scarce or completely eliminated in later development.

2.2 (2) DISORDERS OF STEROID PRODUCTION AND FUNCTION

THE MALE

This section covers so called "male pseudohermaphrodites"; individuals are XY, possess two testes but are phenotypically female or have ambiguous external genitalia. As described in section I, differentiation of the reproductive tract depends on the secretion of steroids by the gonads, testicular secretions determine Wolffian duct development, Müllerian duct regression and male external genitalia. In the absence of these secretions (testosterone and AMH), a female tract and genitalia are permitted to develop.

The production of sex steroids, either in the gonads, adrenals or placenta, depends on a chain of enzymes (Figure 2.1), deficiencies of which will affect sexual differentiation. Steroid hormones, including testosterone, exert their action in target tissues by first becoming attached to an intracellular receptor in the cytoplasm. This steroid receptor complex is then translocated into the nucleus where it may influence protein synthesis or may be further transformed before exerting its action.

The action of steroids also depends upon the response of the target tissue. Failure to respond to androgens results in feminization, and a deficiency in anti-Müllerian hormone leads to incomplete regression of the female reproductive tract.

Further details of the conditions described may be found in Jaffe (1978) and Biglieri and Kater (1987).

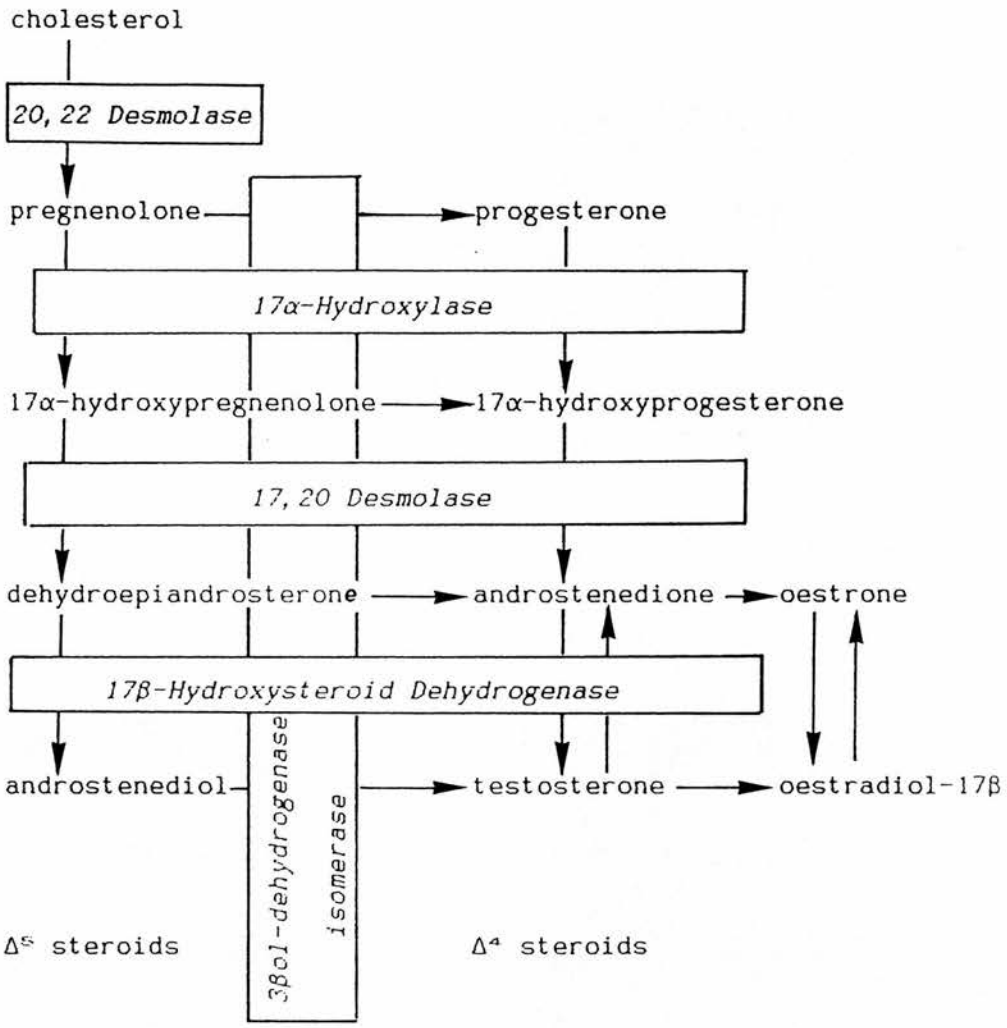


Figure 2.1. Pathways of steroid hormone biosynthesis (enzymes involved in each step are in italics).

Deficiencies in androgen formation

a) Cholesterol desmolase complex deficiency

Deficiency in this enzyme affects both glucocorticoid and testosterone biosynthesis. Glucocorticoid and mineralocorticoid deficiencies are usually severe and the condition is often fatal. Affected males have female (or ambiguous) external genitalia, hypoplastic male genital ducts but no uterus or Fallopian tubes since AMH production is normal.

b) 3β-hydroxysteroid dehydrogenase deficiency

A rare disorder which again affects both adrenal and gonadal function, blocking the conversion of Δ5 to Δ4 steroids. Affected males exhibit incomplete masculinization of external genitalia although Wolffian duct differentiation is normal. Müllerian ducts are absent.

Transmission of the disorder is consistent with determination by an autosomal recessive gene.

c) 17α-hydroxylase deficiency

Patients suffering from deficiency in this enzyme may have normal female external genitalia (with a blind vaginal pouch) or male genitalia with hypospadias. The magnitude of impaired masculinization is correlated with the severity of the block in 17α-hydroxylation and hence the degree of impairment of testosterone synthesis. The testes may be abdominal, inguinal or in the labioscrotal folds. Müllerian ducts are absent and the Wolffian ducts are usually hypoplastic. Other symptoms, such as salt and water retention, are linked to raised concentrations of cortisone and deoxycortisone in the adrenal cortex.

d) 17,20-desmolase (lyase) deficiency

Unlike the previous three enzyme deficiencies which affect synthesis of adrenal corticosteroids as well as androgens, deficiency in this, and the following enzyme, primarily affect testosterone synthesis by the testes. As Figure 2.1 shows, deficiency in 17,20-desmolase lyase results in insufficient androgen formation. Patients have ambiguous genitalia, inguinal or abdominal testes and, usually, show male duct development.

e) 17β-hydroxysteroid dehydrogenase deficiency

Lack of masculinization results from deficiency of this enzyme, which is distributed throughout the body and is the only reversible enzyme reaction involved in testosterone biosynthesis. Patients appear female but have a blind vaginal pouch. At puberty, the voice deepens, muscle mass and hair growth increase and occasionally breast development occurs. Androstenedione and oestrone secretion by the testes increases at puberty to such an extent that their conversion to testosterone and oestradiol in extragonadal sites is sufficient to cause virilization and breast development. Spermatogenesis is impaired due to the lack of testicular testosterone.

f) 5α-reductase deficiency

5α-reductase deficiency is inherited as an autosomal recessive trait and causes feminization due to inability of target cells to convert testosterone to dihydrotestosterone (DHT). Wolffian duct development is not impaired since local concentrations of testosterone are sufficient to overcome competition from progesterone, but, at birth, the affected XY individual has a blind vaginal pouch and a hypospadic phallus. At puberty, in the absence of raised progesterone concentrations, growth of the phallus and body muscle occurs, the testes descend and the voice

deepens, the patient undergoing an apparent "sex reversal". Testosterone levels are normal or elevated and DHT levels remain low.

Disorders in androgen function

a) Testicular feminization

The most common form of male pseudohermaphroditism, this syndrome is an X-linked disorder in which affected XY individuals are phenotypic females. They develop female secondary sexual characteristics at puberty but fail to menstruate. The vagina is blind and no Müllerian structures are present. Testes are abdominal, inguinal or in the labia. Prepubertally, the testes appear histologically normal but postpubertally, the seminiferous tubules are small with few spermatogonia. Leydig cells are hyperplastic.

Administration of methyltestosterone fails to stimulate growth of pubic hair, leading to the original hypothesis that the target organs are insensitive to testosterone. This is supported by the finding that circulating testosterone levels are normal or higher than those of normal men. Studies in testicular feminized mice suggested that the primary defect was a reduced number of androgen receptors for DHT and testosterone (Goldstein and Wilson, 1972). Cultured fibroblasts from the genital skin of affected human males indicated low or undetectable amounts of androgen receptor (Griffin *et al.*, 1976). This defect occurs in the embryo, so that stabilization of the Wolffian duct does not occur, although secretion of AMH is not impaired and the Müllerian ducts regress. At puberty, androgen resistance at the hypothalamic/pituitary level leads to an increase in LH pulse frequency and amplitude with a resultant increase in testosterone secretion by the Leydig cells. Increased oestradiol secretion and the peripheral conversion of testosterone to oestradiol leads to feminization, including breast development, but sexual hair growth, being androgen dependent, is sparse.

There are also cases of partial androgen resistance reported where an XY individual appears as a masculinized female.

b) Persistent Müllerian duct syndrome

Failure of fetal Sertoli cells to secrete AMH leads to persistence of the Müllerian duct and its differentiation into Fallopian tubes and a uterus. The XY individual possesses two normal testes and Wolffian ducts, although the vas deferentia may be attached to, or embedded in the uterine wall. Phenotypically the affected individual is male.

THE FEMALE

Female pseudohermaphroditism, the masculinization of the duct and genitalia in the presence of two X chromosomes and two ovaries, occurs if the individual is subjected to androgens from an extra-gonadal source. The Müllerian duct is not affected since AMH can only be secreted by testicular Sertoli cells, and the degree of masculinization of the external genitalia depends on the stage of differentiation at the time of exposure. Once the vagina has separated from the urogenital sinus (by about the 12th week of fetal life) androgens can only cause clitoral hypertrophy.

Since the adrenal cortex is, in the female, the major source of androgens, overproduction by the adrenocortical cells will result in masculinization. Details of malfunctions of the adrenal cortex are described in Chapter 4.

Ingestion of testosterone or some progestational agents during the first trimester of pregnancy can cause masculinization of the urogenital sinus, but after the 12th week, it is only the clitoris which is affected. Other maternal sources of androgens are virilizing tumours of the adrenal or ovary.

2.3 INTERSEXUALITY IN THE MOUSE

As in other mammals, XX and XO mice are phenotypically female, XY and XXY mice develop as males. However, mice suffer from various abnormalities of sexual differentiation as do humans, including testicular feminization (Goldstein and Wilson, 1972) and masculinization caused by external administration of androgens. The conditions described in this chapter are genetically determined and, as far as is known, exclusive to the mouse. Since this mammal has been so extensively studied in the laboratory, the conditions are well characterised and may throw light on similar conditions in other species.

Y-LINKED SEX REVERSAL CONDITIONS

There are five known Y-linked and two autosomal-linked conditions which interfere with sex determination in the mouse. The Y^{POS} and Y^{ORB} conditions both cause XY individuals to develop as females or hermaphrodites.

Y^{POS}, Y^{ORB} and BALB/cWTt Y

The presence of the Y^{POS} or Y^{ORB} chromosome in a certain inbred strain of mouse seems to cause improper "reading" of the testis determining gene, resulting in the development of varying amounts of ovarian tissue. In ovotestes, ovarian tissue is preferentially found at the cranial end of the gonad and germ cells seem to disappear from the ovarian tissue postnatally, even in the absence of testicular tissue (Eicher, 1982).

BALB/cWt hermaphroditism is caused by non-disjunction of the Y chromosome in some cells during early embryonic development, resulting in XO and XYY cells (Eicher *et al.*, 1980). With a sufficient number of XO cells in the

undifferentiated gonad, ovarian tissue develops, apparently preferentially at the cranial pole. The condition is inherited as a Y-linked trait.

The *Sxr* mutation

This mutation causes XX individuals to develop as males (Cattanach *et al.*, 1971), and was first thought to be an autosomal mutation since it was inherited in a Mendelian fashion, yielding equal proportions of normal XY males, XY*Sxr*-carrying males, normal XX females and sterile XX*Sxr* males. Singh and Jones (1982) then found that a DNA probe specific to the heterologous sex chromosome of a snake, the banded krait, showed a distinct hybridization pattern with XX*Sxr* males as well as with XY male mouse DNA fragments, a pattern not shown with normal female DNA. Furthermore, *in situ* hybridization using this probe showed Y-specific DNA to be present on one end of an X chromosome in XX*Sxr* mice.

Burgoyne (1982) and Eicher (1982) put forward very similar models for the location and transmission of *Sxr* which accounts for the pseudoautosomal transmission of the trait (see Figure 2.2). It seems that the testis determining region in XY*Sxr* mice is duplicated and attached to the distal, pairing region of the Y chromosome. During meiosis, this fragment is transferred to one X chromatid at crossing over, resulting in an X-bearing sperm which also carries testis determining DNA sequences. Because of pseudoautosomal behaviour of loci in the crossover section of X and Y chromosomes, *Sxr* appears to be transmitted as an autosomal trait.

Inactivation of *Sxr*

Since *Sxr* is attached to an X chromosome, inactivation would be expected to affect it in XX individuals. The effect of inactivation can be assessed using Searle's X-autosomal translocation T(X;16)H or T16H, which causes preferential activation of the translocated X chromosome (Lyon *et al.*, 1964). The X*Sxr* chromosome would thus be inactivated in the

presence of the T16H chromosome. T16H/X*Sxr* mice develop as sterile males, hermaphrodites or as fertile females (McLaren and Monk, 1982; McLaren, 1983; Ward *et al.*, 1988). This implies that the *Sxr* region can indeed be inactivated (to produce fertile females) but that inactivation is not complete in all cells, hence the appearance of hermaphrodites. McLaren (1983) postulates that, since inactivation of the X chromosome spreads along the chromosome, the translocated *Sxr* region may be inactivated in some cells but remain active in others, and this is cell heritable. At a critical stage of development (probably at about 11 days *post coitum*) the proportion of cells with the testis determining segment active in the gonad determines whether a testis or an ovary develops. The animal is therefore effectively a mosaic of XX and XX*Sxr* cells. Since about 30% of XY cells in a gonad primordium is enough to ensure development of a normal testis (McLaren, 1983), T16H/X*Sxr* females will be rare.

Gonadal development in XX*sxr* males and hermaphrodites

XX*Sxr* male mice develop testes which are smaller than those of normal male mice, possibly because they are sterile *i.e.* the seminiferous tubules are empty. Germ cells do not undergo spermatogenesis and degenerate soon after birth, presumably because two active X chromosomes do not allow male germ cell development. XO*sxr* male mice, with only one X chromosome in the germ line, undergo spermatogenesis, but are sterile because the sperm are not normal (Cattanach *et al.*, 1971). However, meiotic germ cells are occasionally seen in the testes of XX*Sxr* mice before birth, and they are believed to be XX in chromosome constitution (McLaren, 1980). McLaren (1980) suggests that the meiotic germ cells survive because they are effectively in a mosaic environment where the testis determining sequences are expressed in some cells but not all. The presence of XX meiotic germ cells in testicular tissue may therefore represent the meiosis inducing effect of surrounding somatic tissue rather than the inherent activity of XX germ cells.

Female *Sxr* carrier mice (heterozygotes for T16H), are fully fertile, indicating that the presence of the *Sxr* region in XX germ cells does not prevent oogenesis.

Studies of the gonads of mature hermaphrodite T16H/X*Sxr* mice (Ward *et al.*, 1987 and 1988) revealed that the majority had a male phenotype. They were only identified as hermaphrodites at autopsy. Female genotypes showed some masculinization in the genitalia. Testicular tissue was more likely to be found in the left gonad than the right, and ovotestes were less common than ovaries and testes. An epididymis was associated with testicular development and the testes were abdominal or inguinal. Ovaries usually had an associated uterus and occasionally an epididymis as well, due to the systemic action of testosterone secreted by a contralateral testis.

Normal oocytes were observed in some testicular tubules both in testes and ovotestes. In some ovotestes, the oocytes were surrounded by granulosa-like cells, even in the testicular portion of an ovotestis. However, oocytes were always observed within the seminiferous tubules, never within interstitial tissue, implying that the oocytes were sequestered at an early stage of development, or that intertubular oocytes did not survive.

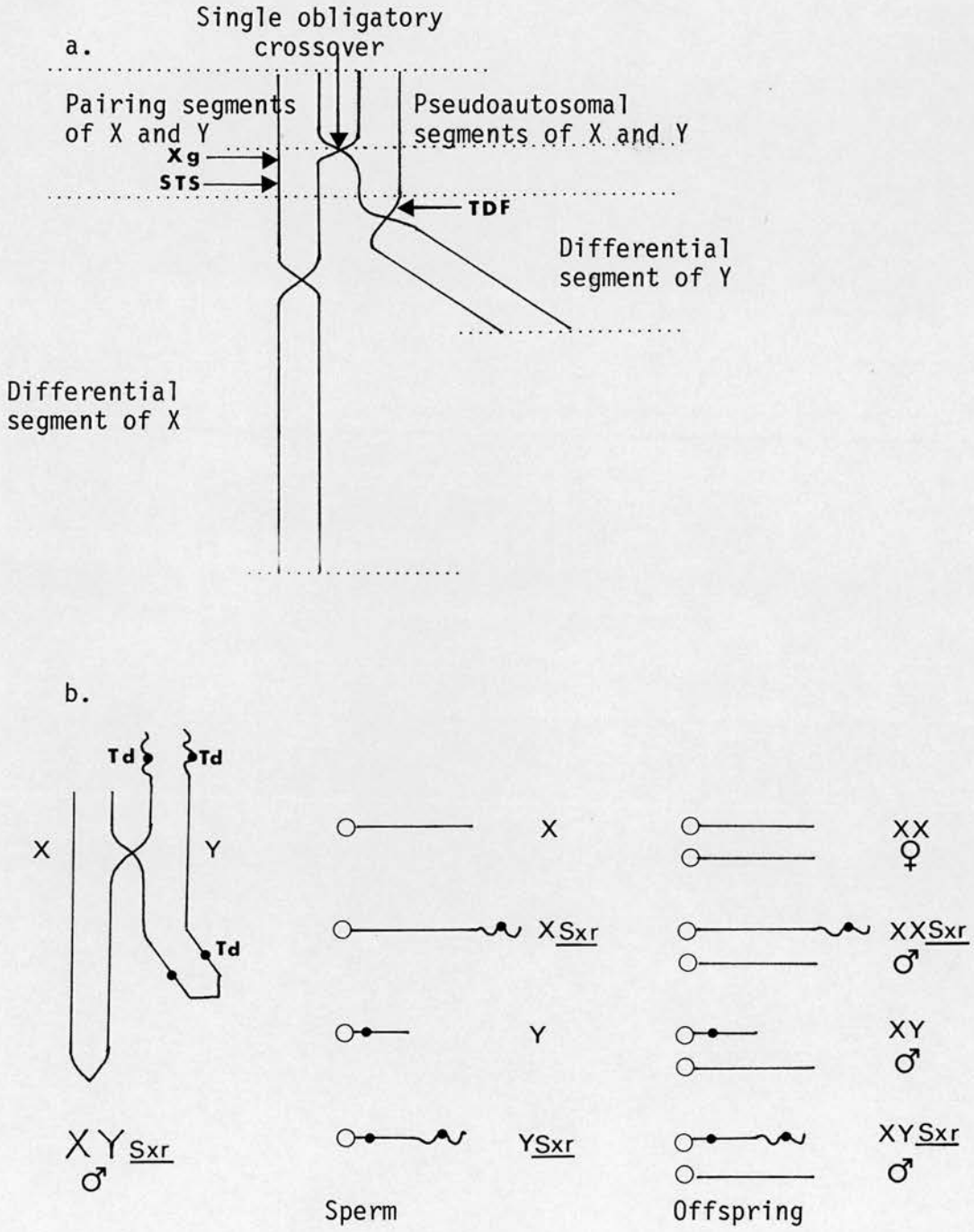


Figure 2.2 a. Burgoyne's X-Y crossover model as exemplified from the human X-Y chromosome pair. b. Sxr interpreted on Burgoyne's X-Y crossover model as the addition of a duplicate of the Y-linked testis-determining region Y(Td) to the distal tip of the Y 'pseudoautosomal' segment. (Burgoyne, 1982)

X-Y translocation

Whilst sex reversed XX mice are the result of crossing over between the X and Y chromosome in an *Sxr* carrier male, Eicher (1982) describes another case of sex reversal caused by translocation of genetic material between the X and Y chromosome. XX males resulted from the translocation of most of the Y chromosome onto the distal region of an X chromosome (X^Y) which was thus cytologically identifiable due to its large size. The XY counterparts possessed one normal sized X chromosome and one very small chromosome containing the centromeric region of the Y chromosome and the distal region of the X (Y^X). XX^Y males were sterile, XY^X females fertile. Obviously the X^Y chromosome carries the testis-determining locus, and the Y^X chromosome does not.

AUTOSOMAL SEX REVERSAL GENES

a) The *Tda-1* locus

Inheritance of an autosomal gene, *tda-1*, with the testis determining gene of *Mus musculus domesticus* Tdy^{DOM}, results in XY fetuses developing as hermaphrodites. It seems that not all strains of mice are affected by the autosomal mutation, and the extent of sex reversal varies (Nagamine *et al., 1987* a and b), from development of an ovotestis to an ovary, but not a testis. The testicular region of ovotestes appears to be concentrated in the mid region of the gonad with ovarian portions at the cranial and caudal poles. BALB/cWt hermaphrodites also show preferential ovarian development at the caudal pole. In the ovarian regions. numerous meiotic germ cells are seen and are also found in seminiferous tubules, although postnatal gonads were found to possess comparatively few or no developing oocytes.

Nagamine *et al.*, (1987a), suggest that a diffusible testis-inducing factor is released by XY cells and the titre of this factor determines the

proportion of ovarian/testicular cells in the gonad. The fetal mesonephros has been implicated in the triggering of the onset of meiosis (Byskov, 1974) and in feminization of a gonad (Byskov and Grinsted, 1981).

b) The *Tas* locus

This autosomal gene is located on chromosome 17 in mice, is inherited as a dominant trait and causes complete or partial sex reversal of XY individuals. As with the *tda-1* gene, ovotestes develop with ovarian tissue predominantly at the cranial end (Washburn and Eicher, 1983). Other loci on chromosome 17 (the *T/t* complex), are also known to be important in the development and fertility of mice (Bennett, 1975), and Kiel-Metzger and Erickson (1984) have shown that the sex-specific Bkm sequences are also localized on the proximal portion of mouse chromosome 17.

2.4 INTERSEXUALITY IN DOMESTIC SPECIES

Freemartin cattle and sheep

This term is usually reserved for cattle, since in this species it is relatively common, but could refer to any species in which sexual development in the female is severely inhibited due to the presence *in utero* of a male twin.

It was Hunter (1779) who first produced a detailed study of the condition although it was recognized some time before - even Roman literature makes reference to *taurae* or *female-bulls* (Marcum, 1974).

The external genitalia of the freemartin are essentially the same as those of the normal female, although vulval hair may be coarser. The reproductive tract shows signs of Müllerian regression and Wolffian duct development, with an epididymis apparent in some animals. The gonads show varying degrees of masculine development, from intra-abdominal ovotestes to inguinal testes (Marcum, 1974; Ali *et al.*, 1981). Spermatogenesis is not observed, but the animals are sterile since the uterus is always adversely affected and is not fully developed.

The cause of the condition lies in the tendency for vascular anastomosis to occur between the placentae of twin fetuses in certain species. A common circulation results, so that "virilizing factors" from the developing male fetus enter the female co-sib circulation at the crucial stages of development. What exactly these virilizing factors are has been a matter for some debate (Marcum, 1974). Whilst male androgens will obviously cause masculinization of external genitalia, repeated attempts to induce freemartinism with injections of testosterone have met with no success, and the H-Y antigen theory could expect little support in the light of findings that testicular differentiation is not dependent on this antigen (see Chapter 5). The presence of XY cells in freemartin cattle points to a "cellular" theory, in other words, the animals are essentially chimaeras of XX and XY cells. Marcum (1974) reviews literature concerning karyotyping of freemartin cattle, the smallest percentage of XY

cells seen in a female freemartin is 1.0%. However, the "cellular" or chimaeric theory does not seem to apply to marmoset monkeys, another species in which vascular anastomosis occurs between twin placentae, and XY cells are found in female co-sibs of male fetuses, but freemartinism does not result (Short, 1982). Why this is so is not known.

A more recent explanation for freemartinism comes from Vigier *et al.*, (1987) who cultured prospective fetal rat ovaries with bovine anti-Müllerian hormone. The result was a reduction in gonadal volume, germ cell depletion and the initial differentiation of Sertoli cells, *i.e.* characteristic freemartinism effects (Jost *et al.*, 1972). It seems possible that it is AMH from the male fetus which causes inhibition of ovarian development and masculinization of the gonad in the female twin. The timing of freemartin development supports this theory, since ovarian inhibition coincides with the onset of Müllerian regression in male fetuses (Jost *et al.*, 1972).

Freemartinism is also reported in sheep, affected female lambs are sterile and often have an enlarged clitoris. Chromosome chimaerism is reported (Breure and Macnab, 1968) and placental vascular anastomosis has been observed (Slee, 1963).

The Intersex goat

These animals are also XX (or very occasionally XX/XY) in chromosome constitution, but do not share the same aetiology as freemartins.

Intersex goats vary in phenotype from "almost" female to "almost" male, with scrotal testes and a penis. In less masculinized animals, the Müllerian ducts are well developed and Wolffian ducts underdeveloped, although an epididymis and vas deferens may be present. The gonads are usually testicular, although Hamerton, Dickson *et al.* (1969) report three cases of true hermaphroditism. Germ cells are not observed at birth, although degenerating germ cells may be present in the seminiferous

tubules in the fetus, at least at 126 days of gestation (Hamerton, Dickson *et al.*, 1969).

The observation that all intersex goats are hornless *i.e.* polled, (Eaton and Simmons, 1939; Kondo, 1955) led Hamerton, Dickson *et al.* (1969) to postulate a genetic mechanism leading to intersexuality. The polled gene *P*, is autosomal and dominant, and apparently affects reproduction in several ways, since polled animals have significantly larger litters than if one or both parents are horned (Soller and Kempenich, 1964) and a higher proportion of intersexes occur among multiple as opposed to single births (Soller and Angel. 1964). According to Hamerton, Dickson *et al.* (1969), the *P* gene (or genes closely associated with this gene) acts like a Y chromosome, inducing an X-borne gene to determine testicular development. This theory follows the supposition that, in a normal XY animal, a gene on the Y chromosome acts on a "male" determiner which is X-borne. The *P* gene in an XX goat therefore acts on this "male" determiner, as would the Y-linked gene. The notion of an X-linked gene being involved in testis determination is supported by Wolf *et al.* (1980 a and b), in connection with H-Y antigen, and the finding that the Y-specific probe detecting the putative *TDF* in man also hybridizes to an X-linked DNA fragment (Page *et al.*, 1987).

Regarding the variable phenotype observed in intersex goats, Hamerton, Dickson *et al.* (1969) suggest that the *P* gene is of variable penetrance, not only in the intersex animals, but also in *PP* males, where some, but not all individuals are sterile (Soller *et al.*, 1963). As yet, there is no evidence to corroborate the genetic theory of Hamerton, Dickson *et al.* although the link between polledness and intersexuality is clear.

Other species

Intersexuality is also reported in horses (Kent *et al.*, 1986) and dogs (Selden *et al.*, 1978; Meyers-Wallen *et al.*, 1987). The condition in horses affects XY animals, which may be phenotypically female (but sterile) or show extensive masculinization. The inherited condition may be

transmitted through the female (X-linked recessive) or male (autosomal or Y-linked with variable expression). Sex reversal in dogs is described in XX cocker spaniels which develop as phenotypic males or as hermaphrodites (Selden *et al.*, 1978). Despite the presence of large amounts of testicular tissue in these animals, the uterine horns persist and Meyers-Wallen *et al.* (1987) have found that this is not due to insufficient production of AMH by testicular tissue, but is possibly due to a deficiency in AMH receptors.

CHAPTER 3 INTERSEXUALITY IN PIGS

3.1 INTRODUCTION

In 1925, Baker described nine "sex-intergrade" pigs which varied in phenotype from "almost female" to an animal with two scrotal testes and upturned vulva. In his paper, Baker mentions that farmers called these animals "wildews" or "wilgils", suggesting that the condition was well recognised amongst pig producers. The name "wilgil" or "Jenny Willick" is still used, at least by Scottish farmers, to describe intersex pigs. One of the animals described by Baker came from the New Hebrides (now Vanuatu), and he later went to study these animals in detail, the islands being a rich source of material since intersex pigs were prized for use in religious ceremonies (Baker, 1928).

Since Baker's first descriptions, there have been several studies conducted on intersexuality in pigs, possibly because the condition is relatively common in these animals compared with other species. Estimates for the incidence of intersexuality in populations of domestic pigs range from 0.2% in Sweden (Bäckström and Henricson, 1971), 0.5% in the Netherlands (Breeuwsma, 1970), 0.5-1.0% on a Scottish farm (Hunter *et al.*, 1982) up to 28% reported on a South African farm (Gerneke, 1973). Perhaps another reason for the interest in intersex pigs is the wide range of anomalies seen, from partial masculinization of the genitalia due to the presence of an ovotestis, to those cases in which two scrotal testes are found.

Before reviewing the literature on intersex pigs, a brief summary of the timing of events in embryonic development of the gonads in the pig will be given.

3.2 EMBRYOLOGY OF THE GONADS

Pelliniemi's description (1975) of the early embryogenesis of the testis and ovary in the pig, is the most detailed for pinpointing gonadal differentiation. At 26 days after artificial insemination, the male gonad in the fetus was identifiable due to the presence of testicular cords which were absent from the female gonad. Primordial germ cells are identified within the cords at this stage and are present in the indifferent common blastema at 24 days, but their exact time of arrival at the gonadal ridge is not known.

At day 26 the gonads appear as longitudinal protrusions along the medial mesonephric surfaces. In the pig, the mesonephros is particularly large at this stage (Patten, 1948), as in other eutherians, it is the embryological source of the urogenital ducts.

Pelliniemi (1975) stated that testicular differentiation is initiated prior to Leydig cell development, inferring that these cells do not play a role in early differentiation, The earliest sign of testicular differentiation was found to be the development of testicular cords, although these are perhaps better described as sheets (Pelliniemi, 1975). These cords do not extend right to the centre of the gonad, the area thought to be the future rete testis. The tunica albuginea can also be identified early on, apparently before Leydig cell differentiation.

Testosterone secretion by the Leydig tissue is reported to occur as early as day 34-39 *post coitum* (Raeside and Sigman, 1975), falling off and then rising again in older fetuses. This pattern of testosterone secretion is matched by observations of Leydig cell growth *in vitro*, mature Leydig cells representing a high percentage of interstitial tissue at crown-rump lengths of 4.5cm and 28cm, corresponding to 39 and 89 days *post coitum* respectively (Moon and Hardy, 1973). Measurement of testosterone in serum from the umbilical artery of male pig fetuses also showed an increase in concentration around day 35 *post coitum* (Ford *et al.*, 1980). Whilst a peak in fetal testosterone secretion seems to occur around day 35, there is evidence that testes of even younger fetuses are capable of steroid biosynthesis; Moon and Raeside (1972) report that hydroxysteroid

dehydrogenase activity is observed within the sex cords of 26-30 day old fetuses.

The early wave of Leydig cell development (around day 34) is apparently pituitary independent (van Vorstenbosch *et al.*, 1982) whereas after day 42, decapitation of the fetus affects Leydig cell development; the tissue is estimated to become pituitary dependent between days 60 and 72 *post coitum* (van Vorstenbosch *et al.*, 1984).

During the time of testicular differentiation, (day 24-27), the female gonad remains devoid of any conspicuous morphological differentiation. Primordial germ cells are present throughout the gonad, primordial follicles first being observed around 68 days *post coitum*. Prior to this, in the differentiated ovary, most of the germ cells become clustered into "egg nests". Near the time of birth, secondary follicles are common, but antral follicles are not observed in pigs younger than 60 days old (Oxender *et al.*, 1979).

Before birth, the ovaries and testes migrate from their mesonephric position. The testes descend to the scrotal pouch through the inguinal canal. taking with them the epididymides and associated vasa deferentia, and also a portion of peritoneum termed the processus vaginalis. Testicular descent occurs because of several factors. Firstly, testicular growth and mesonephric degeneration means that the testes grow to fill the space vacated by each mesonephros. A ligament (the gubernaculum) attached to the caudal pole of the testis, passes through the inguinal canal, and is thought to exert a traction on the gonad because of an increase in its size within the extra-abdominal portion. This gubernacular "swelling" brings about a displacement of the testis towards the internal inguinal opening (Wensing, 1968, 1973 a and b; Wensing and Colenbrander, 1973).

As the ovaries increase in size, the gonads and Müllerian ducts sag progressively further into the body cavity, moving caudally, laterally and ventrally. The peritoneum surrounding the gonads and ducts is thus stretched, becoming reinforced by fibrous tissue and constituting a supporting ligament and a pathway for the nerves and blood vessels (Patten, 1948).

3.3 THE EXTERNAL GENITALIA, MORPHOLOGY OF THE REPRODUCTIVE TRACT AND HISTOLOGY OF THE GONADS IN INTERSEX PIGS.

Intersex pigs are usually identified amongst a herd by the masculinized appearance of the external genitalia. The vulva is often upturned, described as "fish-hook" (Baker, 1925; Pond *et al.*, 1961), the clitoris enlarged and, in some cases, protruding from the vulva (Hunter *et al.*, 1982) and, in animals with at least one testis, this gonad may be scrotal.

Other identifying features of intersex pigs include masculine behaviour such as mounting of gilts on heat, and frothing at the mouth, the salivary glands producing male pheromones (Johnston *et al.*, 1958; Gerneke, 1973; Booth and Polge, 1976), the presence of a mid-ventral penile sheath, toughened skin, and a characteristic urination pattern caused by the upturned vulva (Hunter *et al.*, 1982, 1985 and 1988).

Most intersex pigs possess a morphologically normal uterus, although the urethral opening may be positioned such that urine could pass back into the uterus, distending the uterine horns (Johnston *et al.*, 1958). Proximal to the gonads, the reproductive tract is more influenced by the gonadal tissue so that, adjacent to a testis, the upper portion of the oviduct may not be apparent. The Wolffian duct develops in the presence of testicular tissue, and an epididymis is often apparent. Johnston *et al.* (1958), also found prostate and bulbourethral glands and seminal vesicles in some intersexes, and Pond *et al.* (1961) described a case with a vas deferens running parallel to a uterine horn.

Gonads in intersex pigs vary in the amount of testicular tissue present, so that some authors classify animals as pseudo- or true hermaphrodites, the former possessing no ovarian tissue (Gerneke, 1973). In the case of "true hermaphroditism", testicular tissue more often occurs in the right hand gonad (Breeuwsma, 1970; Hunter *et al.*, 1982 and 1985), in common with hermaphrodite humans (Van Niekerk and Retief, 1981) but apparently differing from hermaphrodite mice (Ward *et al.*, 1987).

Histological examination of the gonads shows that the testicular tissue consists of tubules which are lined by Sertoli-like cells (Krishnamurthy *et al.*, 1971), although Hunter *et al.* (1982) note that these cells are

paler-staining than their counterparts in a normal pig testis. Germ cells are not observed in these tubules, although Krediet (1939) claims that spermatogonia may be found in testicular tubules of new-born animals, but that these degenerate during puberty. Certainly, no spermatozoa are seen in the gonads of adult intersex pigs. There is clear demarcation between the ovarian and testicular portion in ovotestes, (Krishnamurthy *et al.*, 1971) and within this ovarian tissue (and in the ovaries of intersex pigs) follicles are observed. Ovulation may occur (Gerneke, 1967) and intersex pigs can show oestrus (Hunter *et al.*, 1982 and 1985) and become pregnant (Scofield *et al.*, 1969). However, Krishnamurthy *et al.* (1971) noted that many follicles within the ovarian part of an ovotestis appeared atretic and primordial follicles tended to be sparse. Hunter *et al.* (1985) failed to induce a response in follicular growth in ovarian tissue adjoining testicular tissue, by giving a systemic injection of PMSG (pregnant mare's serum gonadotrophin).

3.4 THE KARYOTYPE OF INTERSEX PIGS

Most descriptions of intersex pigs include some reference to the genetic sex of these animals. In almost all cases, blood leucocyte or kidney or liver culture reveals a normal female 38XX karyotype (Johnston *et al.*, 1958; Gerneke, 1967; Breeuwsma, 1970; Bäckström and Henricson, 1971; Basrur and Kanagawa, 1971; Melander *et al.*, 1971; Hunter *et al.*, 1982 and 1985). Basrur and Kanagawa (1971) also karyotyped testicular tissue and state this was of an XX karyotype. There are a few exceptions in which XX/XY chimaerism is found (Bosma *et al.*, 1975; Toyama. 1974) or an XXY karyotype (Breeuwsma, 1968). In the case of blood leucocyte chimaerism, the investigators suggest that placental anastomosis has occurred, allowing the interchange of XX and XY leucocytes between adjoining fetuses resulting in intersexuality, as occurs in freemartin cattle. However, Breeuwsma (1970) found that an intersex fetus could occur flanked *in utero* by two female fetuses, thus excluding the possibility of anastomosis between an XX and XY fetus. The placental morphology in the pig also indicates that placental anastomosis is rare, since the tips of the placenta regress. Hughes (1929) gives evidence of anastomosis between the placentae of two pig fetuses, but the resultant affected female did not show masculinization of the gonads, but rather a lack of female development. Breeuwsma (1970) attempted to show vascular anastomosis by injecting dye into blood vessels of a male fetal placenta adjoining an intersex, but no link was found between the two circulations.

Inheritance of the condition

Observations on the incidence of intersexuality in pig herds indicate that the condition is a hereditary characteristic. Breeuwsma (1970) found that some boars were more likely to have intersex offspring than others. Laus *et al.* (1984) bred from one sire and four dams and produced a frequency of 15.2% intersexes amongst the offspring. Analysis of breeding records have led Sittman (1973) and Sittman *et al.* (1980) to suggest inheritance

of the condition through autosomal recessive genes. Laus *et al.* (1984) agree that a recessive autosomal mutation may be responsible for sex reversal in intersex pigs, possibly through the expression or H-Y antigen in the absence of a Y chromosome. However, as discussed in a later chapter, H-Y antigen is probably not involved.

3.5 THE AETIOLOGY OF INTERSEXUALITY IN PIGS

Since freemartinism in pigs is generally not accepted as being the cause of intersexuality, other suggestions have been made, amongst them, crowding of fetuses, adrenal influence and pineal gland involvement.

Breeuwsma (1970) found a correlation between litter size and incidence of intersexuality, treatment with PMSG increasing litter size with a concomitant rise in incidence of intersexuality. Breeuwsma concluded that an increase in litter size caused increased crowding *in utero*, resulting in brief contact between embryos prior to attachment, and development of intersex gonads due to a "freemartin" effect, although other factors were also involved, and the effect of the male embryos on the females did not extend to an exchange of cells. However, Sittman *et al.* (1980) suggest that litter size does not directly affect the incidence of intersexuality, but influences the detection of intersexes, and that environmental factors (crowding, hormones) are not as important as genetic factors.

The close proximity of the developing adrenal gland and embryonic gonad, and the known masculinizing effect of adrenocortical secretions, has led several authors to suggest an involvement of the adrenal gland in intersexual development (Gerneke, 1967; Breeuwsma, 1970). However, as Gerneke points out, the adrenogenital syndrome (CAH) in humans is known to affect the genital tubercle and urogenital sinus rather than the genital ducts and gonads. The asymmetry of adrenal position would help explain the gonadal asymmetry in intersex pigs, and for this reason Breeuwsma (1970) supports the notion of adrenal involvement, but in combination with the "crowding" effect and a genetic predisposition to the condition.

The pineal gland, usually associated with seasonal breeding, is also implicated in the aetiology of intersexuality. Gerneke (1967) found that hypertrophy of the pineal gland occurred in intersex pigs, and suggested that this may be related to hypertrophy of the adrenal cortex, which has also been observed. However, other studies of the adrenal gland do not support these findings, and whilst pineal tumours are known to delay sexual development in children, the role of the pineal gland in intersex pigs is not known.

CHAPTER 4 INTERACTIONS BETWEEN THE ADRENAL GLANDS AND THE GONADS

4.1 ADRENAL EMBRYOLOGY AND FUNCTION

The adrenal glands, situated at the anterior surface of each kidney, are described as compound endocrine glands since they are derived from different embryonic tissues. The adrenal cortex develops from lateral mesoderm, whereas the medulla is derived from the neural crest, in close contact with the sympathetic nervous system. Links with the nervous system are maintained and the adrenal medulla secretes the catecholamines adrenaline and noradrenaline under sympathetic nervous stimulation.

In the mammalian embryo, the adrenal cortex, mesonephros and gonads develop in close proximity. Upadhyay and Zamboni (1982), studying the relationship between mesonephros, adrenal cortex and gonads in sheep fetuses, concluded that adrenocortical cells are of mesonephric origin. Since the mesonephros is also thought to be the source of granulosa cells in fetal mouse ovaries (Byskov and Lintern-Moore, 1973,), Sertoli cells in fetal mouse testes, and forms part of the genital tract in sheep (Zamboni and Upadhyay, 1982), the relationship between gonadal and adrenal development is apparent. This common embryonic source would help to explain the occurrence of adrenocortical tissue in sites other than its usual location *eg.* in the rabbit ovary (Mori and Matsumoto, 1974) and the ovaries of adrenalectomised 13-lined ground squirrels (Chester Jones and Henderson, 1963; Seliger *et al.*, 1966).

The adrenal cortex is composed of three distinct regions; the capsule, zona glomerulosa and zona reticularis-fasciculata (sometimes classified as two distinct regions, the fasciculata exterior to the reticularis). Since each zona possesses a characteristic enzyme profile, the sub-compartments of the adrenal cortex can function as separate units. The zona glomerulosa secretes aldosterone (involved in regulating salt and water balance in the body) whereas the reticularis-fasciculata produces glucocorticoids (cortisol being the main product in most mammals), androgens (principally androstenedione and dehydroepiandrosterone) and small amounts of progesterone and oestrogens, at least in humans (Tyrrell

and Forsham, 1983). Figure 4.1 shows the relationship between corticosteroids secreted by the adrenal cortex.

In the pig, measurements of adrenal products in the adrenal venous blood of stressed animals shows that concentrations of 11α-hydroxyandrostenedione, pregnenolone, progesterone and 11β-hydroxyprogesterone are raised (Heap *et al.*, 1966). Segal and Raeside (1975) found androstenedione to be the principal androgen present in male and female fetal pig adrenals. Analysis of urine from male and female pigs shows sulphated androgens to be the principal secretory products, presumably of testicular and adrenal origin (Cook *et al.*, 1987).

The secretion of sex steroids such as progesterone and androgens by the adrenal gland obviously has implications in gonadal function and in sexual development. However, this is not the only way in which the adrenals and gonads interact since adrenal glucocorticoids also act on the regulation of the so-called "gonadal axis" i.e. the hypothalamic-pituitary gonadal relationship.

Firstly, stress increases adrenal secretion of glucocorticoids and is known to affect reproduction in many animals, including humans (Peyser *et al.*, 1973), possibly acting to regulate population size of some wild animals (Christian *et al.*, 1965).

Secondly, various adrenal diseases diminish fertility in the affected individual due to raised concentrations of cortisol eg. as in Cushings Syndrome (White *et al.*, 1981).

Thirdly, the adrenal gland is thought to modulate events at puberty (Ramaley, 1974) possibly via an influence of corticosteroids.

Regulation of adrenal function

Cells in the median eminence of the hypothalamus secrete a polypeptide, corticotrophin releasing factor (CRF), which passes to the anterior pituitary by means of the hypophyseal portal vessels. CRF initiates release of ACTH from the anterior pituitary which in turn regulates

steroid secretion by the zona reticularis and fasciculata (Symington, 1969).

There are at least three factors determining pituitary secretion of ACTH:

1) Circadian rhythm (Symington, 1969; Krieger *et al.*, 1971).

2) Stress responses originating in the central nervous system. Stress, physical or psychological induces hypothalamic release of CRF and hence an increase in ACTH secretion (Ganong *et al.*, 1974; Ganong, 1980) and cortisol (Plumpton *et al.*, 1969).

3) Feedback inhibition. Glucocorticoid feedback inhibition occurs at the hypothalamus and pituitary. Prolonged glucocorticoid administration eventually leads to suppression of CRF and ACTH release and atrophy of the zona fasciculata and reticularis. The hypothalamus and pituitary fail to respond to stress (Tyrrell and Forsham, 1983).

Figure 4.2 shows the pathways of adrenal steroid hormone biosynthesis, summarised in Figure 4.3.

The interaction between glucocorticoids and gonadal function will be considered first.

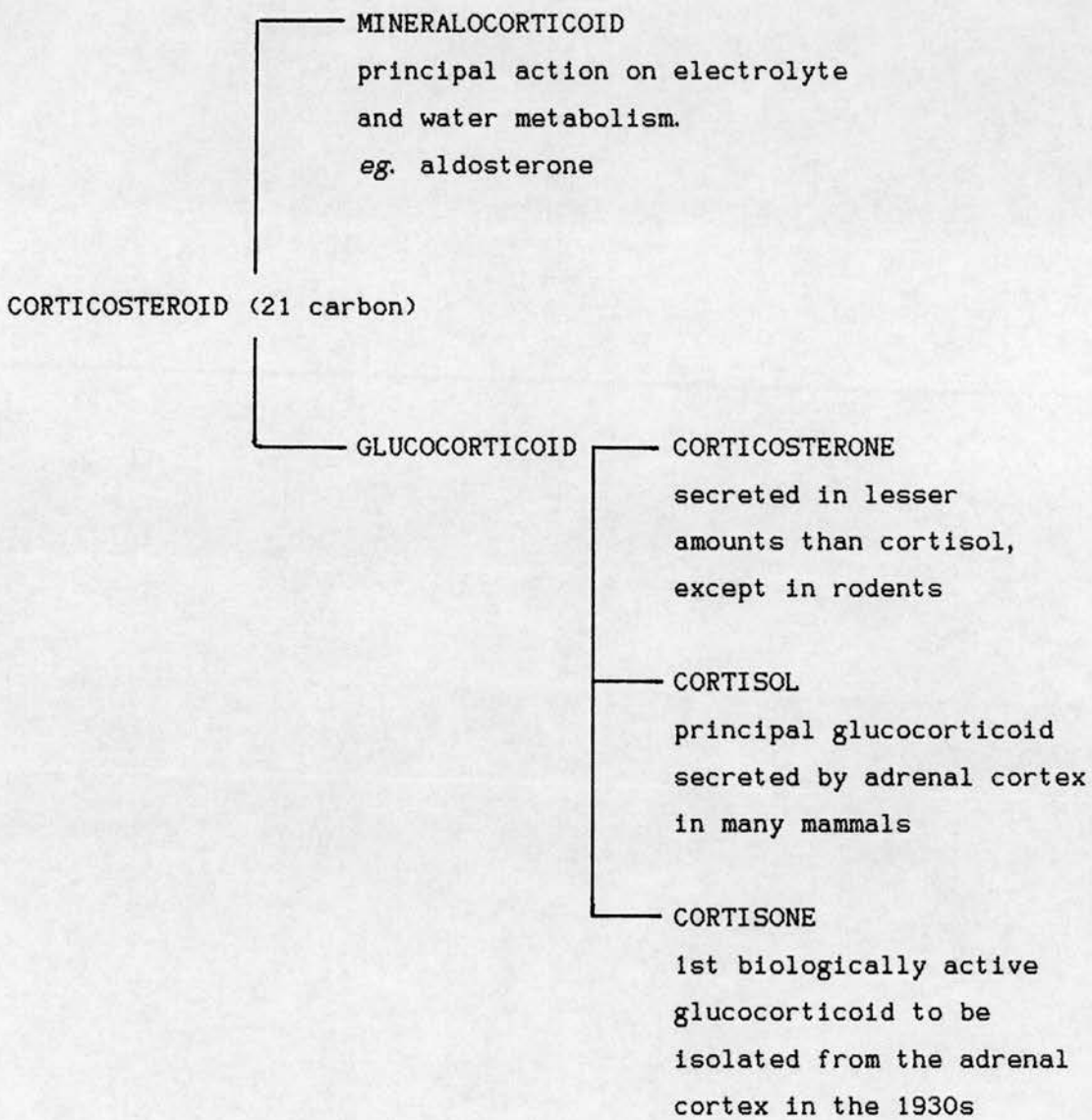


Figure 4.1. Summary of the corticosteroids secreted by the adrenal cortex.

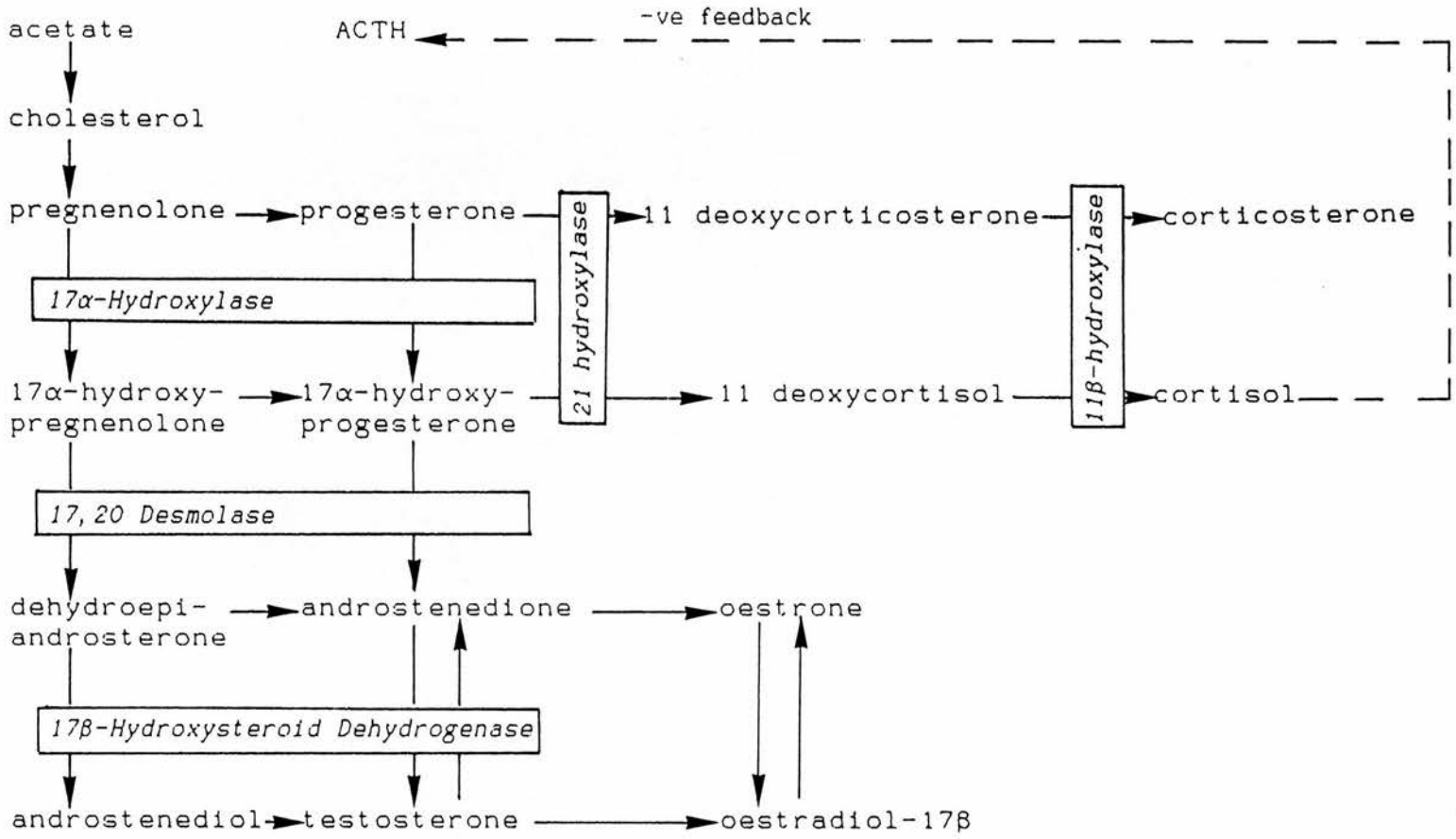


Figure 4.2. Pathways of adrenal steroid hormone biosynthesis.

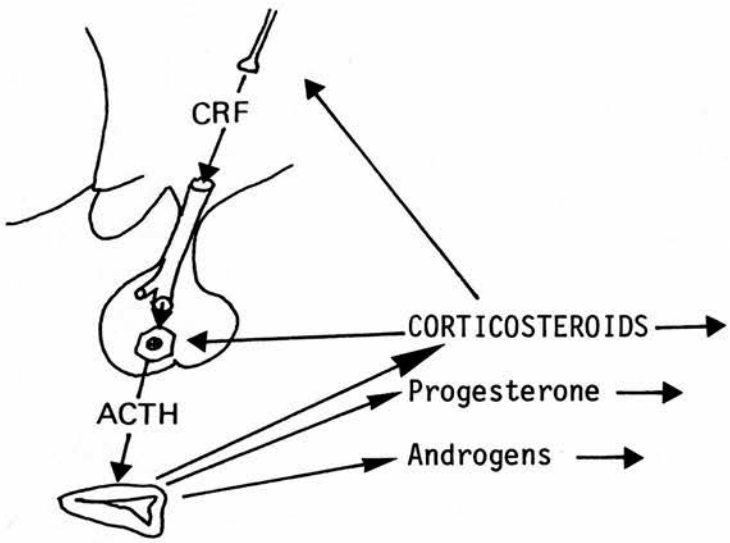


Figure 4.3. Diagrammatic representation of the hypothalamic-pituitary-adrenocortical axis indicating the major hormones involved.

4.2 GLUCOCORTICOIDS AND THE GONADAL AXIS

In the non-stressed animal, corticosteroids (i.e. glucocorticoids and mineralocorticoids, see Figure 4.1) are essential in the regulation of metabolism, immunity, lactation and possibly reproduction (Tyrrell and Forsham, 1983). Under stress, ACTH secretion increases with a concomitant rise in glucocorticoids - cortisol being the principal "marker" used to determine a stress response in mammals. It has been known for many years that fertility is diminished in animals under stress (Selye, 1939), indicating that adrenal secretions are capable of suppressing gonadal function. This suppression can occur at the level of the hypothalamus and pituitary or at the gonad, glucocorticoids or ACTH having a direct effect on gonadal function.

Interactions between the adrenal axis and gonadotrophins

Moberg (1987) reviewed adrenal influence on gonadal function, including the effect of glucocorticoids on gonadotrophin secretion, in detail. This topic will not be considered here. Moberg concludes that the pre-ovulatory LH release can be suppressed by ACTH or corticosteroid administration, basal LH and perhaps FSH secretion being affected if the corticosteroid (here referring principally to cortisol) concentrations are raised for a prolonged period. Examples of suppression of the pre-ovulatory LH release come from rats treated with dexamethasone (a synthetic cortisol), (Baldwin and Sawyer, 1974), cows (Stoebel and Moberg, 1982) and humans (Cunningham *et al.*, 1978). As to the effects of long term elevated plasma glucocorticoid concentrations, patients suffering from Cushing's syndrome (in which cortisol concentrations are raised due to adrenal hyperplasia or overproduction of ACTH, Liddle, 1967) have subnormal levels of circulating LH and FSH (White *et al.*, 1981). Such an effect on the gonadotrophins could be operating at the hypothalamic or pituitary level. Again, Moberg (1987) reviews literature on a variety of species investigating both possibilities. Evidence from the rat indicates

that the adrenal hormones may alter gonadotrophin secretion through the gonadal steroid "feedback" mechanism. Baldwin (1979) suggested that in this species glucocorticoids block oestrogen sensitisation of the pituitary, thereby preventing the pre-ovulatory LH surge. In ovariectomised sows, pretreatment with glucocorticoids prevented exogenous oestrogen from inducing oestrous behaviour (Ford and Christenson, 1981; Barb *et al.*, 1982)

The direct effects of ACTH/glucocorticoids on the gonads

Evidence exists for a direct effect of glucocorticoids and ACTH on the testis and ovary, *i.e.* an effect not mediated via gonadotrophins or adrenal androgen secretion.

a) The Testis

McKenna *et al.* (1979) found that some patients suffering from Cushing's syndrome had reduced testosterone levels despite normal levels of gonadotrophins. This suggests a direct effect of glucocorticoids on testicular secretion without the involvement of gonadotrophins. It seems that elevated corticosteroids act at the cell membrane altering LH binding and hence reducing LH induced testosterone secretion from the testis (Bambino and Hsueh, 1981). Dexamethasone also apparently blocks 17α-hydroxylase activity in testicular cell cultures, leading to reduced 17α-hydroxyprogesterone and testosterone production (Welsh *et al.*, 1982). This reduced testosterone secretion in the face of elevated cortisol concentrations explains the decreased response to HCG (human chorionic gonadotrophin) observed in rats under stress (Charpenet *et al.*, 1982).

There are, however, reports that administration of ACTH can result in elevated plasma testosterone concentrations in pigs although prolonged exposure to ACTH (eg. 5 days) results in testosterone and DHA suppression

(Liptrap and Raeside, 1968; 1975). Within 15 minutes of an intravenous injection of ACTH, boars were found to show a marked increase in plasma testosterone concentration (Liptrap and Raeside, 1975; Hahmeier *et al.*, 1980; Juniewicz and Johnson, 1983) without an increase in LH concentrations. This rise in testosterone was not seen in castrate animals (Juniewicz and Johnson, 1981). Liptrap and Raeside (1975) suggest that the testosterone response to ACTH is brought about by raised cortisol concentrations since an injection of cortisol caused an increase in testosterone concentration. However, Pitzel *et al*, (1979), found that adrenalectomised boars also showed raised testosterone concentrations following ACTH administration, indicating that corticosteroids are not involved, and Juniewicz and Johnson (1984) could not induce a testosterone response in boars with cortisol or progesterone administration. Juniewicz and Johnson (1984) therefore discount the possibility that progesterone produced by the adrenal in response to ACTH administration is acting as a precursor for testosterone production by the testis. In fact Ahmad and Gower (1968) obtained very small yields of testosterone from boar testis minces incubated with progesterone, consistent with findings that the $\Delta 5$ pathway is utilised preferentially in the boar in the biosynthesis of testosterone (Nakajin *et al.*, 1981). Juniewicz and Johnson (1984) point out that administration of ACTH may be inducing pharmacological concentrations of adrenal steroids since the adrenal response to stress (determined by increased levels of adrenal steroids) was of a lesser magnitude and duration than the response to ACTH. Baldwin and Stephens (1973) obtained similar results in that environmental factors did not elevate corticosteroids to the same magnitude as did an ACTH injection. Consequently, testicular secretion of testosterone was not stimulated to the same extent. The reasons for this may lie in the effects of stress-induced elevated catecholamines (secreted by the adrenal medulla) causing vasoconstriction in testicular blood vessels (Setchell *et al.*, 1966) thus counteracting the effects of elevated endogenous ACTH. Alternatively, endogenous ACTH release in a stressed animal and the subsequent rise in plasma cortisol may not be sufficient to induce the increase in testicular testosterone secretion seen in ACTH treated animals. However, the physiological effects of stress on the suppression of testicular testosterone secretion are

documented for man (McKenna *et al.*, 1979), rats (Charpenet *et al.*, 1982), and domestic animals (Moberg, 1987). In the case of rats, the effect has been shown *in vitro* using testicular cell cultures (Charpenet *et al.*, 1982), showing that the effects are not mediated by changes in testicular blood supply.

b) The Ovary

Administration of ACTH or cortisol to cows blocks the pre-ovulatory release of LH (Stoebel and Moberg, 1982) indicating an effect of glucocorticoids at the level of the hypothalamus/pituitary, There is evidence, *in vitro* and *in vivo*, that glucocorticoids may act at the level of the ovary. Hsueh and Erickson (1978) found that glucocorticoids inhibited the synthesis of oestrogen by cultured rat granulosa cells, apparently by binding with receptors to inhibit FSH induction of steroid synthesising enzymes (Schreiber *et al.*, 1982).

Whilst oestrogen synthesis is suppressed, that of progesterone is enhanced by glucocorticoids in granulosa cell culture (Adashi *et al.*, 1981). Women treated with dexamethasone show decreased steroid secretion, except for progesterone (Lachelin *et al.*, 1979). Cows treated with ACTH show an increase in progesterone secretion initially, (Da Rosa and Wagner, 1981) followed by inhibition of secretion by the corpus luteum. Infused hydrocortisone also caused a drop in plasma progesterone concentration and Da Rosa and Wagner (1981) suggested that stress could inhibit corpus luteum function in cows.

4.3 ADRENAL STEROIDS OTHER THAN GLUCOCORTICOIDS

As Figure 4.2 shows, the adrenal cortex secretes progesterone and testosterone, two steroid hormones which could affect the hypothalamic/gonadal axis and, in the case of testosterone, possibly sexual differentiation. In fact, under physiological conditions, there is little evidence that either of these hormones has a significant effect on the gonadal axis. However, there are pathological conditions in which adrenal secretion of testosterone is elevated, either because of pituitary tumours or enzyme deficiencies. If this occurs in the developing female fetus, sexual development is severely affected. In the male, excessive testosterone production is less noticeable but still not without effect.

ADRENOGENITAL SYNDROME

This name covers a variety of conditions brought about by hyperfunction of the adrenal cortex either through adrenal tumours or enzyme deficiency. Baulieu *et al.* (1967) consider four divisions of the syndrome, virilizing or feminizing tumours, congenital adrenal hyperplasia and an ill defined group of patients with hirsutism due to adrenal cortex hyperfunction not fitting into other groups. Of these divisions, congenital adrenal hyperplasia is (in humans) the most common syndrome.

Congenital adrenal hyperplasia (CAH)

As the name implies, this condition is inherited, as an autosomal recessive trait, and induces adrenal hyperplasia due to enzyme deficiencies interrupting the normal feedback mechanism between the adrenal gland and hypothalamus/pituitary gland.

The amount of ACTH produced by the anterior pituitary gland depends upon the amount of cortisol in the circulation. If cortisol concentrations rise, ACTH output falls due to a negative influence acting either directly on the pituitary or via the neurosecretion of corticotrophin releasing factor (CRF) from the hypothalamus. If cortisol production is diminished due to lack of an enzyme in the adrenal cells (Figure 4.2), ACTH secretion rises. This results in an accumulation of steroids immediately preceding the enzyme block. These steroids, which include testosterone, may affect development of the genital tract. The conditions described in the following sections are those in which testosterone production is increased (because of adrenal malfunction), and as such have a more profound effect in female individuals than in males, causing premature sexual development in the latter (Baulieu *et al.*, 1967). However, the biosynthetic pathway shown in Figure 4.2 is common to the adrenal and the testis, deficiencies in enzymes required for testosterone production will result in incomplete masculinization of the genital tract in males, primarily due to testicular malfunction, resulting in male pseudohermaphroditism, Jaffe, 1978).

21-Hydroxylase deficiency

Deficiency of the enzyme 21-hydroxylase is the most common condition found in congenital adrenal hyperplasia. As Figure 4.2 shows, a lack of this enzyme leads to reduced circulating cortisol concentrations and hence an increase in pituitary ACTH release, since there is no negative feedback. As a consequence there is hyperplasia of the adrenal gland and a build up of 17α-hydroxyprogesterone. Androgen production increases, and since (in humans) the adrenal cortex is capable of secreting adrenal androgens by 11.5 weeks of gestation (Villee, 1973), excessive androgen production in the female fetus will cause virilization of the external genitalia which are dependent on testosterone for male differentiation. In humans, the clitoris is known to be sensitive to the masculinizing influences of androgens throughout fetal life (New *et al.*, 1983), but by the 12th week of gestation the vagina has fully separated from the

urogenital sinus and is therefore no longer responsive to the virilizing influence of androgens. However there are reported cases of patients suffering from CAH in whom masculinization has affected the urogenital sinus i.e. the bladder and uterus empty into a common sinus (Biglieri and Kater, 1987), indicating that, despite the timing of onset of adrenal function, the adrenal androgens can cause masculinization of the tract as well as the clitoris. Since testes are absent in female fetuses with CAH, anti-Müllerian hormone is also absent and the uterus and Fallopian tubes develop normally.

11β-Hydroxlase deficiency

Deficiency of this enzyme again causes increased ACTH secretion due to the reduced concentrations of circulating cortisol. Virilization occurs due to overproduction of androgens as in 21 hydroxylase deficiency. Overproduction of 11-deoxycorticosterone results in hypertension (Jaffe, 1978)

Other adrenal enzyme defects are either fatal (3β-hydroxysteroid dehydrogenase) or do not cause virilization and are not considered here (see Symington, 1969).

Adrenal Tumours

Novak and Woodruff (1967), describe conditions in women in which tumours develop from what are apparently adrenal cells in the ovary. Due to the excessive secretion of androgens by the adrenal tissue, virilization of the individual results. Baulieu *et al.* (1967), reported that virilizing tumours occurred in females after birth and caused hirsutism, clitoral hypertrophy and menstrual disorders. Feminizing tumours are rare (in humans), causing breast development in men due to increased oestrogen secretion.

CHAPTER 5 H-Y ANTIGEN

In 1955, Eichwald and Silmser found that, in certain inbred strains of mice, male tissue grafts were rejected when transplanted onto female mice. Since the only genetic difference between the sexes in such an inbred line is the presence of the Y chromosome in the male, Billingham and Silvers (1960) suggested that a gene on this chromosome coded for a weak histocompatability antigen, which they named H-Y antigen (or H-Y) and that it was this antigen which caused graft rejection. Since histocompatibility antigens are detected on the cell surface, H-Y antigen is regarded as a plasma membrane protein.

5.1 H-Y ANTIGEN AND SEX DETERMINATION

With the development of serological methods (Goldberg *et al.*, 1971; Scheid *et al.*, 1972), detection of H-Y antigen in non-inbred species became possible. Mouse antibody directed against H-Y antigen of the same species also recognized the antigen on male cells of other species, including rat lymph node cells and human leucocytes (Wachtel *et al.*, 1974) implying evolutionary conservation of the antigen. Tests on birds using mouse H-Y antibody indicated that the antigen was associated with the heterogametic sex, since ZW females typed H-Y positive and ZZ males were negative (Wachtel *et al.*, 1975).

The persistence of the H-Y antigen across different species, and the observation that, in mammals, testicular development was always associated with expression of the antigen (Wachtel *et al.*, 1976) led to the proposal that H-Y antigen functioned by directing the indifferent embryonic gonad to develop into the mature gonad typifying the heterogametic sex, i.e. the testis in mammals and the ovary in birds. Testicular feminized mice also typed as H-Y positive (Bennett *et al.*, 1975) indicating that sensitivity to androgens is not necessary for expression of H-Y antigen. H-Y antigen was thus hypothesised to be the

mammalian "testis organizer", a product of the Y linked testis determining gene, *Tdy* in the mouse, *TDF* in man (Wachtel *et al.*, 1975b).

To explain the mode of action of H-Y antigen in testis organization, Ohno (1977) proposed that the first step is tubule formation resulting from cell to cell contact, initiated by the binding of H-Y antigen to its receptor site. The conformational change induced by this binding serves to signal the increased synthesis and dissemination of H-Y antigen which then drives out the other organogenesis directing proteins from the anchorage sites of the surrounding gonadal cells. Ohno suggested that once these receptor sites are saturated with H-Y antigen, XX cells in a fetal gonad organize into a typically masculine structure, and even with a minority of XY cells in the indifferent gonad, neighbouring XX cells are induced to engage in testicular differentiation. This would explain why XX/XY mosaic gonads containing less than 10% of XY cells develop as predominantly testicular structures; if H-Y antigen was not disseminated but remained on the plasma membrane, testicular organization would be accomplished through direct cell-cell contacts and gonadal mosaics would invariably develop as ovotestes. Mittwoch (1977) linked H-Y antigen expression with enhanced growth rate of the "dominant" gonad.

Evidence for the "testis organizing" role of H-Y antigen came from Zenzes *et al.* (1978) who obtained suspensions of dissociated rat testicular cells which were then subjected to anti H-Y antiserum. After culture, these cells re-organized into follicular-like structures typical of the ovary; in the absence of antiserum tubular structures formed. Addition of H-Y antigen of epididymal fluid origin caused suspended rat ovarian cells to re-associate into what appeared to be testicular structures (Zenzes *et al.*, 1978). Bovine fetal ovarian cells have shown testicular-like organization *in vitro* with the addition of purified H-Y antigen (Ohno *et al.*, 1979).

Such experiments indicate that both ovarian and testicular cells must be endowed with H-Y antigen receptors, the antigen acting as a differentiation factor for the organization of testicular structures independent of whether the cells are XX or XY.

To determine the distribution of H-Y receptors, Müller *et al.* (1978) investigated the binding of H-Y antigen by various tissues in male and

female rats. In non-gonadal tissue (liver, kidney, brain and epidermis), binding could not be demonstrated in either sex, but ovarian and testicular cells were able to bind exogenously supplied H-Y antigen. Expression of the antigen (i.e. whether a cell is H-Y positive or negative) is confined to male cells and all male cells so far tested, including gonadal cells, type H-Y positive (Müller *et al.*, 1978). Exceptions are the immature germ cells (Zenzes *et al.*, 1978a) which do not express the antigen. Secretion of H-Y antigen is confined to the Sertoli cells (Zenzes *et al.*, 1978a).

5.2 EVIDENCE AGAINST H-Y ANTIGEN AS THE TESTIS DETERMINANT

Evidence indicating a role for H-Y antigen in the organization of testicular tissue from the undifferentiated gonad implies that any mammal possessing testes must be H-Y antigen positive. Studies of animals showing abnormal sexual development suggests that this is not so, and the hypothesis that H-Y antigen is the mammalian testis determining factor is under question. An example comes from H-Y antigen typing of sex reversed mice. XX*Sxr* male mice usually type as positive for H-Y antigen. However, McLaren *et al.* (1984) found that some such males (actually T16H/X*Sxr* males) were H-Y negative (designated *Sxr'*) indicating a separation of testis determination from the expression of H-Y antigen, and hence that the *Tdy* and *Hy* genes were not the same. Ohno (1985) still supported the notion of one gene, pointing out that it was spleen cells which were tested for H-Y expression in these mice, and since the animals consisted essentially of a mosaic of cells (*Sxr*-activated and *Sxr*-inactivated cells), the H-Y status of one organ (the spleen) should not be equated with that of another (the gonad). He also proposed that spreading of X-inactivation into the *Sxr* region was responsible for the non-expression of H-Y antigen, a proposal refuted by the demonstration that XO*Sxr'* mice are also H-Y negative (Burgoyne *et al.*, 1986). Since these animals (which possess testes) have only one X chromosome, X inactivation does not occur. Some XX human males type H-Y antigen negative, and XY females are found who are H-Y positive, endorsing the

view that H-Y antigen alone cannot be responsible for testis determination (Simpson *et al.*, 1987).

5.3 METHODS FOR H-Y ANTIGEN DETECTION

Much of the controversy surrounding H-Y antigen and its putative testis determining role has perhaps arisen due to the methods used in its detection. The term H-Y antigen originally introduced by Billingham and Silvers (1960), strictly speaking refers to the transplantation antigen responsible for rejection of male skin grafts by sensitized female mice of the same inbred strain.

In vitro methods developed for H-Y detection generally fall into two categories. Following from the transplantation assay, Goldberg *et al.* (1971) showed that the sera of C57BL/6 (or B6) female mice which had been grafted four or five times with syngeneic male skin grafts, were able to kill male target cells *in vitro*. This cytotoxicity was removed (absorbed out) by male, but not by female cells, the results being used as an indication of the H-Y status of the cells used for the absorption.

The second category of *in vitro* methods involves female cytotoxic T lymphocytes (CTLs) from animals immunized against syngeneic male cells. These CTLs are then used to lyse ^{51}Cr-labelled male cells. lysis being determined by quantifying chromium release (Goldberg *et al.*, 1973). These H-Y specific T cells are, however, MHC restricted, meaning that they must recognize both the major histocompatability complex antigens and the H-Y antigen on the target cell in order to lyse it.

Since graft rejection is a T cell response, the *in vitro* T cell assays for H-Y and the graft transplantation assay are taken to recognize the same histocompatability antigen (Simpson *et al.*, 1986). However, Hurme *et al.* (1978) state that genes controlling the rejection of skin grafts are different from those detected by the CTLs *in vitro*, so that H-Y antigen

detected by the CTL assay may not be the same as that defined by graft rejection. Wiberg (1987) suggests naming the antigens H-Yt or H-Yc (t=transplantation, c=CTL) according to the method used in typing a subject.

Serological techniques of detecting H-Y antigen have led to more problems of definition since several subjects tested using the serological and transplantation assays have yielded conflicting results (Melvold *et al.*, 1977). XO mice have been found to be H-Y negative by transplantation and cytotoxic T cell tests (Simpson *et al.*, 1982) and H-Y positive by serological methods using liver and spleen cells as target cells (Koo *et al.*, 1983). Due to these discrepancies in results, Silvers *et al.* (1982) suggested naming the antigen detected serologically, SDM (serologically detected male antigen). Wiberg uses the term Sxs (serologically sex specific antigen) since the antigen is not male specific in the bird, a species in which the female is the heterogametic sex and types SDM positive (Wachtel, 1983).

5.4 THE GENETIC DETERMINANTS OF H-Y ANTIGEN

H-Yt, the antigen detected by graft transplantation, was assumed to be coded for by a Y-linked gene since the presence of the Y chromosome was the only genetic difference between male and female mice of a syngeneic strain. However, human females suffering from Turner's syndrome (XO karyotype) have since been found to be H-Yt positive (Wiberg, 1985) as have female XO wood lemmings, (Wiberg and Gunther, 1985) indicating an autosomal, or X chromosome location for the gene. H-Yc typing of XY females lacking that part of Yp which determines testis development, shows them to be positive, whereas XX males carrying TDF type H-Yc negative (Simpson *et al.*, 1987). Since it is assumed that H-Yc and H-Yt are the same antigen, Simpson *et al.* (1987) conclude that the gene for this antigen maps to the long arm (Yq) or centromeric region of the human Y chromosome. Burgoyne (see "comments" in Wiberg, 1987) points out that this gene may be structural or regulatory, and if regulatory, the

structural gene for H-Yt and H-Yt may still be located on an autosome or X chromosome.

Wiberg (1987) details findings relating to the chromosomal location of the H-Ys gene in man, mouse and the wood lemming. It seems unlikely that the H-Ys gene is on the Y chromosome since XO women type H-Ys positive (Wolf *et al.*, 1980b), as do XX true hermaphrodites who lack detectable Y chromosome sequences (Waibel *et al.*, 1987). Wolf *et al.* (1980a) found that deletion of Xp in XX humans resulted in synthesis of reduced amounts of H-Y antigen compared with individuals with Xq deletions (even normal XX individuals typed, very weakly, H-Y positive). This led these authors to suggest that the H-Ys gene is autosomal and under the control of X- (specifically Xp) and Y-linked genes, a testis developing when a threshold titre of H-Y antigen is reached.

Lau *et al.* (1986) identified a male specific gene present in various mammalian species and believed to code for H-Ys. The gene was mapped to human chromosome 6. Wiberg (in press) suggests that this gene codes for a precursor protein which is then modified by gene products coded for by Y-linked genes. These Y-linked gene products determine structural changes in the protein such that different antibodies recognise different forms of the H-Y protein, thus accounting for discrepancies found using different methods for H-Y detection. Since XX humans would also possess the postulated structural gene on chromosome 6, Wiberg (in press) suggests that the X chromosome carries a gene coding for its repression. This X-linked gene, it is postulated, would escape inactivation in the female who would then carry a double dose of the repressor gene. The finding that XX true hermaphrodites, humans and dogs, type H-Ys positive (Waibel *et al.*, 1987; Selden *et al.*, 1978) suggests that in these individuals the double dose of X-linked repressor genes is not sufficient to override the autosomal structural gene.

Wiberg's model (in press) thus assumes that H-Y antigen is a single antigen structurally determined by an autosomal gene but modified by gene products carried on the X and Y chromosomes. Different epitopes of the H-Y antigen would be recognised by different immunological tests - a protein epitope would initiate a T cell response (identified by the CTL

and transplantation assays), whereas carbohydrate epitopes would activate B lymphocytes, assayed in serological tests.

5.5 H-Y ANTIGEN AND SPERMATOGENESIS

Since *Sxr'* male mice are H-Y antigen negative and infertile, it has been suggested that H-Y antigen plays a role in spermatogenesis (McLaren *et al.*, 1984).

In the presence of two X chromosomes, spermatogenesis does not occur due to the perinatal loss of germ cells (in the mouse) whereas this does not happen in XO*Sxr* mice. Histological examination of the testes of XO*Sxr* male mice shows that spermatogenesis is much more severely affected in XO*Sxr'* mice than in XO*Sxr* individuals, although both are infertile (Burgoyne *et al.*, 1986). It is suggested that H-Y antigen is the product of the spermatogenesis gene or that the two genes are closely linked (Burgoyne *et al.*, 1986). This may be so in mice, but in humans a spermatogenesis gene is located on Yq, close to the centromere (Tiepolo and Zuffardi, 1976) whereas the H-Ys structural gene is assigned to chromosome 6 (Lau *et al.*, 1986). If the spermatogenesis gene in humans does regulate expression of H-Ys, then only those individuals possessing Yq should type H-Ys positive, a condition not met by those patients who are XO or XX yet type H-Ys positive (Wolf *et al.*, 1980b; Waibel *et al.*, 1987).

PART B EXPERIMENTAL

INTRODUCTION

Fifteen animals were used for the following experiments, the pigs being supplied by three farms in the Edinburgh area. The animals were between 6 days and 5 months old at the time of identification and were aged up to 20 months at the time of slaughter. All the animals were identified as intersexes by the appearance of their external genitalia *i.e.* upturned vulva, enlarged clitoris and, in some cases, one or two scrotal sacs.

Housing was in a large building with individual barred pens so that each animal could see the other pigs. Feed was a barley based concentrate with added minerals and vitamins. Straw bedding and water were provided. All animals were slaughtered at the local abattoir, except for two small pigs which were slaughtered on alternative, registered, premises.

Since the extent of masculinization differed between each intersex, Chapter 6 describes observations made on each animal, including descriptions of tract morphology and of gonadal type. Data on gonadal type *i.e.* testis, ovary or ovotestis, and size are collated, as are litter size of intersex and normal litters. Chapters 7 and 8 (section I) cover the histology and karyotypes of selected animals.

Sections II and III describe experiments carried out on intersex animals to ascertain brain sex and the aetiology of the condition respectively. The results from all the experiments are considered in the discussion, and some suggestions are made as to the possible aetiology of intersexuality in pigs.

SECTION I MORPHOLOGICAL, HISTOLOGICAL AND GENETIC ANALYSIS OF SOME INTERSEX PIGS

CHAPTER 6 THE ANIMALS DESCRIBED

For each animal, a description will be given of the animal's external appearance and the gonadal type as ascertained at slaughter or mid-ventral laparotomy. In some cases, the gonads were weighed so that comparison could be made between left and right gonads. Of the intersexes for which parentage was known, none was found to have the same sire or dam.

Abbreviations used are; EH, Easter Howgate, the University pig farm.

ABRO, Animal Breeding and Research organisation pig farm, now Institute of Animal Physiology and Genetics Research station, Edinburgh.

Madderty, a commercial pig farm in Fife, Scotland.

M = male, F = female. CL = corpus luteum or corpora lutea .

Animal LE

ABRO. Approximately one month old. Two abdominal testes. Uterus, normal female.

Animal MM

ABRO. Slaughtered at 6 days old. Two testes, left abdominal, right scrotal. Uterus, normal female but right uterine horn pulled towards scrotum.

Animal 2

EH. Born 15/12/85. 2nd litter, 1 of 11 born, 7F 4M. Upturned vulva, penile clitoris, tusks. Two abdominal testes and epididymides. Fluid-filled uterus. Bulbo-urethral gland, uterus and bladder emptied into common opening.

Animal 4

EH. Born 25/11/84. 8th litter, 1 of 12, 5F 1M 6 dead. Upturned vulva, penile clitoris, tusks. Two abdominal gonads, left ovotestis; 90% testicular, luteal tissue identified during histological studies, epididymis. Right testis with adjoining epididymis.

Animal 5

EH. Born 4/11/84. 8th litter, 1 of 10, 4F, 6M. Upturned vulva, enlarged clitoris. Preputial sheath. Tract and gonads couldn't be exteriorised at laparotomy nor identified at slaughter.

Animal 7

EH. Born 19/10/84. 7th litter, 1 of 13, 8F 5M. Upturned vulva, clitoris hypertrophied. Tusks. Two abdominal ovotestes. Left 90% ovarian with >12 follicles, some 8-9 mm diameter. No corpora lutea. Small piece of testicular tissue near stalk with adjoining small epididymis. Right gonad 30% ovarian, some cystic follicles (>10 mm diameter) on ovarian portion (next to stalk). Testicular 70% with adjoining epididymis. Tract apparently normal uterus although ampullary portion of Fallopian tubes not well developed. See Figure 6(a).

Animal 9

EH. Born 23/10/84. 2nd litter, 1 of 11, 5F 6M. Upturned vulva, tusks. Ridge running up to vulva from ventral position between back legs. penile clitoris revealed on deflection of vulval lips. Penile musculature revealed at mid-ventral laparotomy. Gonads- two abdominal testes both

with adjoining epididymis and pampiniform plexus. Right gonad positioned as if descending into inguinal canal.

Animal 93

EH. Born 25/11/85. 1st litter, 1 of 12, 2F, 9M, 1 unrecorded. Upturned vulva. Gonads- abdominal, left ovary, right testis. Ovary with 16 follicles. many cystic (>10 mm diameter). Some luteal tissue found during histological examination. Right, testis, large epididymis and pampiniform plexus. Tract, normal uterus but on right only 3cm of isthmus apparent. Upper Fallopian tubes vestigial.

Animal 096

ABRO. Upturned vulva. hypertrophied clitoris exposed on deflection of vulva. Gonads. both abdominal testes with red patches on surface. Each gonad with adjoining epididymis and pampiniform plexus. Uterus, fluid-filled.

Animal 100

Madderty. Upturned vulva. hypertrophied clitoris. Left gonad, abdominal testis (migrating towards inguinal canal). Right, scrotal testis. Each with associated epididymis and pampiniform plexus. Uterus normal but upper portion of Fallopian tubes vestigial.

Animal 307

EH. Born 17/4/86. Upturned vulva, hypertrophied, penile clitoris. Gonads, left 70% testicular, right 75% testicular. No CL on ovarian portions but approx. 5 follicles, some up to 9mm in diameter. Tract, uterus fluid-filled, cervix normal, Fallopian tubes normal as far as isthmus, ampullae under-developed.

Animal 433

ABRO. Born approx 2/86. Upturned clitoris, penile clitoris, tusks. Gonads both scrotal testes with associated epididymides. Uterus small. horns pulled towards inguinal canals. Vasa deferentia running parallel to uterine horns.

Animal 502

Madderty. Born approx 8/87. Upturned vulva. Left gonad scrotal testis, right abdominal testis, both with associated epididymides and pampiniform plexus. Uterus apparently morphologically normal.

Animal 753

ABRO. Born approx 8/87. Upturned vulva. Two abdominal testes, right larger than left. Uterus fluid-filled.

Animal 1161

Madderty. Born approx 8/87. Gonads, left, abdominal ovary with 6 large pre-ovulatory follicles. Right, testis with mottled appearance and associated epididymis. Fallopian tube interrupted by epididymis but apparent again next to the testis. See Figures 6 (b, c and d).

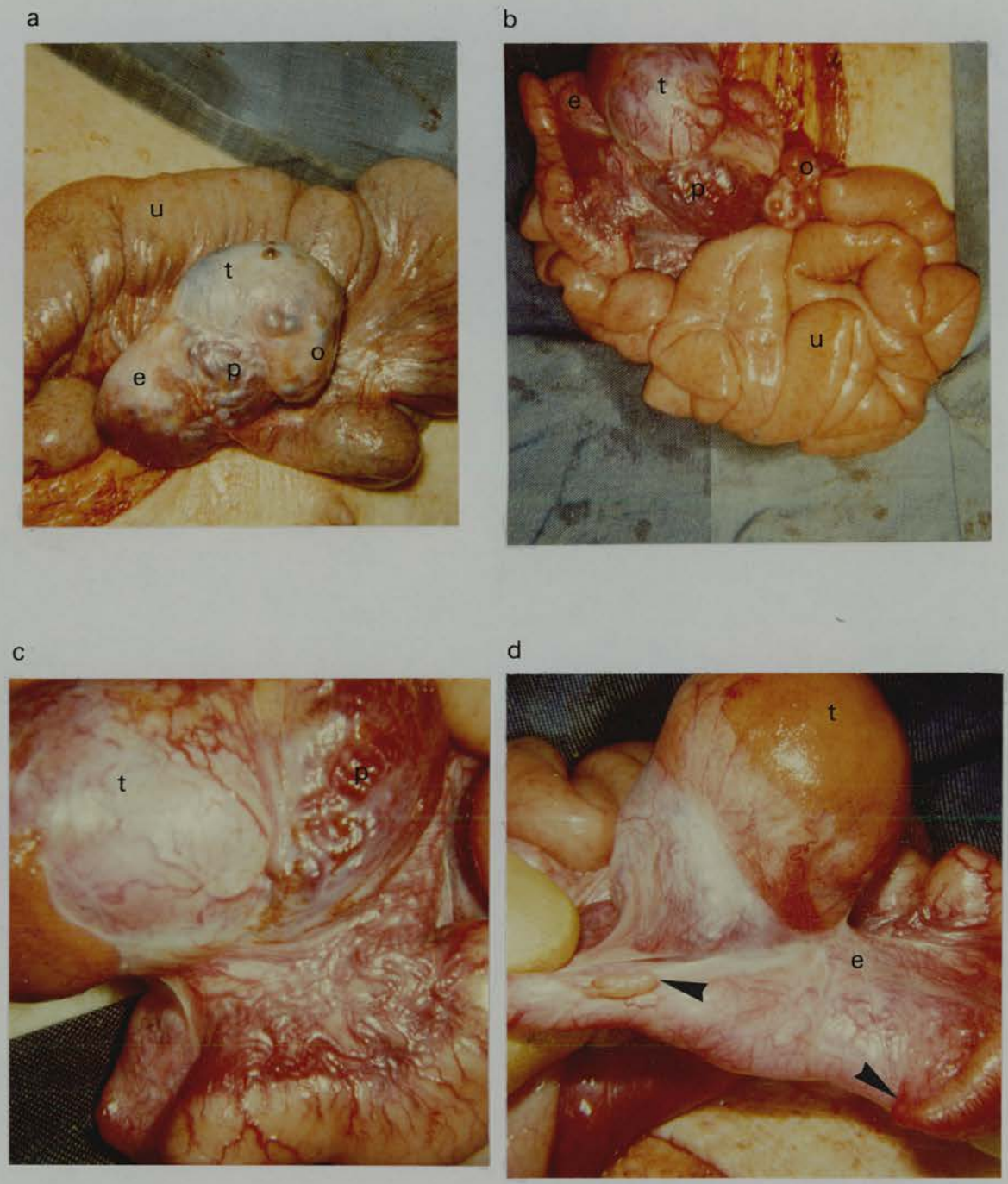


Figure 6. Photographs taken at mid-ventral laparotomy. a) Right ovotestis, intersex 7. b) Right testis, left ovary, apparently normal uterus, intersex 1161. c) Uterine blood supply adjacent to right testis, intersex 1161. d) Fallopian tube (arrowed) interrupted by epididymis, intersex 1161. o = ovarian, t = testicular, p = pampiniform plexus, e = epididymis, u = uterus.

6.1 Gonadal types and weights

Table 6.1 Summary of gonadal types, distribution and weight in the intersex pigs.

ANIMAL	LEFT GONAD	WEIGHT (g)	RIGHT GONAD	WEIGHT (g)
LE	testis, A	-	testis, A	-
MM	testis, A	-	testis, S	-
2	testis, A	-	testis, A	-
4	ovotestis, A	-	testis, A	-
5	unknown	-	unknown	-
7	ovotestis, A 10% testicular	43	ovotestis, A 70% testicular	78
9	testis, A	-	testis, M	-
93	ovary, A	-	testis, A	-
096	testis, A	77.6	testis, A	102.6
100	testis, M	55.7	testis, S	103.5
307	ovotestis, A 70% testicular	38	ovotestis, A 75% testicular	62
433	testis, S	-	testis, S	-
502	testis, S	-	testis A	-
753	testis, A	28.5	testis, A	78.7
1161	ovary, A	11.5	testis, A	39.3

S = scrotal A = abdominal

Table 6.2. Gonadal distribution in 14 intersex pigs

GONAD	LEFT	RIGHT
Testis	9/14 (64.3%)	12/14 (85.7%)
Ovary	2/14 (14.3%)	0/14 (0%)
Ovotestis	3/14 (21.4%)	2/14 (14.3%)

Total number of testes	21	(75%)
Total number of ovaries	2	(7.1%)
Total number of ovotestes	5	(17.9%)
Total	28	

The gonadal weights show that the right gonad is always heavier than the left irrespective of gonadal type.

Table 6.2 shows that a testis is the most common type of gonad, accounting for 75% of the gonads. Of these testes, 12/21 (57%) were found on the right, 43% on the left, however, this difference in distribution of testes is not significant when tested with the χ^2 test (χ^2=0.428 d.f=1, 0.2< p >0.5). A testis was found in 85.7% of the right gonads but only 64.3% of the left gonads.

Discussion

Of the intersex pigs studied by Breeuwsma (1970), 38% were found to be true hermaphrodites *i.e.* possessing some ovarian and testicular tissue. Of the 14 animals described here (discounting animal number 5), 35.7% possessed some ovarian tissue, the remainder had two testes.

The proportion of testes found in the right compared with left gonads does not differ significantly. In the animals described here, a testis was more often found on the right hand side, and ovotestes possess more testicular tissue when found on the right, than in left gonads. The distributions of testes (57% on the right) is similar to that seen in human true hermaphrodites (van Niekerk and Retief, 1981) in which 59.5% of testes were found to occur on the right hand side. This does not seem to be the case in true hermaphrodite mice in which the left gonad is significantly more masculine than the right (Ward *et al.*, 1987) although this figure refers to animals which were phenotypically male rather than phenotypically female, as is the case in intersex pigs. Regarding gonadal size, the testis of the normal boar is some 30 times heavier than an ovary from a female pig of the same age. Measurements of gonadal weights of prepubertal pigs at slaughter weight (approx 60 kg) showed the mean weight of ovaries to be 2.5g and of testes, 75g (unpublished observations). The weights of the gonads in the intersex pigs obviously reflects the gonadal type, the average weight of the intersex testes being 69.4 (+/- 11.1) g, similar to that found for prepubertal testes, although direct comparison cannot be made due to the range in age of the intersex pigs. The difference in gonadal size between males and females led Mittwoch and Mahadevaiah (1980) to study human fetal gonads in an attempt to show that gonadal growth rate could influence gonadal sex. These same authors proposed that a higher growth rate in the right gonad led to development of a testis in hermaphrodite individuals, and human fetal testes were found to be larger than ovaries, right gonads larger than left. However, Mittwoch and Buehr (1973) failed to find a difference in gonadal size between right and left gonads of fetal mice, although testes were larger than ovaries. In the prepubertal pigs, no significant difference was found between right and left gonadal

weights, and a study of gonads from new-born piglets failed to show right-left differences although testes were heavier and larger than ovaries (unpublished observations).

That the right gonad is heavier than the left supports Mittwoch's views (1971) *i.e.* that right gonads grow faster than left gonads and consequently are more likely to develop as testes (Mittwoch, 1986).

6.2 Litter size

Introduction

Breeuwsma's extensive study (1970) of intersex pigs revealed that the mean litter size of affected litters (containing at least one intersex) was significantly larger than that of normal litters ($p < 0.001$). Further analysis of the data revealed a positive correlation between incidence of intersexes and increasing litter size, leading Breeuwsma (1970) to postulate a "crowding" effect *in utero* contributing to the occurrence of an intersex fetus. The sex ratio of normal litters did not differ from affected litters, provided that the intersexes were assumed to be XX *i.e.* genetic females.

Litter sizes were known for 6 of the intersex pigs in this study, and comparison was made with the size of normal litters from the same farm. Since litter size increases with parity in pigs (French *et al.*, 1979) comparison was made between litters of similar parity.

Table 6.3. Litter sizes of affected litters. Parity 1 or 2

PARITY	LITTER SIZE	NUMBER OF MALES	NUMBER OF FEMALES
1	12 *	9	2
2	11	6	5
2	11	4	7
Mean	11.3	6.3	4.7

* sex of 1 piglet unrecorded

Table 6.4. Litter size of affected litters. Parity 7 or 8

PARITY	LITTER SIZE	NUMBER OF MALES	NUMBER OF FEMALES
7	13	5	8
8	12 (6 dead)	1	5
8	10	4	6
Mean	11.7	3.3	6.3

Table 6.5. Mean litter size of normal litters

PARITY	NUMBER OF LITTERS	MEAN LITTER SIZE	MEAN NUMBER OF MALES	MEAN NUMBER OF FEMALES
1 or 2	16	11.5	5.6	5.8 *
7,8 or 9	12	11.1	5.0	6.1

* sex of 2 piglets not recorded

Table 6.6. Comparison of litter size of affected and normal litters, matched for parity

PARITY	1 or 2 (+/-s.e).	7, 8 or 9 (+/-s.e)
Mean litter size of affected litters	11.3 (0.3)	11.7 (2.3)
Mean litter size of normal litters	11.5 (5.1)	11.1 (4.9)
Student's t-test:		
Degrees of freedom =	17	13
p =	> 0.3	> 0.3

Table 6.6 shows that there is no significant difference between the sizes of normal and affected litters ($p > 0.3$) when matched for parity.

Discussion

From these results, there is no evidence to support Breeuwsma's findings (1970) that intersexuality is associated with larger litter size, although the sample number of affected litters (6) is low. Breeuwsma used the data from 105 affected litters. Moreover, Breeuwsma found no link between parity and incidence of intersexuality, despite the observation that litter size did increase with parity.

Sittman *et al.* (1980) studied litter size in animals from the farm used in Breeuwsma's work, but found no relationship between this and incidence of intersexuality. Sittman *et al.* (1980), do not attempt to explain Breeuwsma's findings.

CHAPTER 7 HISTOLOGY OF THE GONADS OF INTERSEX PIGS

7.1 Introduction

Previous studies of gonadal histology in intersex pigs state that the seminiferous tubules of testicular tissue were devoid of germ cells, although Sertoli-like cells lined the tubules and Leydig cells were present between the tubules (Pond *et al.*, 1961; Basrur and Kanagawa, 1971; Krishnamurthy *et al.*, 1971; Gerneke, 1973; Hunter *et al.*, 1982 and 1985). One exception is noted by Baker (1925), who stated that one of the animals studied (case 8) possessed spermatocytes in the seminiferous tubules. However, this animal came from the New Hebrides and was morphologically different from intersexes found in Britain. Ovarian tissue has been studied and oocytes, follicles and corpora lutea observed (Pond *et al.*, 1961; Krishnamurthy *et al.*, 1971; Hunter *et al.*, 1985), although several authors noticed that small follicles were scarce, and the number of atretic follicles was greater than expected for normal ovarian tissue (Gerneke, 1967; Krishnamurthy *et al.*, 1971).

Most authors state that epididymides adjoin testes, but that the uterine horns are unaffected by the presence of testicular tissue. However, there are no reports of histological examination of the ducts in close proximity to the gonads.

The following studies were conducted to 1) confirm that testicular tissue was devoid of germ cells, even in young intersex animals. 2) to assess whether ovarian tissue contained oocytes and follicles. 3) to study the effect of testicular tissue on the proximal portion of the Müllerian duct.

Because of the small numbers of animals used, and to the paucity of follicles in ovarian tissue within intersex animals, quantitative studies on follicular health could not be made.

7.2 Materials and Methods

Gonadal tissue was collected at biopsy (during laparotomy), from the abattoir or, in the case of the 6 day old normal control, after castration. Tissue was fixed in Bouin's fixative for 24 hours, dehydrated in alcohol and embedded in paraffin wax (Paraplast). Sections were cut at 7μm on a Reichert-Jung microtome and were stained with haemotoxylin and eosin. Representative photographs were taken of sections from the intersex gonads and control male and female pigs using Kodak Plus-X-pan film.

7.3 Results

Figures 7.1 a) and b) are photographs of sections of large, antral follicles seen in the ovarian portion of an ovotestis (animal 7). The junction between ovarian and testicular tissue in the same animal is shown in Figure 7.1 c), and d) shows the "nests" of primordial follicles characteristic of normal ovarian cortical tissue. Figure 7.2 a) is of seminiferous tubules seen in a scrotal testis (animal 433) compared with b), testicular tissue from a mature boar. Figure 7.2 c) shows a section of testicular tissue taken from a normal, 6 day old piglet and d) is a section of testicular tissue from an intersex pig of the same age, showing a cell in the process of dividing. Figure 7.3 a) shows a section of what appear to be hilar cells, seen in the ovarian portion of an ovotestis from animal 307. Figure 7.3 b) shows a dividing cell seen in the testis of a normal 6 day old pig and c) is a section of the epididymis adjoining the Fallopian tube in animal LE. Figure 7.3 d) is a part of the Fallopian tube in this animal at a higher magnification.

Figure 7.1. Histological sections, stained with haematoxylin and eosin, of a) Graafian follicle in left ovotestis of intersex 7, x100. b) Graafian follicle in left ovotestis of intersex 7, showing signs of degeneration, x50. c) Junction between testicular (left) and ovarian portions of left ovotestis, intersex 7, x50. d) Primordial follicle "nests" in the ovarian cortex of a normal gilt, x100.

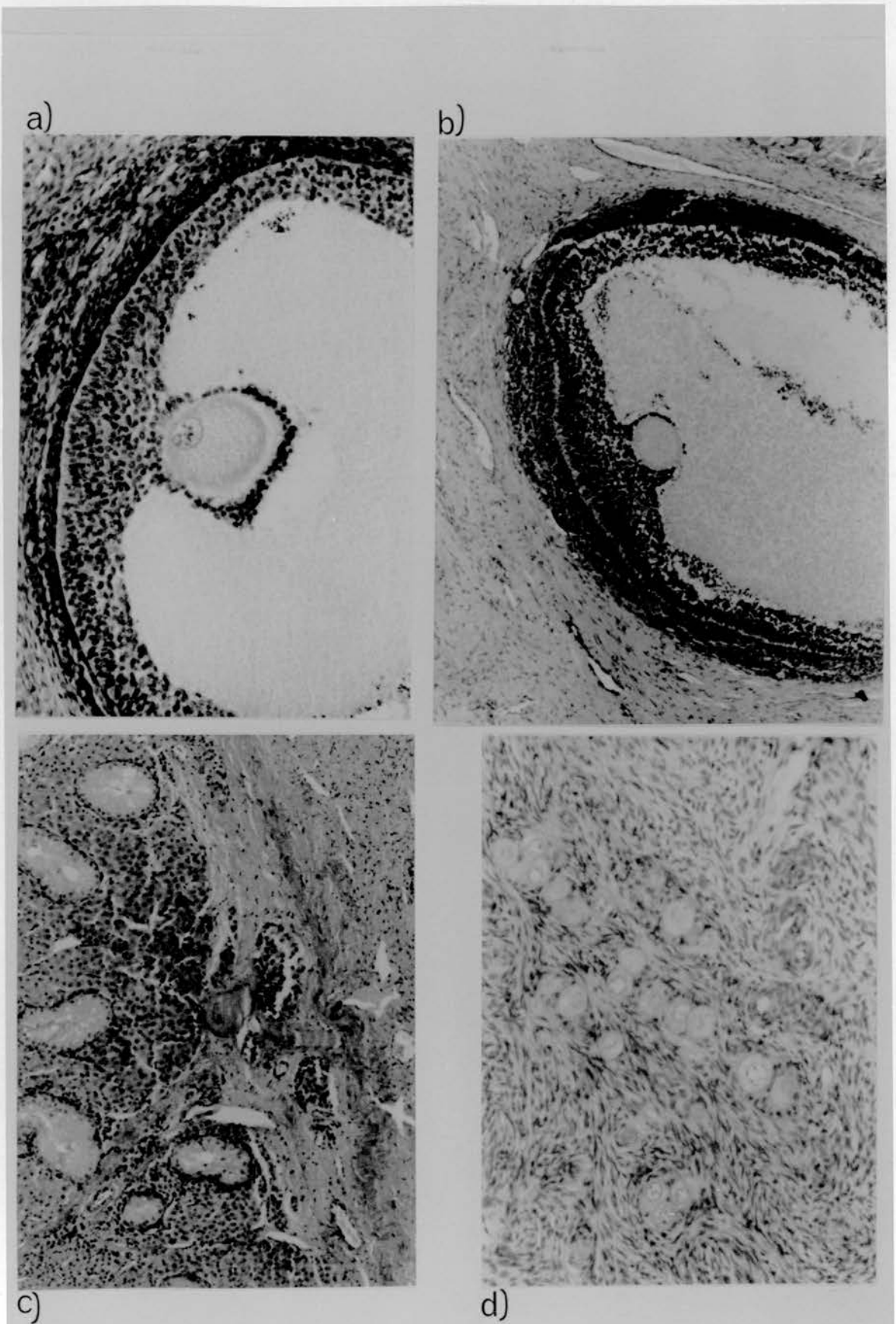

a)
b)
c)
d)

Figure 7.2. Histological sections, stained with haematoxylin and eosin, of seminiferous tubules from a) a scrotal testis, intersex 433, x200, b) a normal boar, x400, c) a 6 day old normal male pig, primordial germ cell arrowed, x400 and d) a 6 day old intersex gonad (MM) showing a dividing Sertoli or germ cell (arrowed), x400.

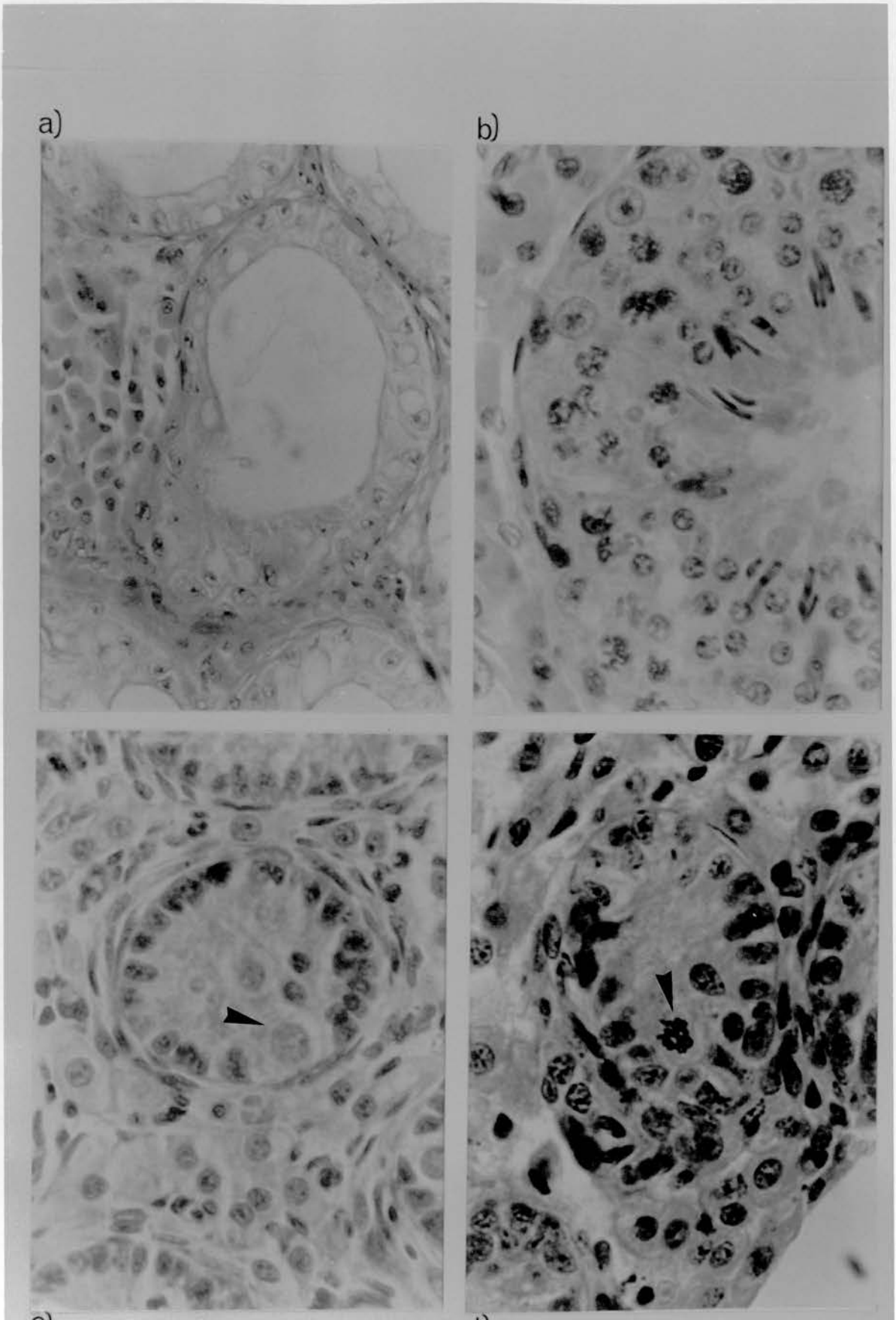
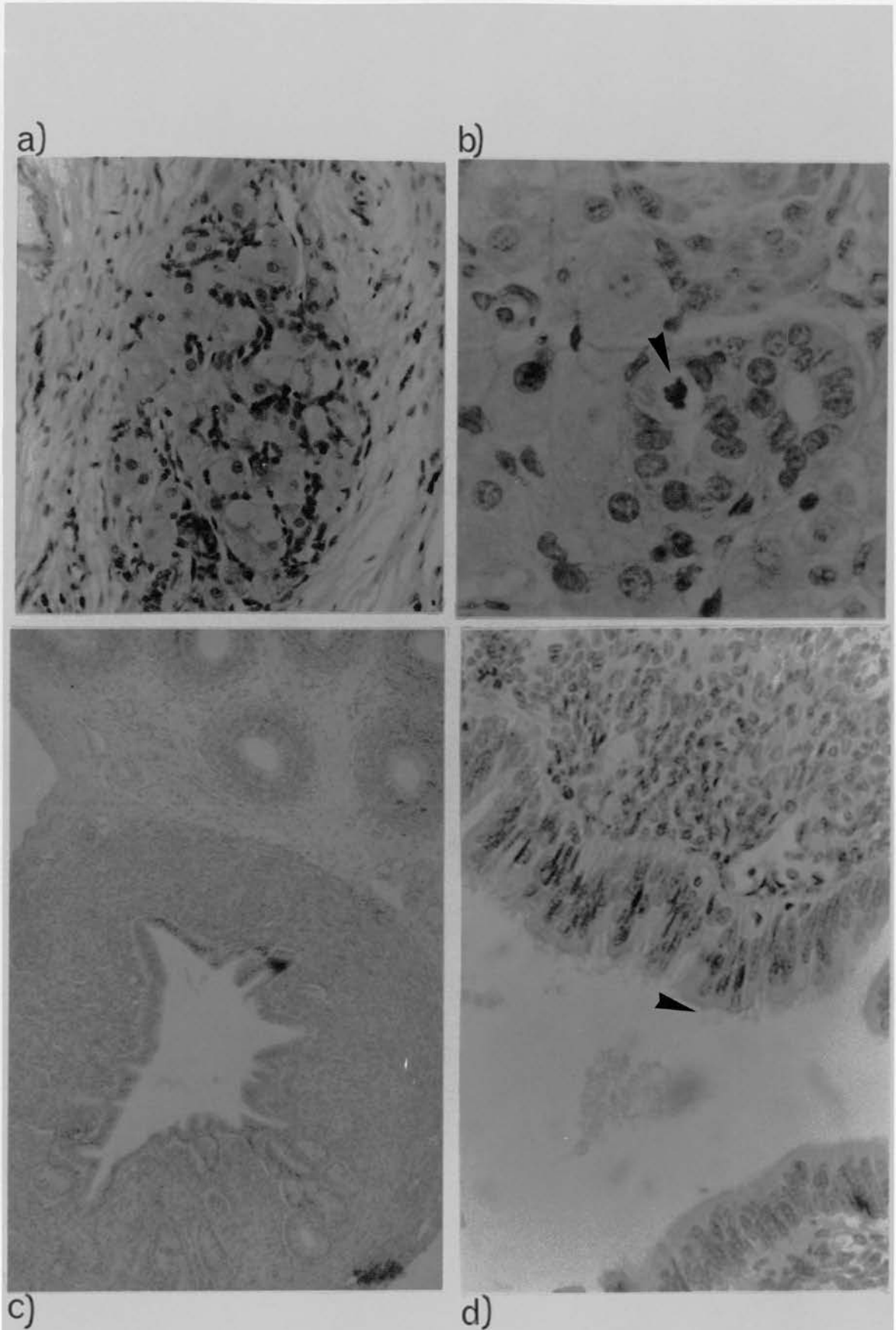

a)
b)
c)
d)

Figure 7.3. Histological sections, stained with haematoxylin and eosin, of a) "hilar-like" cells from the ovarian portion of an ovotestis (pig 307), x100. b) A dividing Sertoli or germ cell (arrowed) in a seminiferous tubule of a 6 day old normal male pig, x100. c) Fallopian tube adjoining the epididymis in intersex LE, x50. d) Higher magnification of the female duct seen in (c), showing cilia (arrowed), x400.


a)
b)
c)
d)

7.4 Discussion

Ovarian tissue in intersex pigs, whether from an ovary or an ovotestis, was found to contain oocytes and follicles, including large antral follicles. However, there seemed to be a lack of primordial follicles in the intersex ovarian sections. In a normal pig ovary these primordia appear in clusters around the periphery of the ovary (see Figure 7.1 d). The paucity of primordial follicles made any quantitative assessment of follicular health difficult, although, in agreement with other workers (Krishnamutrthy *et al.*, 1971) many of the larger follicles observed were atretic. Luteal tissue was observed in animal 4 (in the ovotestis) and animal 93 (in the ovary), implying that these animals had ovulated, and that ovulation can occur from ovotestes.

Testicular tissue, whether within a testis or an ovotestis, resembled testicular tissue observed in cryptorchid testes of pigs (Liptrap and Raeside, 1970). Interstitial tissue appeared abundant, possibly because of tubular shrinkage (Clegg, 1961) although extensive interstitial tissue is a characteristic of boar testis (Setchell, 1978). The high concentrations of testosterone in tissue homogenates (Hunter *et al.*, 1982 and 1985) and in peripheral blood plasma (see Chapter 11) as well as the masculinized external genitalia, indicate that the Leydig cells are secreting testosterone.

Junctions between testicular and ovarian portions of an ovotestis were well defined but follicles were not seen in close proximity to testicular tissue (see Figure 7.1 c). In the ovarian portion of one intersex (307) a region resembling hilar tissue was identified (Figure 7.3 a). These groups of polyhedral cells, found near the hilus of the ovary in humans and certain other mammals, are morphologically similar to Leydig cells and are thought to produce androgens (Baird, 1984). They are common in women and are especially prominent at the end of fetal life, puberty, pregnancy and the menopause. Hilar tumours may cause masculinization due to excessive androgen secretion. Adrenal "rests" (groups of adrenocortical cells) are morphologically similar to hilar cells, but are distinguished by encapsulation of the former. Since hilar cells have previously been identified in the pig (Unsicker, 1970, cited in Harrison

and Weir, 1977), and because no extensive areas of these cells were found in the intersex gonads in the present study, it is unlikely that hilar tissue alone is responsible for gonadal masculinization.

Lining the seminiferous tubules (Figures 7.1 c and 7.2 a) are Sertoli-like cells which, in some animals, appear to be vacuolated. The testes of cryptorchid boars showed similar Sertoli cell degeneration (Liptrap and Raeside, 1970). However, in the intersexes, the degenerative appearance is not confined to abdominal testes. Figure 7.2 a) shows seminiferous tubules from a scrotal intersex testis.

The lack of germ cells, even in scrotal testes, indicates that the abdominal location is not the cause, although cryptorchid testes are known to lose spermatozoa, spermatocytes and spermatogonia (Liptrap and Raeside, 1970). These authors also demonstrated that a cryptorchid testis was capable of producing normal amounts of testicular steroid hormone *i.e.* an abdominal position does not render Leydig tissue non-functional. It is possible that scrotal testes of intersex pigs are sterile because of late descent of the testis and hence early loss of germ cells, but Nishimune *et al.* (1978) found that, if artificial cryptorchidism in mice was surgically reversed, persistent type A spermatogonia were able to survive in the abdominal testis and normal spermatogenesis returned 60 days after surgical reversal. Intersex testes which were abdominal might then be expected to recover, even if testicular descent occurred later than the normal, prenatal time (Wensing, 1968).

The section of the 6 day old intersex gonad MM (right = scrotal, left = abdominal) reveals what appears to be a dividing cell within the tubules (Figure 7.2 d). Figure 7.3 b) shows a dividing cell observed in the testis of a normal 6 day old pig, presumably undergoing mitosis, since meiosis (in the male germ cells) does not occur until puberty.

The questions arise as to whether the dividing cells seen in the intersex animal are undergoing meiosis or mitosis and, if the latter, are the cells Sertoli or germ cells? In the rat, Sertoli or "supporting" cells apparently cease to divide around day 18 after birth (puberty is reached at approximately 60 days), and no DNA labelling can be demonstrated after day 15 (Steinberger and Steinberger, 1971). In 4 day old rats, two types of mitotic figures are seen; small cells which are thought to be germ

cells, and large cells thought to be Sertoli cells (Clermont and Perey, 1957). Thus, according to the rat model, mitotic figures seen in 6 day old pig testes could be Sertoli or germ cells. However, germ cells in the pig testis could, at this stage, be in a "resting" phase. Again, in the rat, germ cells are known to enter a period of rapid mitotic division between days 14.5 and 20 *post coitum*, after which little or no mitosis is observed until around day 4 after birth, when another wave of mitotic activity is accompanied by degeneration and atresia of many germ cells (Clermont and Perey, 1957; Beaumont and Mandl, 1963). In the human testis, Wartenberg (1981) described the germ cells in the human newborn testis as T-prospermatogonia (T for transitional). These diploid cells are derived from M- (multiplying) prospermatogonia which in turn are derived from the primordial germ cells which undergo mitosis within the seminiferous tubules. M-prospermatogonia divide further to form the T-prospermatogonia around the 13th week of life. It seems that these T-prospermatogonia are then relatively quiescent until puberty, when further division occurs, spermatogonia resulting. However, without a differential stain to distinguish between mitotic Sertoli and germ cells, it is impossible to say which is which in the intersex animal.

Since non-mitotic germ cells were not observed, several possibilties exist to explain sterility in the intersex testis. 1) A scarcity of germ cells in the embryonic testis, linked with degeneration (as in the rat) during post natal mitosis of existing germ cells, results in a sterile testis. 2) The testicular tissue differentiates in the absence of a primordial germ cell population and mitotic figures observed are Sertoli cells not germ cells. This implies that the Sertoli cells, even in sterile tubules, are healthy. 3) The germ cells, being XX, do not survive in a testicular environment (McLaren *et al.*, 1972). The dividing cells observed may then be XX germ cells undergoing meiosis, following their inherent genetic developmental pathway regardless of the testicular surroundings. The fact that intersex pigs may ovulate and become pregnant shows that at least some germ cells are XX.

Figures 7.3 c) and d) are of the intersex LE, aged approximately 1 month and possessing two abdominal testes. The Wolffian and Müllerian ducts have both persisted and developed into an epididymis (devoid of spermatozoa) and Fallopian tube (identified by the presence of cilia).

Obviously the developing testicular tissue, whilst secreting sufficient testosterone for development of the Wolffian duct, is not producing AMH required for regression of the Müllerian duct. The Sertoli cells are the source of AMH (Josso, 1973) and, since regression of the Müllerian duct normally occurs by the time the pig embryo has reached 30 or 40mm in length, persistence of the ducts infers inadequate functioning of the Sertoli cells early on in development. Whether the Sertoli cells are non-secretory due to the lack of germ cells, or whether germ cells fail because of insufficiency in the Sertoli cells, is not clear. However, there is evidence that, whilst depletion of germ cells *eg.* through irradiation or in "Sertoli-cell only" syndrome, may affect the Sertoli cell numbers, there are no reports of such individuals possessing well developed Müllerian ducts (Del Castillo *et al.*, 1947; Erickson and Blend, 1976). Burgoyne *et al.* (1988) have suggested that Sertoli cell differentiation is triggered by cell-autonomous activity of a gene on the Y chromosome and that subsequent steps in testicular development are a consequence of Sertoli cell differentiation. The possibility therefore exists that the Sertoli cells in these testes carry part of the Y chromosome, causing cell-autonomous differentiation, and that Leydig cell development occurs as a consequence. AMH produced by the Sertoli cells, is known to cause elimination of oocytes from ovarian tissue (Ożdżeński *et al.*, 1976; Vigier *et al.*, 1987; Burgoyne *et al.*, 1988), AMH production by the Sertoli cells in intersex pigs may therefore be sufficient to cause regression of germ cells without affecting the adjoining Müllerian duct. Alternatively, a deficiency in germ cells colonising the gonadal ridge may itself be the cause of tubule formation, even in an XX animal. The suggestion that inadequate germ cell colonisation is the cause of development of an intersex gonad (Hunter *et al.* 1988) goes against conventional thoughts that germ cells are not required for differentiation of the gonad, and is discussed in more detail in the final discussion.

CHAPTER 8 THE KARYOTYPES OF INTERSEX PIGS

8.1 Introduction

Crew (1923) believed intersex pigs to be genetic males, since all the specimens which he studied possessed two testes. Observing the presence of fertile ovarian, but sterile testicular tissue, Baker (1925) concluded that the animals were masculinized genetic females. Cantwell *et al.* (1958) determined the genetic sex of intersex animals using a cytological method and found all 6 animals to be XX. Since then, several reports have confirmed that the majority of intersex pigs do have a normal female, 38,XX karyotype, determined by culture of leucocytes from peripheral blood (Melander *et al.*, 1971; Miyake, 1973; Hunter *et al.*, 1982 and 1985) or fibroblasts from liver, kidney, bone marrow, spleen or spinal cord (Pond *et al.*, 1961; Gerneke, 1967; Melander *et al.*, 1971).

McFee *et al.* (1966) reported a case of XX/XY mosaicism in an intersex pig. Toyama (1974) found five such cases and Breeuwsma (1968) described an affected animal with an XXY chromosomal constitution. Since testicular differentiation is thought to occur only in the presence of a Y chromosome, karyotypes of 8 animals were ascertained by means of blood leucocyte culture. An attempt was also made to culture testicular tissue from an intersex pig. Metaphase spreads of five animals were G-banded to facilitate classification of the chromosomes.

8.2 Materials and Methods

Blood leucocyte cultures

Ear vein blood samples (10 ml) were collected, taking aseptic precautions, into heparinised tubes. Whole blood was cultured using the micro-culture procedure reported by Lin *et al.* (1976 and 1980). Approximately 1 ml of whole, heparinised blood was added to 10 ml of sterile F-10 culture

medium (Flow laboratories) supplemented with 20% fetal calf serum (Gibco) and 10% tryptosephosphate broth (Oxoid). Phytohaemagglutinin (0.05 ml,Wellcome) was added to the culture, which was then incubated at 37° C for 72 hours. At 1.5 hours before harvest, Colcemid (Sigma) was added to the culture to give a final concentration of 0.4 µg/ml. The medium was removed by centrifugation (10 min at 700*g*). The supernatant was removed and the cells fixed in freshly prepared fixative (methanol:acetic acid, 3:1 volume:volume), overnight at 4° C and then once more at room temperature for 15 min. The second fixative was removed and the cells resuspended in approximately 0.5 ml of fresh fixative for spreading. Two drops of suspension were pipetted onto clean, moist slides. Slides were then stained with 5% Giemsa for 15 min, or G-banded after drying for 2-3 days.

G-banding

Slides were incubated at 60° C for 1 hour in a solution of SSC (0.3M sodium chloride, 0.03M tri-sodium citrate in distilled water), rinsed with de-ionised water and stained for 15 min in 5% Giemsa.

Testicular culture

The method used is described by Rong *et al.* (1988). Small pieces of testicular tissue were placed on the scored surface of culture bottles, and once they had adhered to the plastic (approx 2 hours) 30 ml of medium were added (F10 containing 20% fetal calf serum and antibiotics). The flasks were incubated at 37° C, the medium changed every 3 days, subsequent medium containing 10% fetal calf serum. Approximately 3 weeks later, the cells were harvested, Colcemid (10 µg/ml) was added to the culture 2 hours prior to harvest, trypsin was used to suspend the cells in the medium which was then centrifuged for 5 min at 700*g*. Preparation then proceeded as for blood leucocyte harvest.

Photography

Photographs of the chromosomes were taken using Kodak Plus-X-pan film, 125 ASA.

8.3 Results

Blood leucocytes

G-banding of chromosomes enabled karyotypes of five intersex animals to be produced (Figures 8.1 to 8.5) based on Ford, Pollock and Gustavsson (1980). The karyotypes appear normal, with no obvious translocations or deletions affecting the sex chromosomes or autosomes. The diploid number of chromosomes in the domestic pig is 38. The Y chromosome is easily identified since it is much smaller than any of the others (Hansen, 1977). Metaphase spreads (50) from animals 4, 7 and 433 were studied; all revealed an XX chromosome constitution. In animals 2, 5, 9, 93 and 307, 10 spreads observed from each showed a 38,XX karyotype.

Testicular tissue culture

Cell growth in the culture bottles was very poor, despite good results when growing fibroblasts from normal animals. Harvest of the cells produced no metaphase spreads of a quality suitable for identification of sex chromosomes.

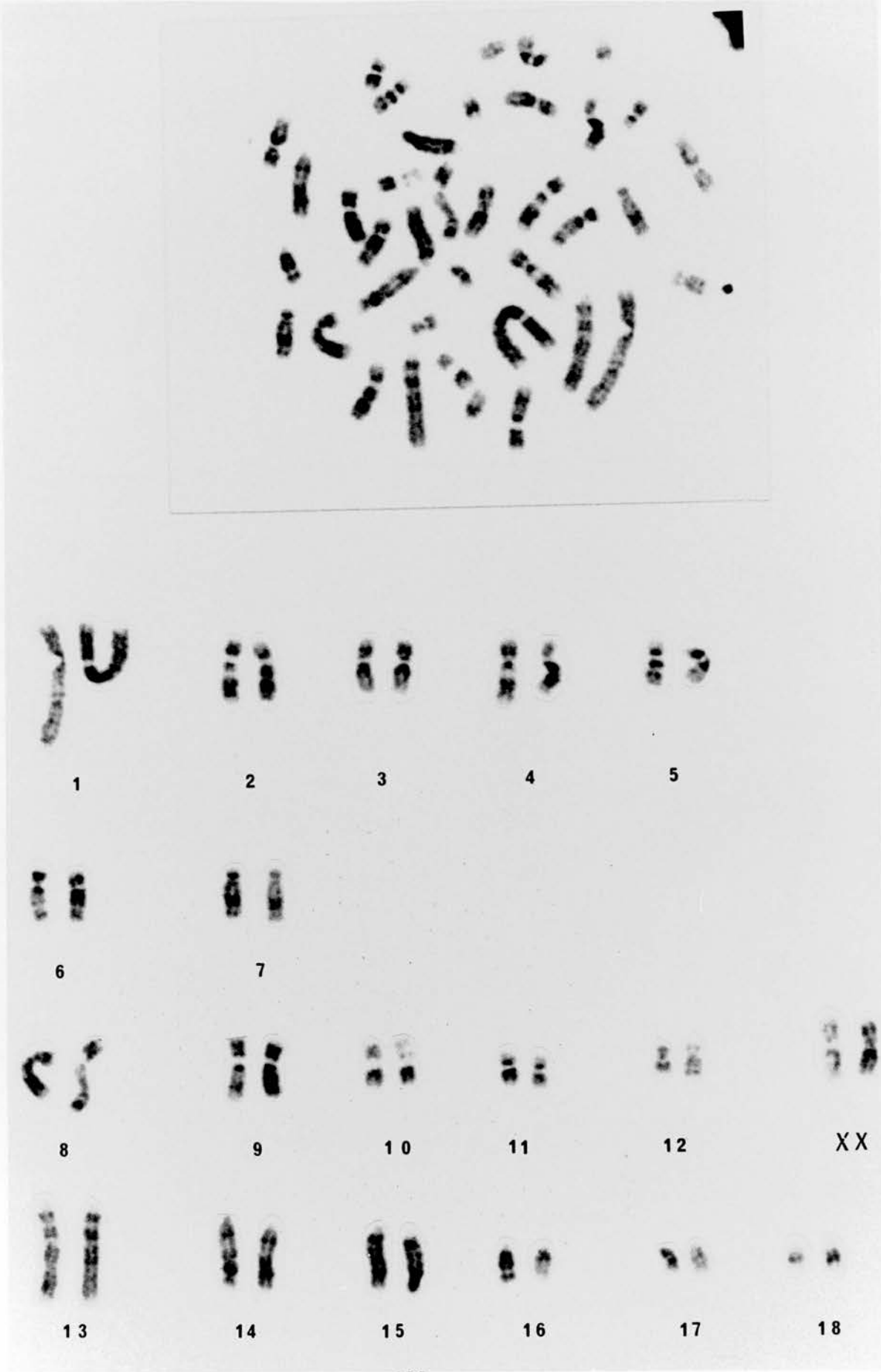


Figure 8.1. G-banded karyotype of intersex 4.

Figure 8.2. G-banded Karyotype of intersex 5.

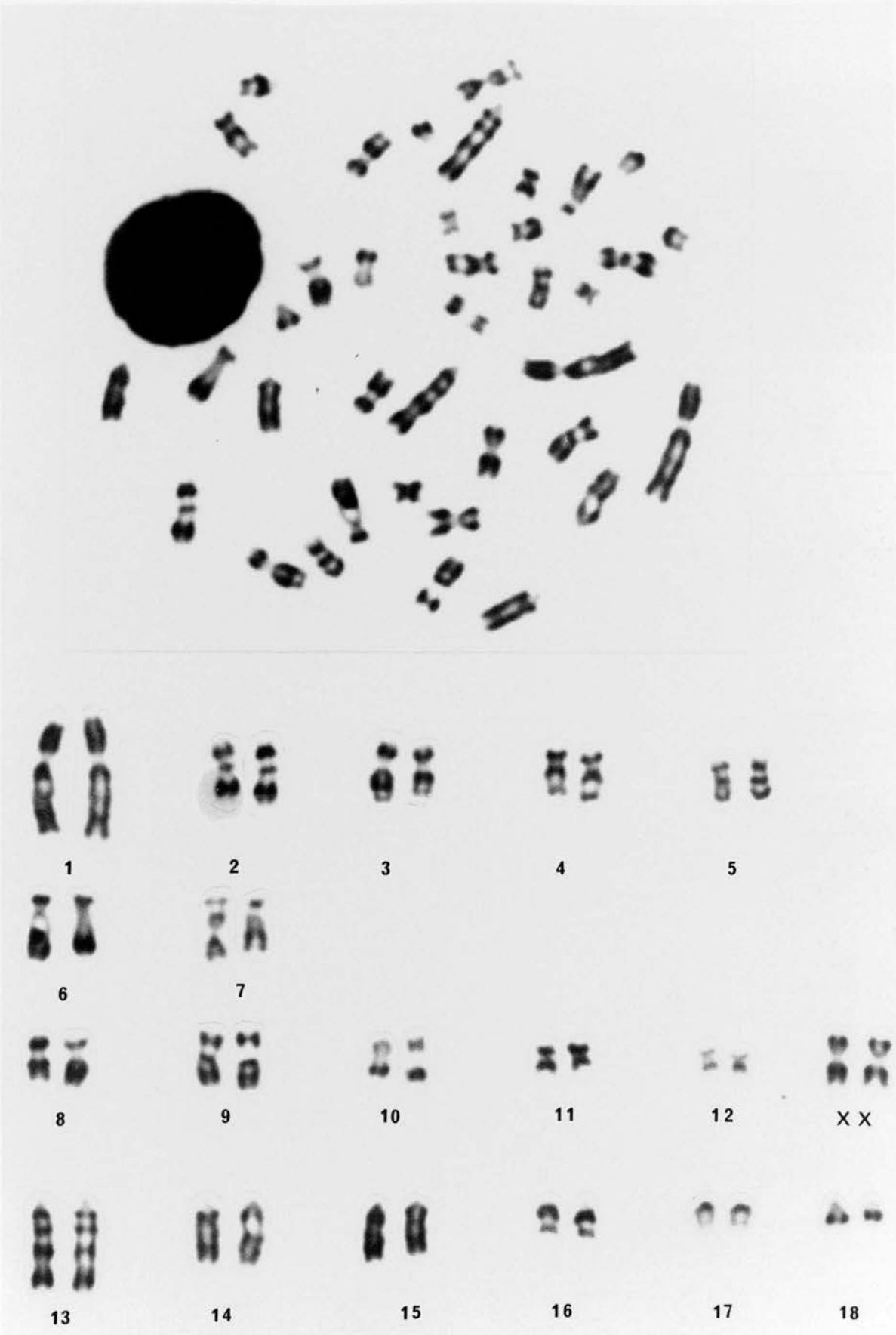


Figure 8.3. G-banded karyotype of intersex 7.

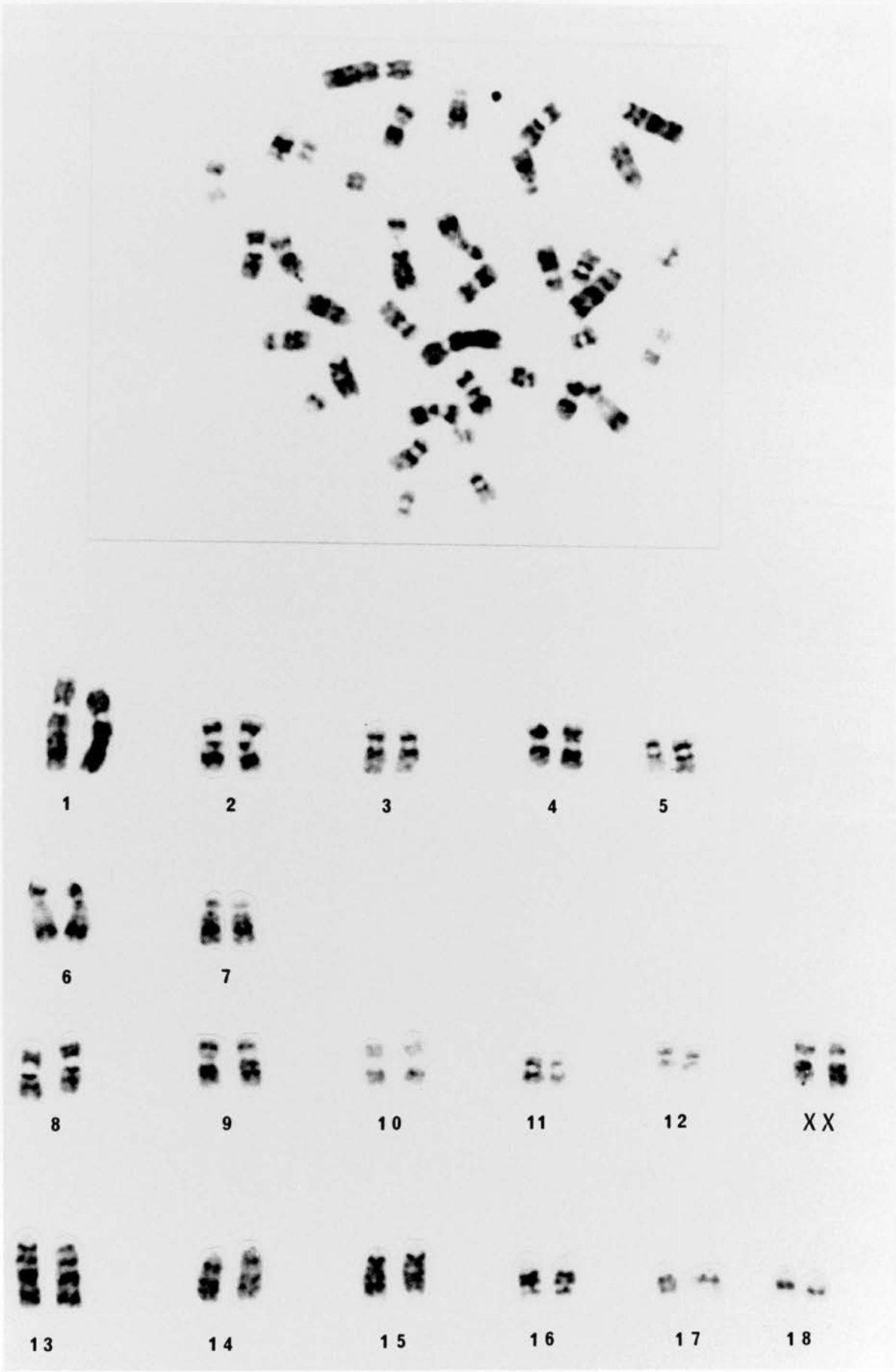


Figure 8.4. G-banded Karyotype of intersex 9.

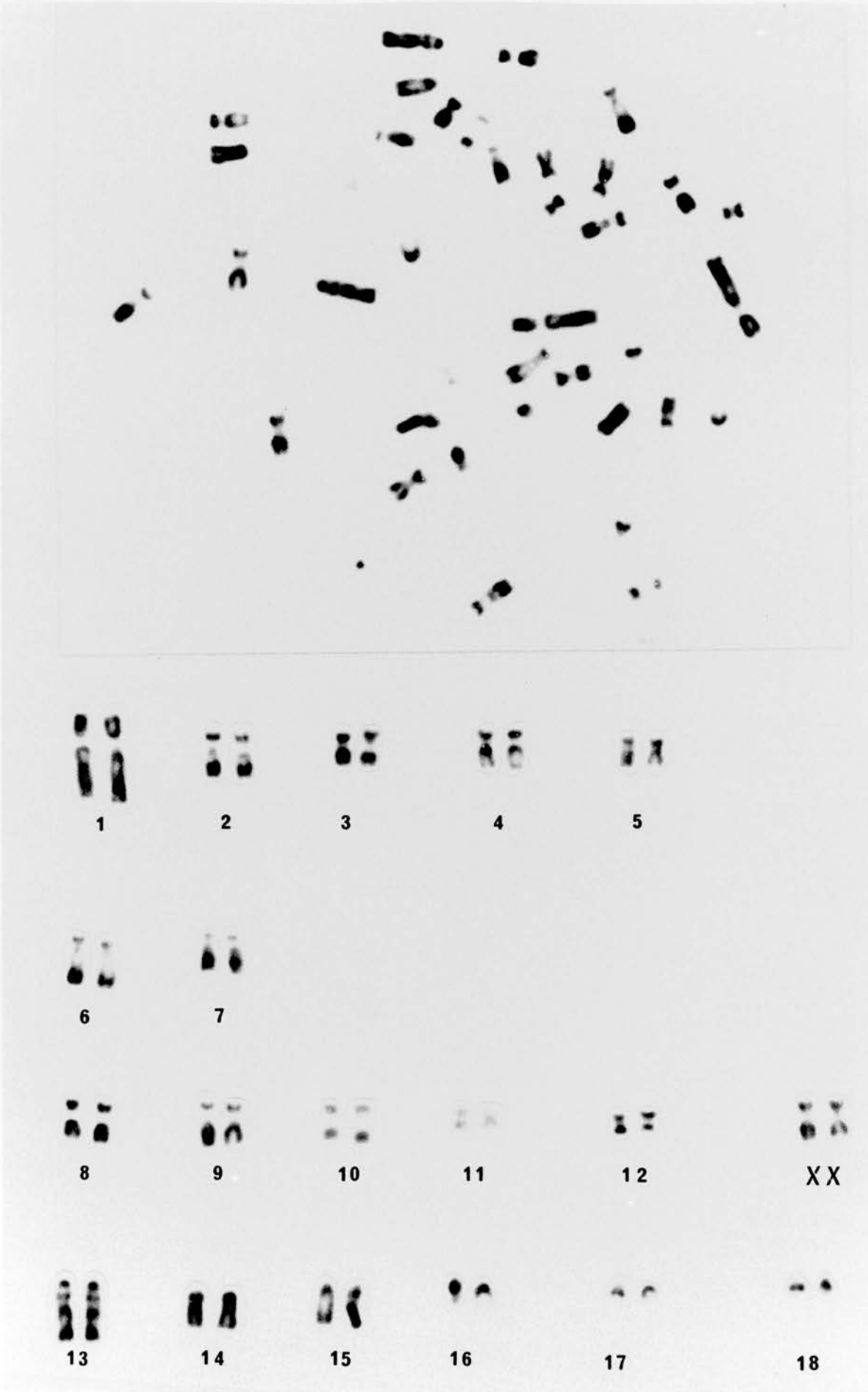


Figure 8.5. G-banded Karyotype of intersex 307.

8.4 Discussion

In order to eliminate the possibility that an intersex individual is a mosaic of XX and XY cells. at least 100 cell karyotypes should be studied. De la Chapelle (1972) gives figures for probabilities that a minor cell line will not be detected in counts of 10, 20, 50, 100 or 200 cells. If the frequency of the minor cell line is 0.5% there is a 95% probability that it will not be found if 10 cells are studied; the probability drops to 8% if 500 cells are counted. Obviously, an assessment of 50 cells from the intersex pigs is unlikely to detect an XY cell, if the animal is a mosaic with few XY cells present. However, the wealth of literature on karyotypes of intersex pigs, including chromosome counts in over 100 cells/animal from leucocyte and fibroblast cultures (Breeuwsma, 1970; Melander *et al.*, 1971), supports the findings that intersex pigs are XX. Mosaicism, when it is observed, is found in counts of 25 blood leucocyte spreads (Bosma *et al.*, 1975). Analysis of blood leucocyte karyotypes of freemartin cattle reveals that, on average, 47.6% of the cells are XY (Marcum, 1974) and hence are easily detected.

The G-banded karyotypes failed to show any evidence of translocation of a portion of Y chromosome onto the X or onto an autosome, as is believed to occur in XX human males (de la Chapelle, 1972). Evans *et al.* (1979) used photographs of X chromosome preparations from XX human males to show a significant increase in the size of the p arm of one X chromosome, concluding that this resulted from an Xp-Yp exchange. The advent of Y-specific probes has enabled identification of Y chromosome material in XX human males with apparently normal sex chromosomes (Müller *et al.*, 1986; Magenis *et al.*, 1987). Screening of DNA from intersex pigs, using a Y-specific probe, would identify Y-chromosome material which may be present. There is a possibility that the probe developed by Page *et al.* (1987) will identify the testis determining region of the Y chromosome (*TDF*) in intersex pigs. This probe is known to identify DNA sequences found in males but not females of a wide variety of mammalian species. However, Page *et al.* (1987) do not mention whether the probe is sex-specific in the pig.

Whilst a Y-specific probe is seen as an important tool in analysis of DNA from the intersex animal, the presence of *TDF* is apparently not a pre-requisite for testicular development; Waibel *et al.* (1987) failed to find Y-specific DNA sequences in human true hermaphrodites. Previous analysis of DNA in "intersex humans" involved sex-reversed subjects (*eg.* XX males) rather than individuals possessing ovarian and testicular tissue (Magenis *et al.*, 1987). The presence of *TDF* in the genetic content of every cell might be expected to produce complete sex reversal since its function in the male is to induce testicular differentiation. Explanation of the development of ovotestes is thus difficult, unless non-random inactivation (of the X chromosome bearing *TDF*) is involved. The possibility that XY cells are present only in the testicular tissue of intersex pigs, without being detected in other organs, should not be overlooked, and culture and genetic analysis of this tissue should be a priority.

In the present study, analysis of the karyotypes of peripheral blood leucocytes of eight intersex pigs identified them as XX and not XY individuals. G-banded karyotypes produced for five of the animals, again using peripheral blood leucocytes, revealed apparently normal, 38XX chromosome constitutions.

SECTION II ASSESSMENT OF BRAIN SEX

CHAPTER 9 THE SEXUAL BEHAVIOUR OF INTERSEX PIGS

9.1 Introduction

Boars and gilts reach puberty at about 6 months of age. Environmental factors such as contact with other pigs, crowding, or transport can act to influence the timing of first oestrus (Kilgour and Dalton, 1984).

The courtship ritual of the boar is shown best by males that have been reared with other pigs rather than in isolation (Hemsworth *et al.*, 1978). Head to head meeting is followed by naso-genital contact and/or nosing under the belly. The male grinds and chomps his teeth and salivates profusely, frothing at the mouth. Boar's saliva is known to contain pheromones, 16-androstenes, which are not present in the female, and are produced by the submaxillary gland (Booth *et al.*, 1973). These pheromones play an important role in initiating the "standing" response in an oestrous female (Signoret and Du Mesnil du Buisson, 1961; Melrose *et al.*, 1971). The boar also emits a "courting song", successive low pitched grunts which have been shown to induce a standing response in otherwise non-standing oestrous females (Signoret and Du Mesnil du Buisson, 1961).

This "standing response", seen in pigs in oestrus, is characterised by an arched back, pricking of the ears and a ridged stance. Pigs in mid-oestrus can be identified by applying pressure to the back; the animal responds by becoming immobile (Signoret, 1971). A series of experiments carried out by Signoret and Du Mesnil du Buisson (1961) showed that several factors are important in initiating the standing response in the absence of the boar. Pressure on the back alone will induce a response in 48% of gilts. Olfactory and auditory stimuli from the boar (pheromones and the "courting song") were the most effective in increasing the percentage of oestrous gilts showing a standing response, the addition of visual and tactile stimuli increasing the number responding to back pressure by 7% and 3% respectively.

Signoret (1967) showed that, within a herd, an animal in oestrus will seek out the boar. This activity can be assessed by means of a T-maze in which an oestrous female is given the choice of a penned male pig or another female. Oestrous, but not anoestrous females will spend significantly longer standing next to the pen holding the male.

Anoestrous females, introduced to strange male or female pigs, may show aggression towards each other, as will two boars unless they have been reared and kept together. The two animals circle round strutting shoulder to shoulder and, in the case of two boars, chomping and frothing as seen during courtship behaviour. Sideways pressure applied with their shoulders increases and the two pigs (male or female) may slash with their teeth (Kilgour and Dalton, 1984).

The sexual behaviour of four intersex pigs was observed in this study, animals 4, 5, 7 and 9 being used. The observations were made before any laparotomies had been performed so that definition as an intersex was made on external appearance alone. None of the pigs had shown signs of oestrus (swelling and reddening of the vulva, lordosis *etc.*) although a mature boar was kept in the same room.

Observations were made of a) the response of each intersex to a mature boar, b) the response to an oestrous gilt and c) the response of an oestrous gilt to an intersex in a T-maze experiment.

9.2 Materials and Methods

The intersex pigs were aged between 15 and 17 months, 150-180 kg. The boar (approximately 2 years old) was of proven fertility and was housed in the same building, separated by a barred pen 5 yards away. Gilts were group-housed in a different building and were aged approx 7 months. They were checked daily with the boar for signs of oestrus.

Interactions between individual intersexes and an oestrous gilt, or the boar, were observed in a central area of the building housing the boar and intersexes. The animals were put together for ten minutes, unless

fighting started in which case they were separated. According to Signoret (1972) the average time taken for a boar to mount a female in oestrus, from the time of the female exhibiting the "standing response", is 43 seconds. Ten minutes were therefore considered long enough for intersex animals to show a response to a gilt in oestrus.

Figure 9.1 is a plan of the T-maze used in part c). The subject was shut into the maze for 5 minutes with a potential sexual partner in pen A and pen B. Observations were made from behind pen B and the time spent by the subject in zones A and B (estimated to be of one pig length) was recorded with a stopwatch. To confirm Signoret's findings (1967) that the oestrous pig discriminates between a boar and gilt whereas anoestrous pigs do not do so, a control experiment was undertaken in which an oestrous gilt was used as the subject, being given the choice between a boar and an anoestrous gilt. or two anoestrous gilts. An anoestrous gilt was then used as the subject, the choices being an anoestrous gilt and boar.

Oestrous gilts were used as subjects to test the attraction to an intersex pig, the choices in the T-maze being an intersex or an anoestrous gilt. Different subjects were used in each observation and the pens were cleaned between observations. Two or three tests were made with each intersex using different subjects. The student's t-test was used to test for significant differences between time spent by the subject next to different pens.

9.3 Results

Table 9.1 gives the results of observations of intersex pig behaviour with a boar and an oestrous gilt. Table 9.2 shows the results of the T-maze experiment, with an oestrous or anoestrous gilt being given the choice between a boar and an anoestrous gilt, or two anoestrous gilts.

Results of the T-maze experiment using intersex pigs and anoestrous gilts in pens A and B are given in Table 9.3.

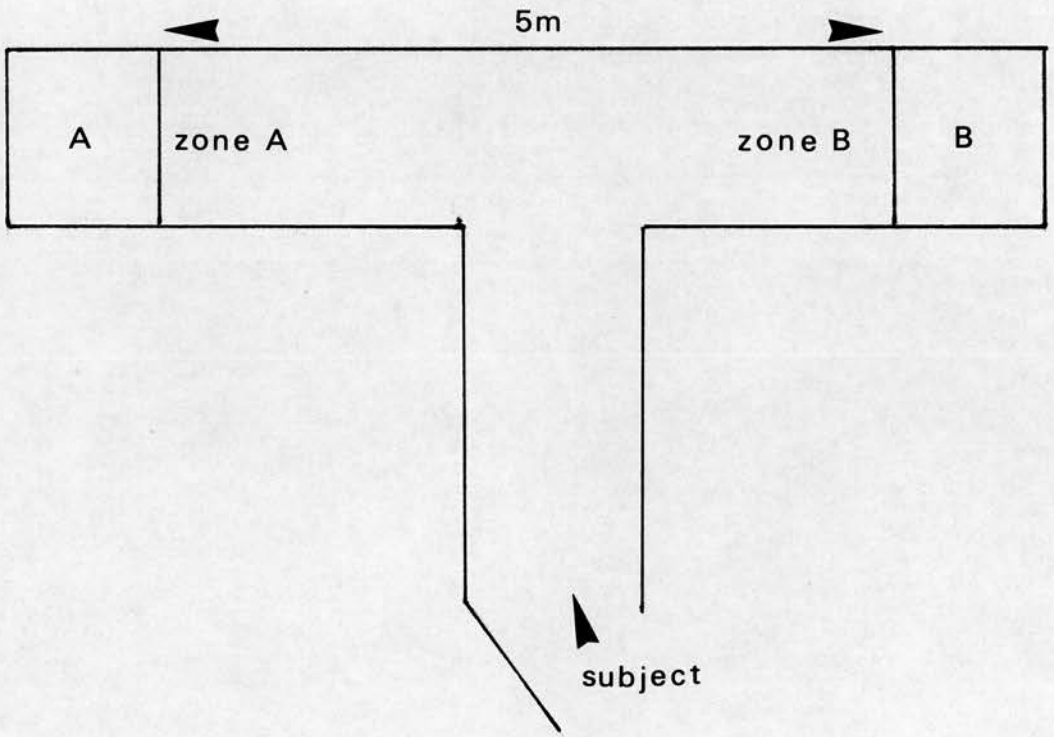


Figure 9.1. Design of the T-maze

Table 9.1. Behaviour of intersex pigs with a boar and with an oestrous gilt.

NUMBER	BEHAVIOUR WITH	
	BOAR	OESTROUS GILT
4	4 chomping, little frothing Aggressive towards boar.	4 avoided gilt. No chomping no frothing. No sexual interest
5	5 chomping, boar attempted to mount but 5 wouldn't stand. No aggression.	Uninterested in gilt. No chomping, no frothing.
7	7 chomping, frothing and grunting. Aggression, some fighting.	7 chomping, frothing. Gilt showed "standing response". 7 mounted gilt and took up stance of normal male.
9	9 chomping, frothing, grunting. Aggression.	9 chomping, frothing. Gilt showed "standing response". 9 mounted. Some ejaculate collected from penile clitoris No sperm present.

Table 9.2. Mean time spent by subjects in each arm of a T-maze given the choice between a) a boar and an anoestrous gilt, b) two anoestrous gilts.

PHYSIOLOGICAL STATE OF TEST SUBJECT	MEAN TIME (+/-se), IN SECONDS PER 5 MINUTE INTERVAL, SPENT NEXT TO:-	
	a) BOAR	ANOESTROUS GILT
oestrus	239.5 (24.5)	25.5 (9.5) *
anoestrus	46.0 (21.4)	56.7 (19.6) n.s
	b) ANOESTROUS GILT	ANOESTROUS GILT
oestrus	42.0 (4.7)	54.0 (19.4) n.s

* $p < 0.05$ n.s not significant

Table 9.3. Mean time spent by an oestrous gilt in each arm of a T-maze, given the choice between an intersex pig and an anoestrous gilt.

INTERSEX NUMBER	MEAN TIME (+/-se), IN SECONDS PER 5 MINUTE INTERVAL, SPENT NEXT TO:-	
	INTERSEX	ANOESTROUS GILT
4	238.2 (4.6)	10.0 (2.5) **
5	69.7 (14.5)	95.2 (37.1) n.s
7	162.5 (22.8)	65.7 (6.7) *
9	190.5 (31.7)	64.8 (15.6) *

* $p < 0.05$ ** $p < 0.01$ n.s not significant

None of the intersexes, whilst held in the pen, produced the same "courting song" as the boar. Intersexes 4 and 7 grunted occassionally in response to the approach of the oestrous gilt, and 7 and 9 showed chomping and frothing though not to the same extent as was seen in the boar.

9.4 Discussion

From the observations of individual intersexes with an oestrous gilt and with a boar, pigs 7 and 9 behaved as if they were males, both showed chomping and frothing and mounted the oestrous female. Whilst normal gilts may mount each other, chomping and frothing are rarely seen, although treatment of gilts with testosterone propionate may induce chomping (Hafez *et al.*, 1962).

The T-maze controls (Table 9.2) confirmed that an oestrous gilt can distinguish between a boar and a gilt. This allowed the use of an oestrous gilt to indicate the "apparent" sex of an intersex. Despite very little frothing of pig 4, her female appearance and lack of interest shown towards an oestrous gilt, in the T-maze she was observed to be attractive to oestrous females (Table 9.3). Presumably olfactory stimuli from the intersex pig were sufficient to convince the oestrous gilt that the intersex was a boar. Since the animal never urinated in the maze, it is assumed that pheromones in the saliva were responsible for the attraction. The compounds acting as pheromones (and those responsible for boar taint) are 16-unsaturated C_{19} steroids (or 16-androstenes), produced in large quantities by the boar testis (Patterson, 1968; Reed *et al.*, 1974). Some of these steroids are taken up by the salivary gland and secreted in the saliva. High levels of 16-androstenes have been found in the submaxillary glands of intersex pigs, indicating the presence of secretory testicular tissue (Booth and Polge, 1976). However, these authors noted that, in contrast to an XX/XY intersex pig, there was little salivation in sexually excited XX intersexes, leading to the suggestion that the absence of a Y chromosome may render the submaxillary glands

refractory to androgen stimulation. From the T-maze observations it appears that an XX chromosome complement does not inhibit the release of pheromones in the saliva, although the quantity of saliva produced was less than that produced by the boar.

Comparing the behaviour of the intersexes in the T-maze with that of the boar, it seems that the lack of a "courting song" did not affect the response of the oestrous gilt. This is not in complete agreement with the findings of Signoret and Du Mesnil du Buisson (1961) in which a recording of the "courting song" alone elicited a standing response in 51% of oestrous females which were negative, in the absence of the male, when tested by exerting pressure on their backs. Apparently the rhythm of the grunts emitted by the boar is also important (Signoret and Du Mesnil du Buisson, 1961), but in the case of pig 4 it seems that olfactory and possibly visual stimuli were sufficient to attract and elicit the standing response in oestrous gilts.

The inability, or reluctance, of the intersexes to emit a "courting song" also raises questions as to the development of sexual behaviour. In the intersex, at least, the neural pathways involved in salivation and pheromone production are obviously independent of those necessary for courtship vocalization. Even when sexually excited during mounting of the females, none of the intersexes emitted any sounds resembling the "song" of the boar.

Very little is known about the sexual differentiation of the pig's behaviour patterns. The sexual development of gilts injected with testosterone propionate, within 48 hours of birth, is not affected (Zimbelman, 1964) whereas male pigs exposed to exogenous oestrogens *in utero* subsequently show abnormal sexual behaviour (Dorner *et al.*, 1977). This suggests that hormonal exposure of the brain prenatally is important in determining sexual behaviour, but the effect of continuous (and simultaneous) exposure to ovarian and testicular hormones is not documented. The possession of an ovotestis presumably subjects the brain of intersex pigs to such influences both before and after birth, making them excellent models for the study of brain sex and of the ontogeny of sex behaviour. As with gonadal development, for normal sexual development, demasculinization (*i.e.* loss of male behavioural

characteristics) and defeminization of the brain must occur as well as feminization and masculinization. The predominantly male behaviour of intersexes 7 and 9 suggests that, despite their XX genotype, the brain in these pigs has undergone masculinization as well as defeminization, whereas the lack of any overt sexual behaviour in pig 5 indicates defeminization without masculinization.

Regarding the association between behaviour and masculinization of the gonads; the gonads in animal 5 were never identified, 7 had two ovotestes, animal 4 had an ovotestis on the left and testis on the right and pig 9 had an abdominal testis on the left and scrotal testis on the right, *i.e.* in the order 5, 7, 4, 9 with number 9 possessing the most testicular tissue. In terms of "attractiveness" to an oestrous gilt, the animals could be ranked, in order of increasing "masculinity", 5 7, 9, 4, and the order of increasing interest in the oestrous gilt (Table 9.1) would be 5, 4, 7, 9, *i.e.* neither ranking of behaviour corresponding to the rank of masculinity as defined by gonadal type. This suggests that the circulating testosterone concentration in the adult (and presumably in the fetus) does not determine the "pitch" of sexual behaviour, environmental factors *eg.* rearing conditions, contact with other animals, may well play a role. However, whether the response to certain stimuli is "male" or "female" depends on the prenatal programming of the brain. Whilst administration of testosterone to an adult female pig may suppress oestrous behaviour, it will not result in the same behavioural responses observed in intersex animals.

CHAPTER 10 THE LUTEINIZING HORMONE RESPONSE TO AN OESTRADIOL CHALLENGE IN INTERSEX PIGS

10.1 Introduction

The stimulatory (*i.e.* positive) oestrogen feedback mechanism that causes a surge of luteinizing hormone (LH) release prior to ovulation in mature mammals has been shown to be absent in males of several species, including rats (Neill, 1972), hamsters (Buhl *et al.* 1978) and sheep (Bolt, 1971; Karsch and Foster, 1975). In monkeys, a sexual dimorphism of the stimulatory oestrogen feedback mechanism does not seem to exist since administration of oestradiol benzoate to both males and females elicits an LH surge (Karsch *et al.*, 1973). Ford and Schanbacher (1977) suggested that an oestradiol challenge would elicit an LH surge in both female and castrated male pigs. However, Elsaesser and Parvizi (1979) showed that, whilst peripheral blood LH concentrations in the pre-pubertal male pig will return to pre-oestradiol treatment levels 72 hours later, females respond, 48-72 hours after an oestradiol benzoate challenge with a surge release of LH not seen in the males. This stimulatory oestrogen feedback mechanism was significantly impaired by prenatal testosterone treatment. The response to oestradiol differs between male and female miniature pigs even at fourteen days of age (Elsaesser *et al.*, 1978b). However, Foxcroft *et al.* (1984) suggested an ovarian (possibly oestrogen) dependent maturation of the feedback response in domestic gilts, such that 60 day old gilts show a smaller LH peak and a greater interval between oestradiol benzoate administration and the gonadotrophic surge than 160 day old gilts.

Neonatal exposure of female rats to appropriate doses of androgen abolishes the positive feedback effect of oestradiol and prenatal exposure influences this response in mice (Barraclough, 1955) and sheep (Clarke and Scaramuzzi, 1978). An assessment of sexual receptivity in female pigs showed that pre- and postnatal testosterone treatment was more effective in delaying oestrus than was prenatal treatment alone (Ford and Christenson, 1987).

Observation of five intersex pigs failed to detect oestrous behaviour, indicating defeminization (*i.e.* loss of traits inherent to females) of sexual behaviour. The following experiment was conducted in order to test the hormonal, rather than behavioural, response of the brain in order to assess the effect of pre- and postnatal exposure to testosterone.

10.2 Materials and Methods

The animals used were numbers 2, 4, 5, 7 and 93. They weighed between 90 and 150 kg at the time of the experiment. All animals were karyotyped as XX.

Oestrous cycles were not detected in any of the animals. Mid-ventral laparotomies were performed in order to determine the gonadal type. Anaesthesia was induced with pentobarbitone sodium (Nembutal; Ceva Ltd, UK) and maintained, after intubation, by a mixture of halothane (Fluothane; ICI), nitrous oxide and oxygen. The reproductive tract and gonads were exteriorised, when possible, and inspected for signs of previous cyclic activity. Tracts and gonads were recovered at slaughter for more detailed examination.

Animals received intramuscular injections (60 µg/kg body weight) of oestradiol benzoate (Intervet laboratories Ltd, Cambridge) and were observed for signs of oestrus, vulval reddening and swelling being taken as an indication of an oestrogenic response.

Blood sampling

Indwelling jugular catheters were fitted to permit frequent bleeding. Samples were collected in heparinized tubes and centrifuged at 1500*g* for 10 minutes at 4° C, and plasma stored at -20° C until assay.

For LH analysis, samples (4ml) were taken every 24 hours for 48 hours before,and every 4 hours for up to 112 hours after, the oestradiol injection. 10 ml samples were taken every 12 hours for oestradiol analysis, except for 6 samples taken even hour immediately after the challenge. Daily samples were also assayed for progesterone and testosterone.

Assays

Plasma LH concentrations were quantified by a homologous double antibody radioimmunoassay (RIA). The primary antiserum was raised in a goat immunized against a purified porcine LH (pLH) preparation, SDG-2-65 (0.96-1.18 X NIHoLHS19, by bioassay), kindly provided by Dr S G Glenn. The first booster injection, six months after initial immunization using Freund's complete adjuvant, produced a series of sera with good titres and specificity. The antiserum characterized in the present assays, GRF-G 81/1, bound approximately 20% of radiolabelled pLH in the absence of unlabelled antigen, at an initial dilution of 1:60,000. Other assay methodology was as described previously (Foxcroft *et al.*, 1984) with the following minor modification. To enhance precipitation of antibody bound hormone, second antibody (donkey anti-goat gamma globulin, AGGG, raised locally) at 1:40 dilution was pre-incubated for 24 hours with 10% w/v polyethylene glycol 6000 (PEG; BDH Ltd, Poole); 200µl of PEG-AGGG was then added to assay tubes and a minimum period of 8 hours incubation at 4° C then followed before centrifugation and aspiration. Reproducible standard curves were obtained in this assay with a range of standard potencies from 0.01 to 0.5 ng/tube and the overall limit of sensitivity, defined as 90% of total binding, was 0.02 ng/tube. A standard plasma pool, routinely assayed at 50, 100 and 200 µl showed parallelism to the standard curve. The recovery of pLH when added to porcine plasma of known potency ranged from 92% to 102% confirming accuracy. The specificity of the antibody was checked by carrying out cross-reaction studies with pPRL and pFSH; at 50% binding these hormones showed 0.17% and 0.88% cross-reactivity,

respectively. All samples were run at 200 µl in duplicate in a single assay for which the intra-assay coefficient of variation was <10%.

Radioimmunoassays for oestradiol, progesterone and testosterone were as described by Cook *et al.* (1977) except that bound and free steroid were separated using a second antibody. Oestradiol antiserum was obtained from Immunodiagnostics, UK.

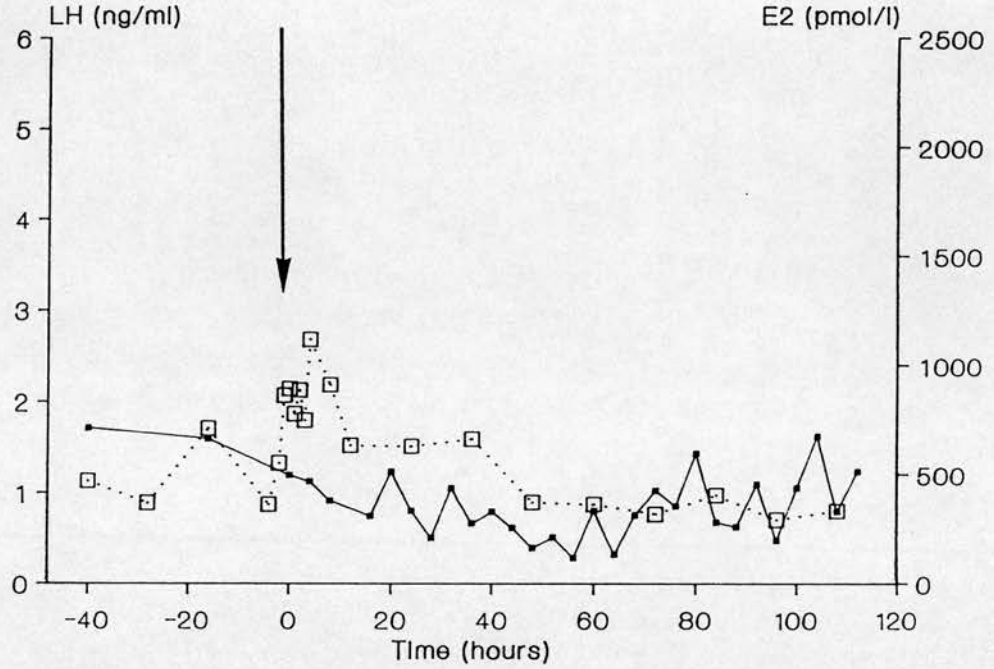
Cross reactions were 8.7% with oestrone for the oestradiol antiserum, 4.3% and 1.8% with deoxycorticosterone and corticosterone respectively for the progesterone antiserum and 33.8% and 6.4% with DHT and androstenedione respectively for the testosterone antiserum. All other cross-reactions were <1%. The assay sensitivities were 59pmol/l, 2.2nmol/l and 1.0nmol/l for oestradiol, progesterone and testosterone respectively. Coefficients of variation were <16% between assays.

10.3 Results

In all animals, plasma oestradiol rose following the oestradiol benzoate (O.B) challenge, the mean concentration 84 hours after the injection was 402 (+/- 115) pmol/l. Following the oestradiol injection, progesterone concentrations remained below 10 nmol/l in all animals, except number 93, in which the progesterone concentrations were between 40 and 75 nmol/l. Testosterone concentrations were, in all 5 animals, <3.0 nmol/l throughout the sampling.

Figures 10.1 to 10.3 show the LH and oestradiol profiles before and after the oestradiol injections in the 5 animals, Figure 10.4 shows testosterone and progesterone concentrations. Table 10.1 gives the maximum LH concentrations recorded after the O.B injection. The mean of the maximum LH concentrations in the animals following the injection (time 0) was 2.10 (+/- 0.41) ng/ml at 86 (+/- 6.8) hours. In animals 2 and 4, higher LH concentrations were recorded before than after the O.B injection.

Pig 2



Pig 4

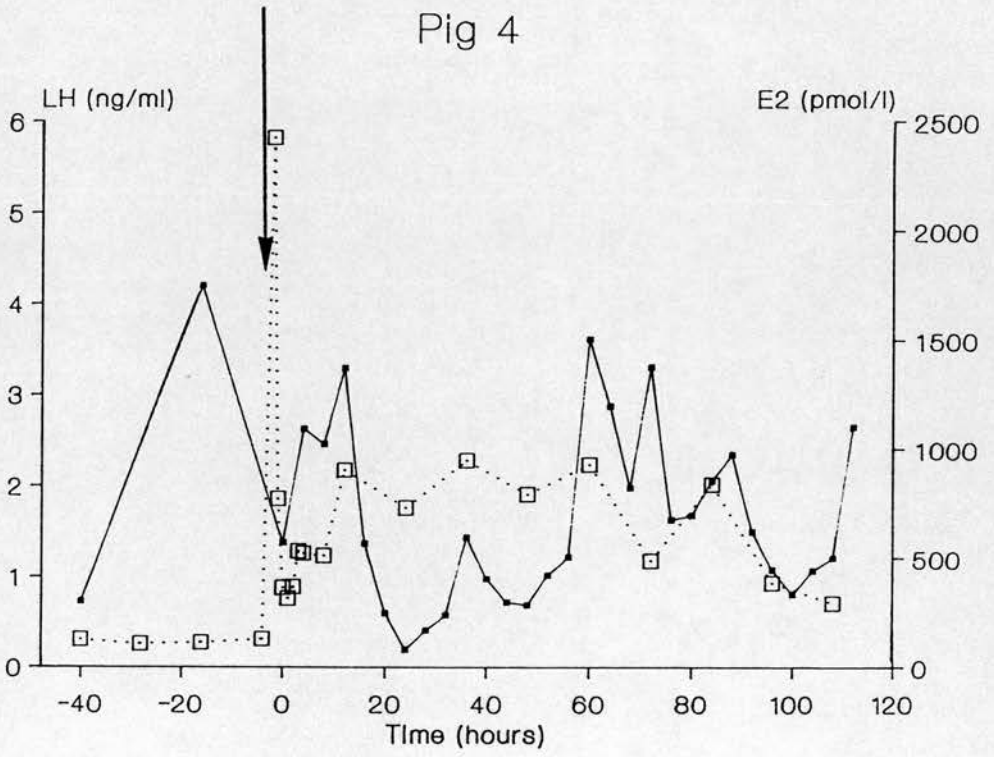
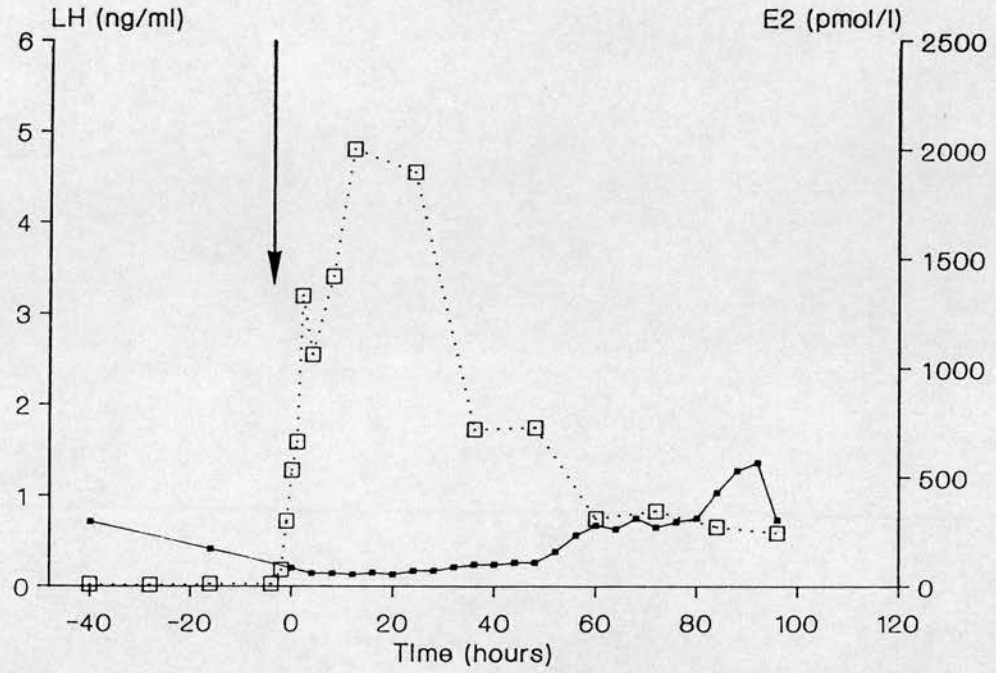


Figure 10.1. Concentrations of LH (▪——▪) and oestradiol (⊡·····⊡) before and after an injection of oestradiol benzoate (arrow).

Pig 5



Pig 7

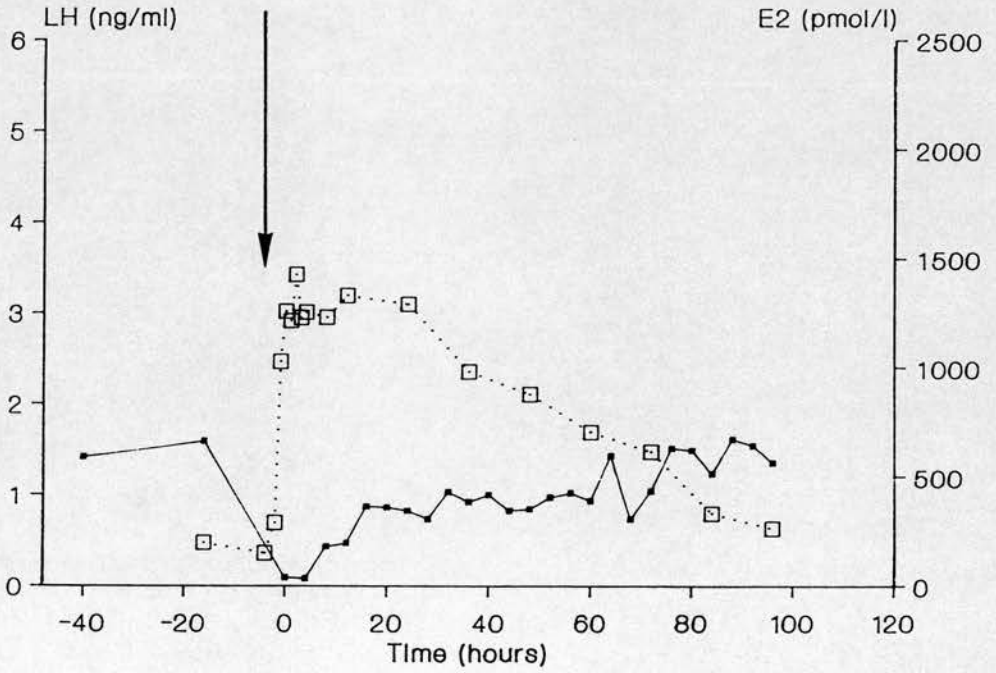


Figure 10.2. Concentrations of LH (■——■) and oestradiol (⊡·····⊡) before and after an injection of oestradiol benzoate (arrow).

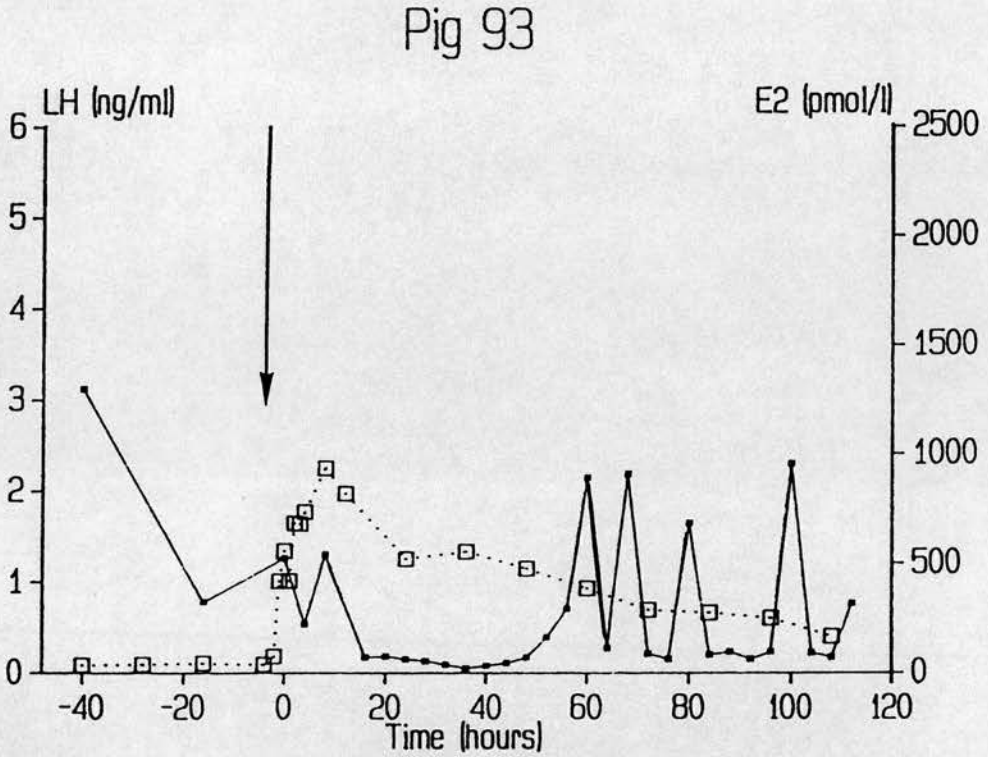
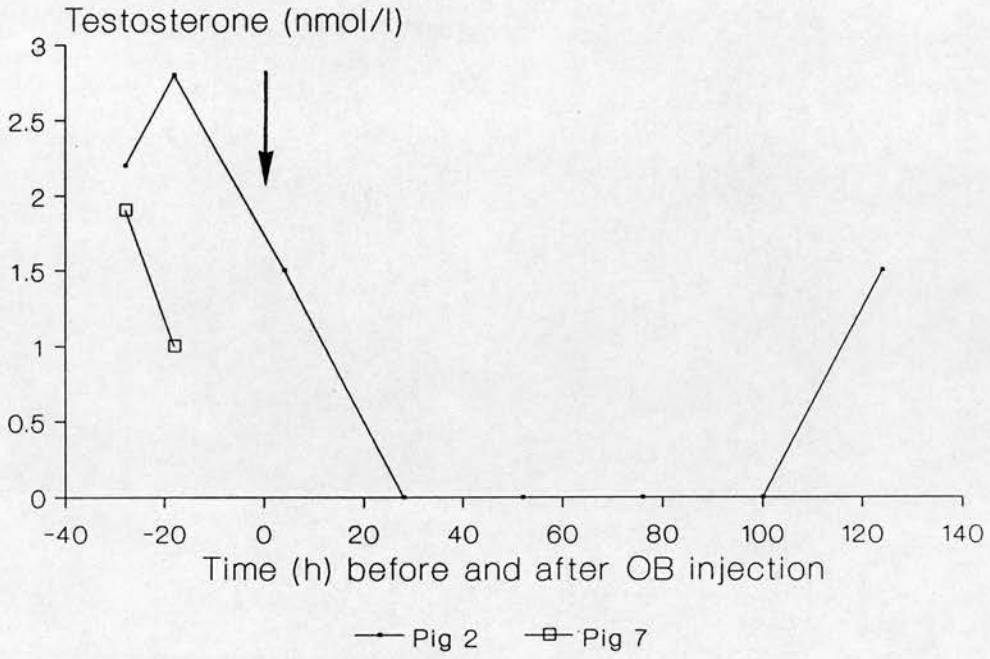


Figure 10.3. Concentrations of LH (■——■) and oestradiol (⊡·····⊡) before and after an injection of oestradiol benzoate (arrow).



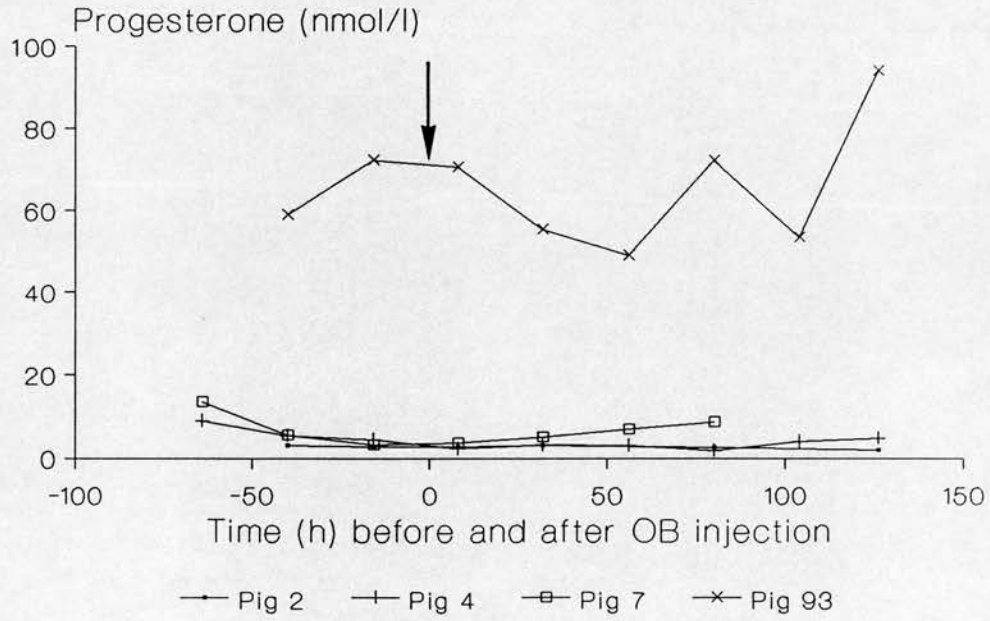


Figure 10.4. Concentrations of testosterone and progesterone before and after an injection of oestradiol benzoate (arrow). Animals not represented had steroid concentrations below the sensitivity of the assay.

Table 10.1. Maximum LH concentrations (ng/ml) recorded in jugular blood samples, following oestradiol benzoate (O B) treatment.

ANIMAL	MAXIMUM CONC OF LH (ng/ml) AFTER O B INJECTION	TIME (HOURS) TO LH PEAK AFTER O B INJECTION
2	1.62	88
4	3.61	60
5	1.36	92
7	1.61	88
93	2.29	100
MEAN (+/- se)	2.10 (0.41)	

MEAN VALUES FOR:-

normal female (Foxcroft *et al.*, 1984)

8.9 ng/ml 37 hours

normal male (Ford and Schanbacher, 1977)

approx 1.7 ng/ml approx 90 hours

MEAN VALUE FOR :-

pre-injection LH concentration in intersex pigs ng/ml (+/- se)

1.62 (0.38)

10.4 Discussion

By 160 days of age, domestic gilts are capable of responding to an oestradiol benzoate challenge with a surge release of luteinizing hormone 37 to 55 hours later (Elsaesser and Foxcroft, 1978; Foxcroft *et al.*, 1984), the average concentration of the surge being 8.9 ng/ml. Using this as a standard, none of the intersex animals in the present study could be said to have shown an LH surge, despite the raised oestradiol concentrations. The mean pre-treatment LH concentration in the present study was 1.62 ng/ml and the mean peak concentration was 2.10 ng/ml. In all animals, following the oestradiol benzoate challenge, peripheral oestradiol remained within the range of peak pre-ovulatory concentrations reported for cyclic gilts (Henricks *et al.*, 1972; Van de Wiel *et al.*, 1981).

Animal 5 showed an LH rise above pre-treatment levels, which reached a maximum 96 hours after the oestradiol challenge. Such a response suggests the operation of an immature feedback mechanism similar to that seen in 60 day old domestic gilts (Foxcroft *et al.*, 1984), a mechanism which these authors suggest matures following exposure to ovarian follicular secretions, possibly oestrogen. The low pre-injection levels of oestradiol in animal 5 support this view, but the masculinized external genitalia indicate prenatal exposure to testosterone, which may also be responsible for the diminished LH release. Mid-ventral laparotomy failed to reveal any gonads although the tract was female.

In four animals, progesterone values were within the range corresponding to follicular phase concentrations in cyclic gilts (Stabenfeldt *et al.*, 1969; Edqvist and Lamm, 1971). In animal 93, however, a high progesterone concentration (presumably secreted by luteal tissue discovered during histological examination) may have prevented an LH surge following the oestradiol injection; oestradiol given during the luteal phase of the oestrous cycle will not elicit an LH surge in rats, sheep or women (Tapper *et al.*, 1974; Karsch *et al.*, 1978; Leyendecker *et al.*, 1972). In all five intersexes, the pattern of LH release, following an oestradiol benzoate injection, bears more similarity to that seen in castrated adult male pigs (approximately 1.7 ng/ml at 90 hours, Ford and Schanbacher,

1977) than to a normal female response (Elsaesser and Foxcroft, 1978; Foxcroft *et al.*, 1984) despite the XX karyotypes.

Prenatal exposure of the brain to testosterone is thought to be the cause of sexual differentiation of the feedback response to oestradiol in rats, treatment with testosterone propionate during pregnancy abolishing the LH response in the female offspring (Neill, 1977). In rhesus monkeys, however, prenatal testosterone does not affect adult LH secretion, both sexes responding to an oestradiol injection with a surge release of LH (Steiner *et al.*, 1976). In the sheep, the effect of prenatal testosterone treatment on the female is said to be quantitative rather than qualitative, since the LH response to oestradiol in the adult is reduced but not abolished (Clarke and Scaramuzzi. 1978). Evidence suggests that this is also the case in pigs (Elsaesser and Parvizi, 1979).

Prenatal treatment of pigs with testosterone between days 30 and 70 of gestation, is more effective in masculinizing the adult LH response to oestradiol in the female than treatment during the second half of pregnancy (Elsaesser and Parvizi, 1979). Testosterone is present in measurable amounts in testes from 26 day old fetuses but maximum testosterone content is reached between day 35-38 (>4 ng/pair) (Raeside and Sigman, 1975) and in umbilical arterial serum on day 35 (>4 ng/ml), concentrations in the female fetus being undetectable at this stage (Ford *et al.*, 1980). These changes in testosterone concentration follow the morphological development of Leydig cells within the testes (Moon and Hardy, 1973; Pelliniemi, 1975). It is thus assumed that differentiation of the brain in the pig is due to exposure to testosterone around day 35 of preganancy (Ford *et al.*, 1980). However, exposure of the 30 day old fetus to testosterone, either via the pregnant sow (Elsaesser and Parvizi, 1979) or directly (Elsaesser *et al.*, 1978a) does not completely abolish the LH response to oestradiol, nor ovulation, in the adult. The presence of testicular tissue in the intersex pigs of this study apparently leads to masculinization of the brain, despite testosterone concentrations being below those reported for mature intact (miniature) or castrated (domestic) pigs (Ellendorf *et al.*, 1975; Ford and Schanbacher, 1977). This indicates either (1), that brain exposure to testosterone before day 30 of gestation is important (unlikely considering the stage of brain development at this age, Patten, 1948; Marrable, 1971), or 2), that

testosterone is not the sole determinant of brain gender. Since development of a normal male reproductive tract involves regression of the Müllerian ducts due to the influence of anti-Müllerian hormone (Jost 1946-47), this hormone could also be implicated in brain defeminization.

Foxcroft *et al.* (1984) have suggested that the lack of an LH surge mechanism in the male pig is not due to the presence of neonatal androgen secretion, but rather to the absence of an ovary during prepubertal life. Whilst the presence of a sexually dimorphic LH response to oestradiol in newborn pigs (Elsaesser *et al.*, 1978b) would seem to refute this, prenatal ovarian influences may be important in counteracting the defeminizing/masculinizing effects of testicular secretions, resulting in the development of a mature feedback response and ovulation in some intersex pigs (Hunter *et al.*, 1982 and 1985). In the intersex pigs used in the present study, testicular secretions are presumably sufficient to cause complete masculinization of the feedback mechanism.

The mechanism involved in masculinization is not clear. It is known that oestrogen is as effective as testosterone in masculinizing neonatal rat brains, aromatization of testosterone to oestradiol in the brain playing a crucial role in masculinization (MacLusky and Naftolin, 1981). An LHRH challenge in male pigs (Pomerantz *et al.*, 1974), and the intersexes of the present study (results not shown), invokes an LH response implying that pituitary insensitivity is not a problem. The lack of hypothalamic/pituitary steroid receptors has been suggested (Elsaesser and Parvizi, 1979) with reference to the rat, where the stimulatory oestrogen feedback mechanism does not operate until 22 days of age (Caligaris *et al.*, 1972) when maturation of hypothalamic receptors occurs (Plapinger and McEwen, 1973). A lack of oestrogen receptors may explain why none of the intersexes studied here showed oestrous behaviour following the oestradiol benzoate injection. However, inhibition of LH secretion was evident in all animals, indicating at least some hypothalamic/pituitary sensitivity to the steroid, albeit as a negative feedback effect. Oestrogen insensitivity is one explanation for infertility in intersex pigs, although the failure of attempts to induce ovulation from an ovotestis, with pregnant mares' serum gonadotrophin (PMSG), in an anoestrous intersex (Hunter *et al.*, 1985), suggests that the gonads may be unresponsive to gonadotrophins. Recorded pregnancies in

intersex pigs are usually the result of ovulations from an ovary rather than an ovotestis (Scofield *et al.*, 1969) although Basrur and Kanagawa (1971) report finding luteal tissue in ovotestes.

SECTION III AETIOLOGY OF THE INTERSEX CONDITION

CHAPTER 11 THE HORMONAL RESPONSE TO AN ACTH CHALLENGE

11.1 Introduction

The asymmetry of gonadal development in intersex pigs points to a non-systemic aetiology, such that the right gonad in the affected animal is more likely to develop as a testis or ovotestis than the left gonad. It seems improbable that maternal hormones could effect such a localised action, and attempts to alter gonadal sex in eutherian mammals via administration of hormones to the pregnant dam have failed.

Under certain circumstances, the adrenal glands are known to cause virilization in female mammals, due to the adrenocortical secretion of androgens, including testosterone (see Chapter 4). The close proximity of developing gonads and adrenal glands, and the asymmetry in position and blood supply (see Figure 11.1) are all factors contributing to the suggestion that the adrenal glands may play a role in the aetiology in pigs. Breeuwsma (1970) proposed that crowding of embryos *in utero* contributed to the incidence of intersex pigs, and that the adrenal glands were involved in asymmetrical testicular development. Gerneke (1967) discussed the similarities and differences between intersexuality in pigs and the "adrenogenital syndrome" (CAH) in humans. However, as Gerneke (1967) concludes, the human condition mainly affects the urogenital sinus and genital tubercle, and not the gonads, as occurs in intersex pigs. Study of adrenal histology in eight cases (Gerneke, 1967) identified only two animals in which adrenal hyperplasia had occurred, and Johnston *et al.* (1958) state that the adrenal glands in 25 intersex pigs were normal.

The experiments described in this chapter aimed to investigate the possibility that early incorporation of adrenal tissue into the developing gonad could lead to masculinization. Assuming that the adrenocortical tissue, once incorporated, would continue to respond to ACTH, a challenge with this hormone might be expected to produce a characteristic response

from the gonad. A rise in cortisol secretion from the gonad would indicate the presence of adrenocortical cells, and in order to determine whether the response was from the gonad or the adrenal glands, an ACTH challenge was given before and after gonadectomy.

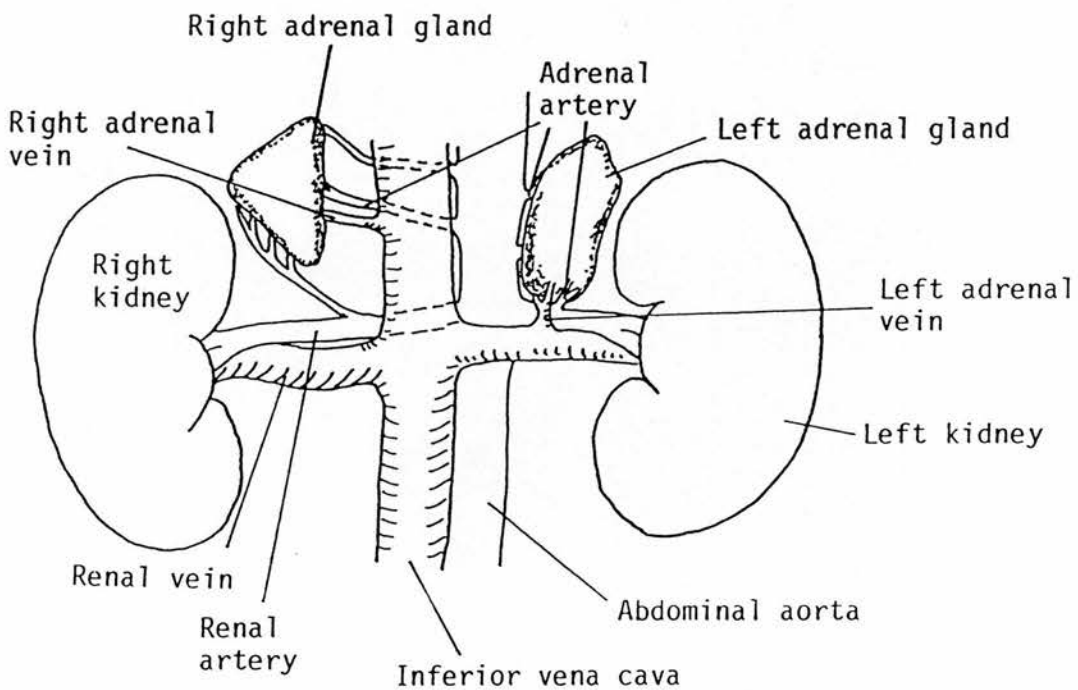


Figure 11.1. Location and blood supply (in the human) of the adrenal glands (schematic). From Tyrrell and Forsham (1983).

11.2 Materials and Methods

To enable frequent blood sampling, all animals were fitted with an indwelling catheter inserted into an ear or a jugular vein. Anaesthesia was induced with pentobarbitone sodium (Nembutal; Ceva Ltd, UK) and, after endotracheal intubation, maintained by semi-closed circuit administration of halothane (Fluothane, ICI), nitrous oxide and oxygen. Animals were grouped according to treatment.

Group 1 Intersex controls

Animals 2 and 753 were sampled under anaesthetic, once the catheter was in place. Mid-ventral laparotomy was performed on pig 753 to enable blood sampling from the right abdominal testis. Animal 2 received no challenge, 753 received 1 ml of Eagle's medium (Flow laboratories) via a jugular catheter.

Group 2 ACTH challenge, conscious animals

Surgery is known to cause an increase in cortisol secretion in humans (Plumpton *et al.*, 1969). Three pigs (2, 93 and 753) were therefore challenged with ACTH after recovering from surgery to fit indwelling catheters, in order to test the response to the hormone in the absence of surgical stress. ACTH was administered in the form of 1 ml (250 µg/ml) of Synacthen (Ciba), a synthetic polypeptide consisting of the first 24 amino acids of ACTH.

Group 3 ACTH challenge, anaesthetised (unconscious) animals

Animals 93, 100, 307 and two normal gilts (aged approximately 6 months) each received 1 ml Synacthen under anaesthesia and during mid-ventral laparotomy to enable gonadal blood sampling. ACTH challenges were given via indwelling ear vein or jugular catheters. Pig 100 received 1 ml of saline prior to the Synacthen challenge. Two 0.5 ml doses of Synacthen were given to pig 93.

Group 4 ACTH challenge before and after gonadectomy

Animals 096, 1161 and 502 were anaesthetised, fitted with indwelling catheters and subjected to mid-ventral laparotomy. A 1ml dose of Synacthen was given before, and 1ml after gonadectomy. 096 possessed two abdominal testes, 1161 a left ovary and right (abdominal) testis, and 502 a left scrotal and right abdominal testis. Blood samples were taken from the gonads of pig 502, together with systemic blood from the jugular catheter.

Blood sampling and assay of samples

Blood samples were taken, if possible, every five minutes, and were collected into heparinised tubes kept on ice. Plasma was separated by centrifugation at 1500*g* for 10 minutes, at 4° C, and stored at -20° C until assayed.

Radioimmunoassays for oestradiol, progesterone and testosterone were as described by Cook *et al.* (1977), except that bound and free steroid were separated using a second antibody.

Assay details for cortisol, progesterone, oestradiol, testosterone, dehydroepiandrosterone and androstenedione are given below.

Cortisol Antibody (AB) raised in sheep
Supplier: SAPU (Scottish antibody production unit)
Label: ^{125}I

	Cross reactions (%)	Assay sensitivity	Between batch CV
6β-hydroxycortisol	1.0	25 nmol/l	< 8%
17α-hydroxy progesterone	5.0		
21-deoxycortisol	34.4		

Progesterone (AB): sheep
Supplier: Glasgow Royal Infirmary (GRI)
Label: ^{125}I

corticosterone	1.8	2.2 nmol/l	< 10%
deoxycorticosterone	4.3		

Oestradiol AB: rabbit
Supplier: Immunogenetics
Label: ^{125}I

oestriol	1.3	59 pmol/l	< 12%
oestrone	11.7		

Testosterone AB: sheep (Y6)
Supplier: GRI
Label: ^{125}I

androstenedione	6.4	1.0 nmol/l	< 12%
dihydrotestosterone	33.8		

Dehydroepiandrosterone AB: rabbit (M99)
Supplier: Brian Rudd, Birmingham hospital for women.
Label: ^{3}H

androsterone sulphate	1.86	300 nmol/l	< 20%

Androstenedione AB: rabbit
Supplier: GRI
Label: ^{125}I

1,4-androstadiene-3,17-dione	43	1.0 nmol/l	<10%
4-androstene-3,11,17-trione	5		
5α-androstane-3,17-dione	40		
5β-androstane-3,17-dione	6		

All other steroids tested showed a cross reaction of less than 1%.

11.3 Results

The concentrations of testosterone and cortisol are given for all animals in groups 1, 2, 3 and 4 in Figures 11.1 to 11.12. Concentrations of all six steroids assayed are given for one animal from each group (Figures 11.13 to 11.16), results for the remaining animals are in the appendix.

Group 1 Intersex controls. Figures 11.1 to 11.2

Maximum jugular cortisol concentrations in the two animals were 522.1 nmol/l (pig 2) and 404.5 nmol/l (pig 753) attained at 60 and 50 minutes respectively from the start of sampling. The maximum recorded gonadal blood cortisol concentration in pig 753 was 359.6 nmol/l.

Group 2 ACTH challenge, conscious animals. Figures 11.3 to 11.4

For the three animals in this group, the mean (+/- se) jugular cortisol concentration at time 0 was 75.5 (11.4) nmol/l, and the mean of the maximum cortisol concentrations after the ACTH challenge was 370.8 (14.7) nmol/l. Testosterone concentrations in the jugular venous samples from pigs 753 and 93 were below assay sensitivity; for pig 2 concentrations were 1.16 nmol/l (before ACTH challenge) and 3.24 nmol/l (15 minutes after challenge) nmol/l.

Group 3 ACTH, anaesthetised animals. Figures 11.5 to 11.9

For the intersex pigs, 93, 100 and 307, the means of the maximum cortisol concentrations following the ACTH challenge in jugular and gonadal venous blood respectively were 470.5 (+/- 64) and 390.9 (+/- 32) nmol/l. Corresponding values for the two normal gilts were 507.8 (+/- 67) and 418.7 (+/- 24) nmol/l.

Group 4 ACTH, gonadectomised animals. Figures 11.10 to 11.12

At the start of sampling, the mean (+/- se) concentration for cortisol in jugular venous blood was 110.8 (31.7) nmol/l. 25 minutes after the first

ACTH challenge, the mean concentration was 397.1 (88.6) nmol/l, and 25 minutes after the second challenge, the concentration was 454.3 (67.5) nmol/l.

The means of the maximum testosterone concentrations recorded after the first and second challenges were 5.3 (2.6) and 2.9 (1.8) nmol/l respectively.

Key to Figures 11.1. to 11.12.

cortisol
testosterone
A ACTH challenge (1 ml Synacthen unless otherwise stated)
E challenge with Eagle's medium.
gonadectomy

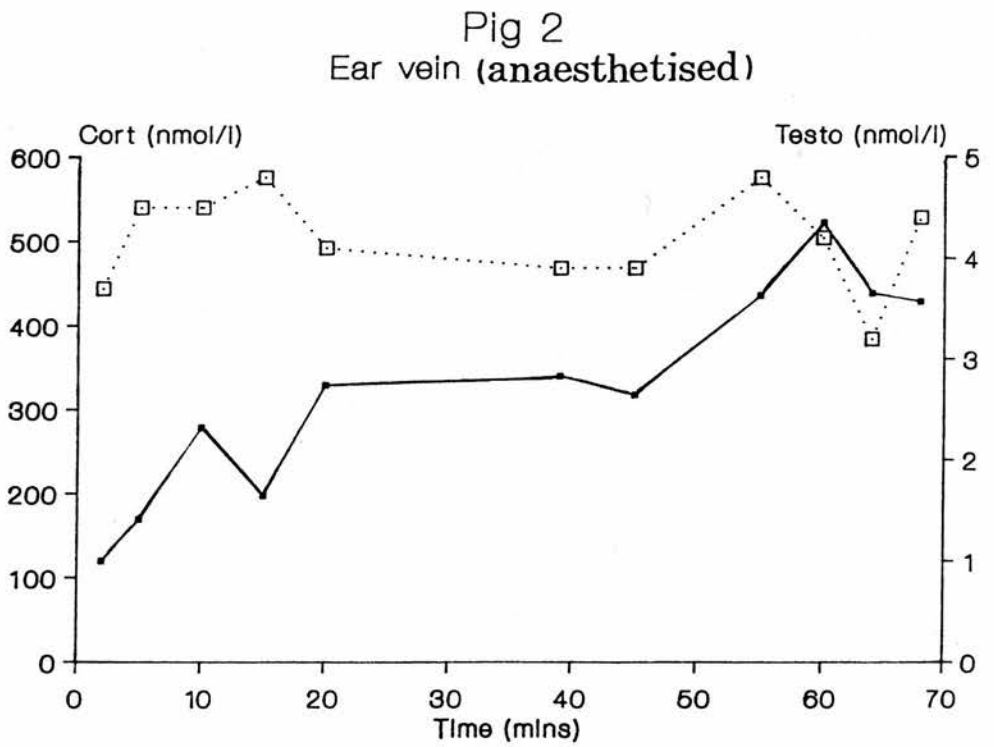
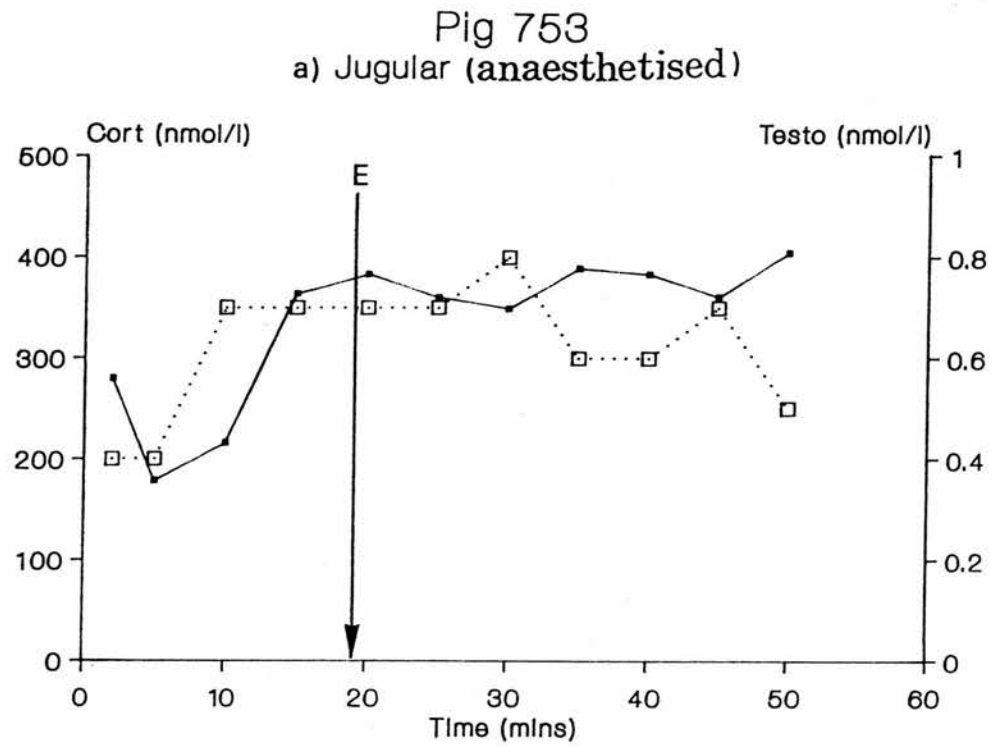


Figure 11.1. Group 1, control. Concentrations of cortisol and testosterone in pig 2, under anaesthetic, no challenge given.



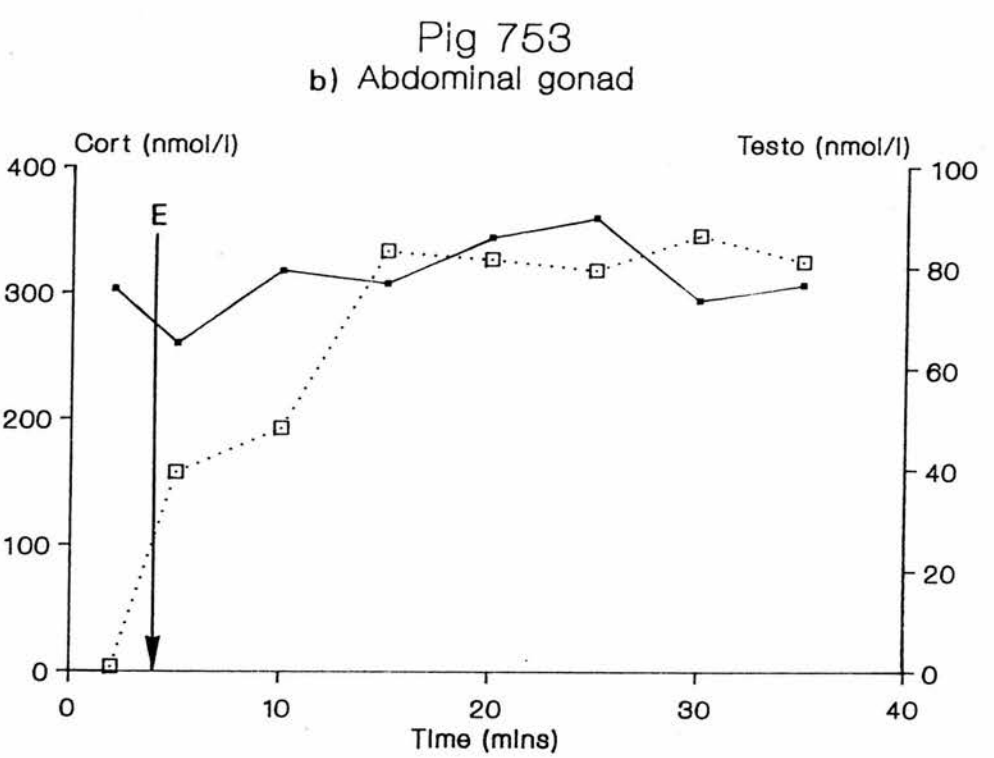
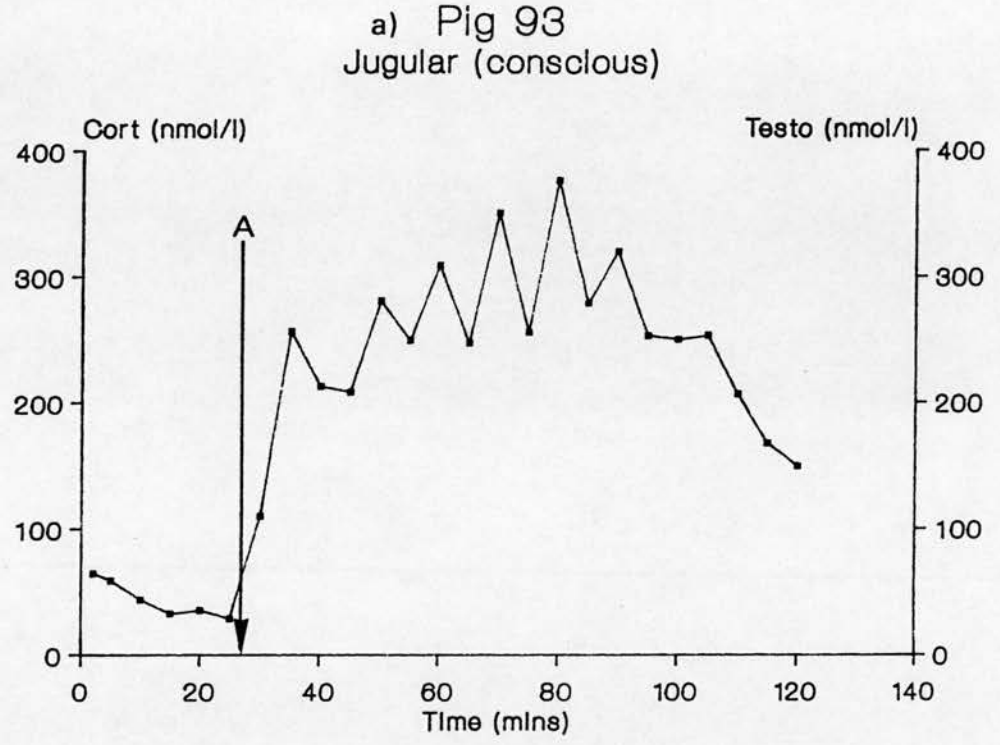


Figure 11.2. Group 1, control. Concentrations of cortisol and testosterone in a) jugular vein and b) gonadal vein in pig 753, under anaesthetic, before and after an injection of Eagle's medium.



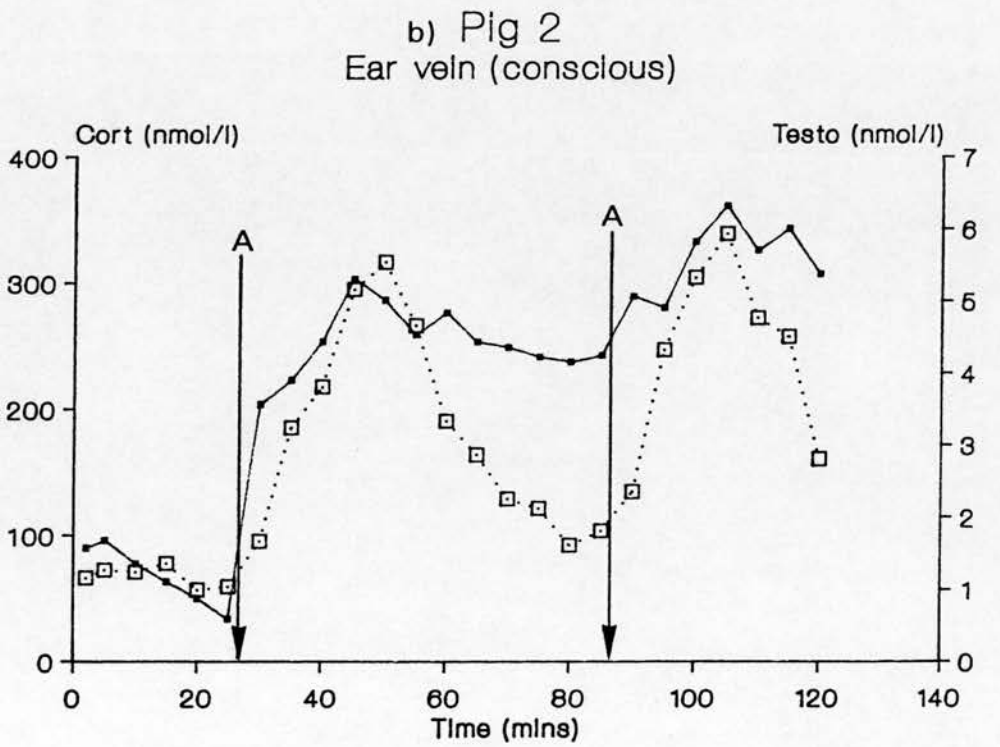


Figure 11.3. Group 2, ACTH conscious animals. Pig 93, testosterone concentrations below assay sensitivity, pig 2, two ACTH challenges given.

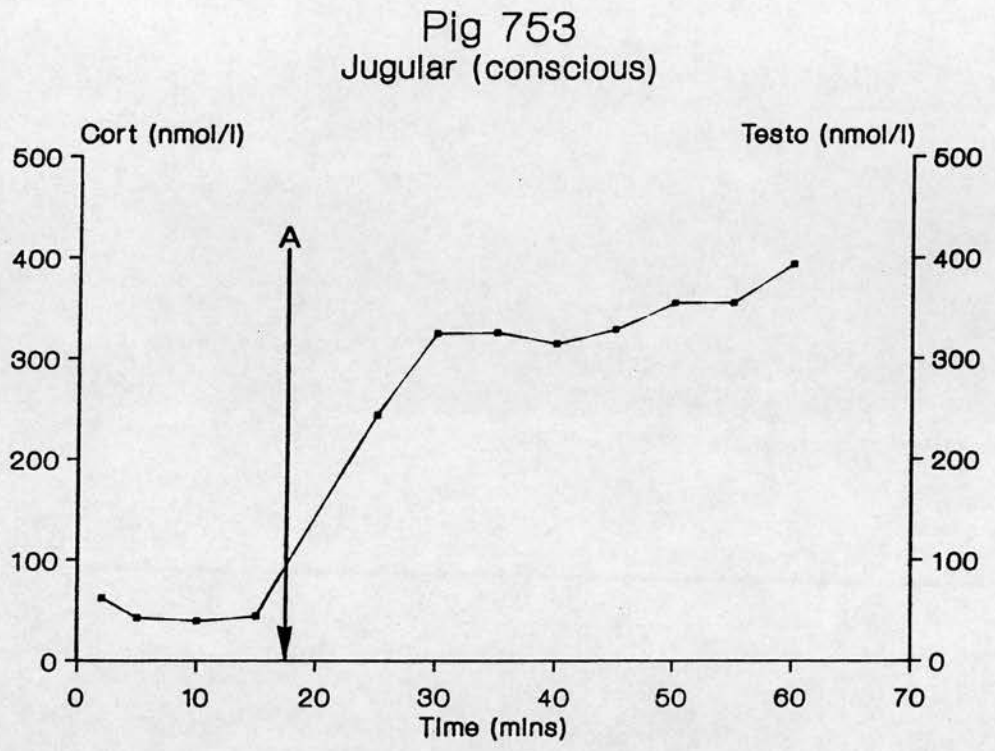
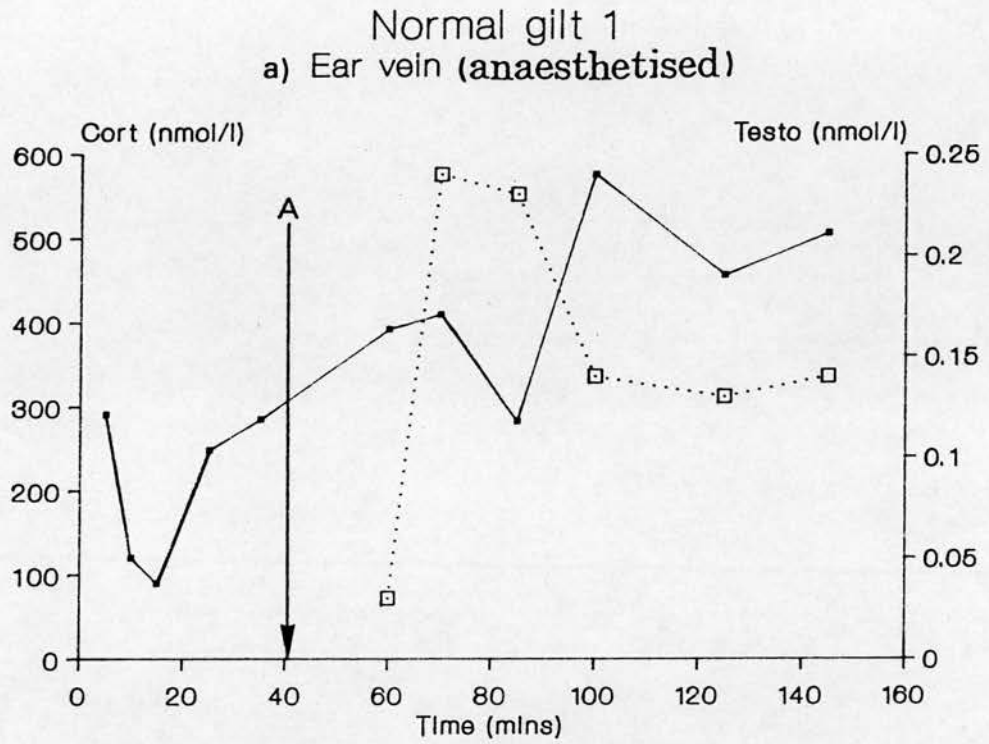


Figure 11.4. Group 2, ACTH, conscious. Testosterone concentration below assay sensitivity.



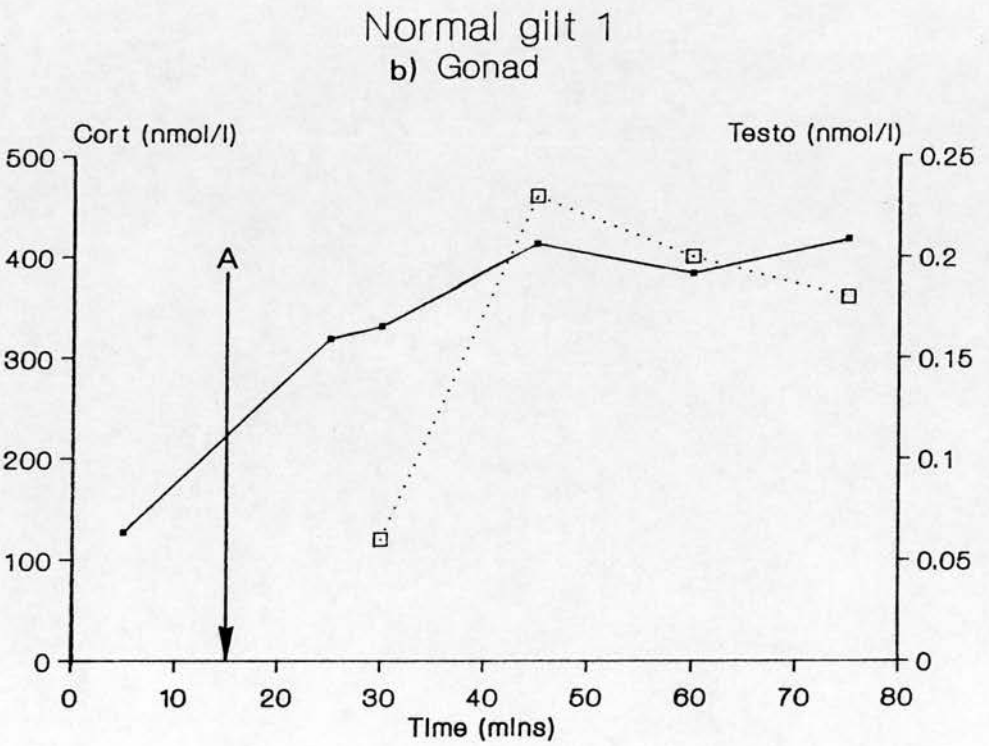
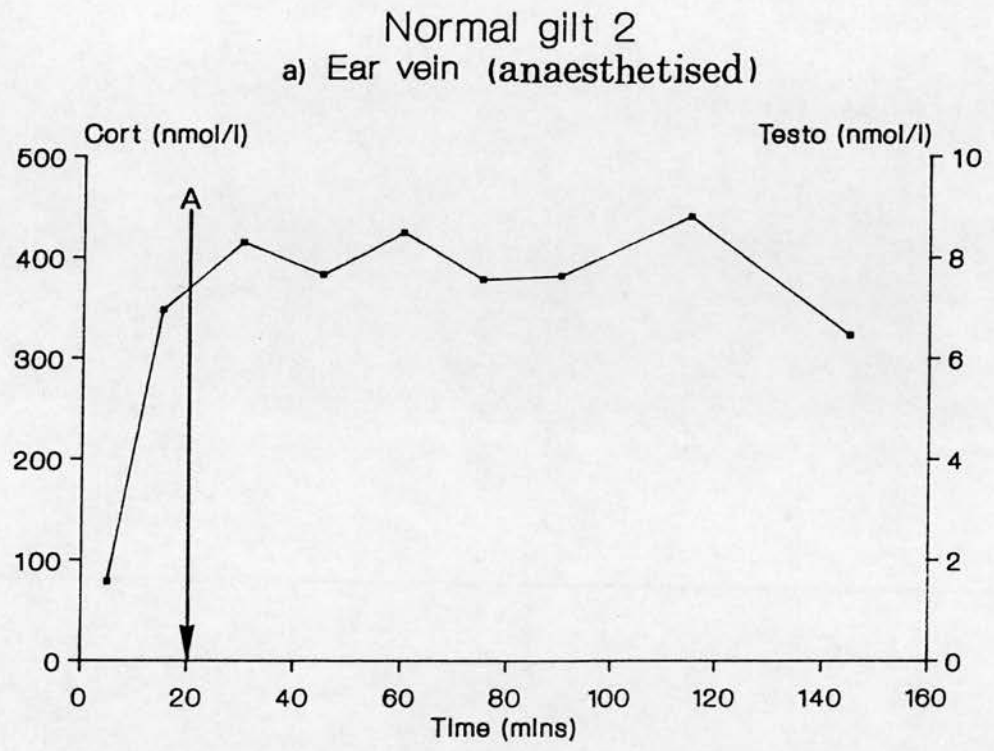


Figure 11.5. Group 3, ACTH challenge, anaesthetised animal.



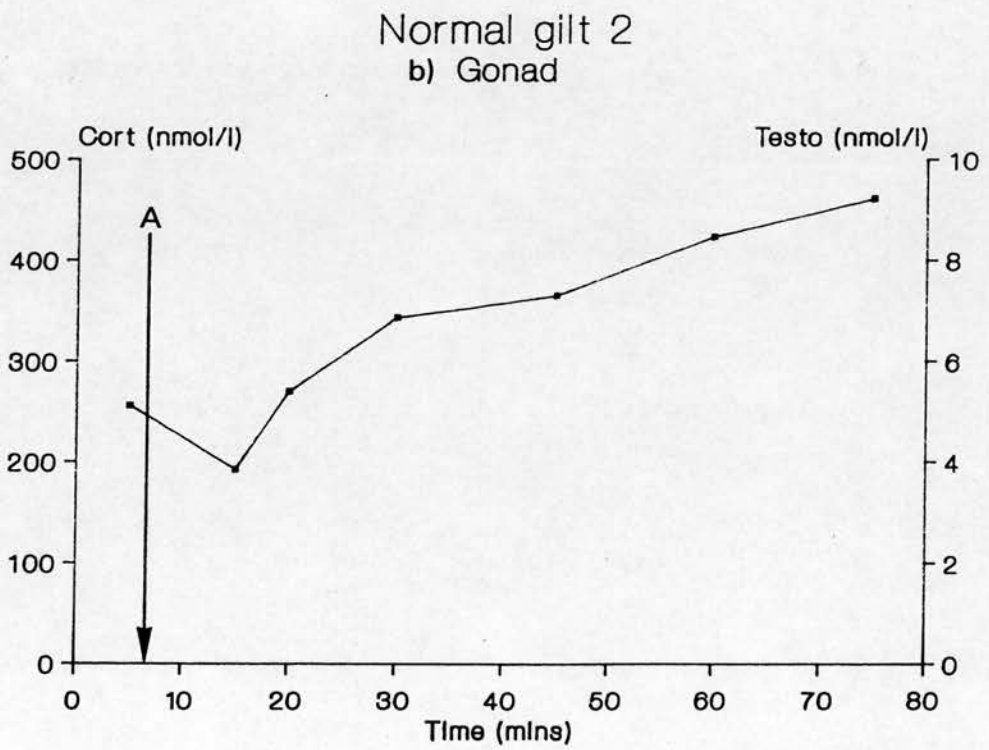
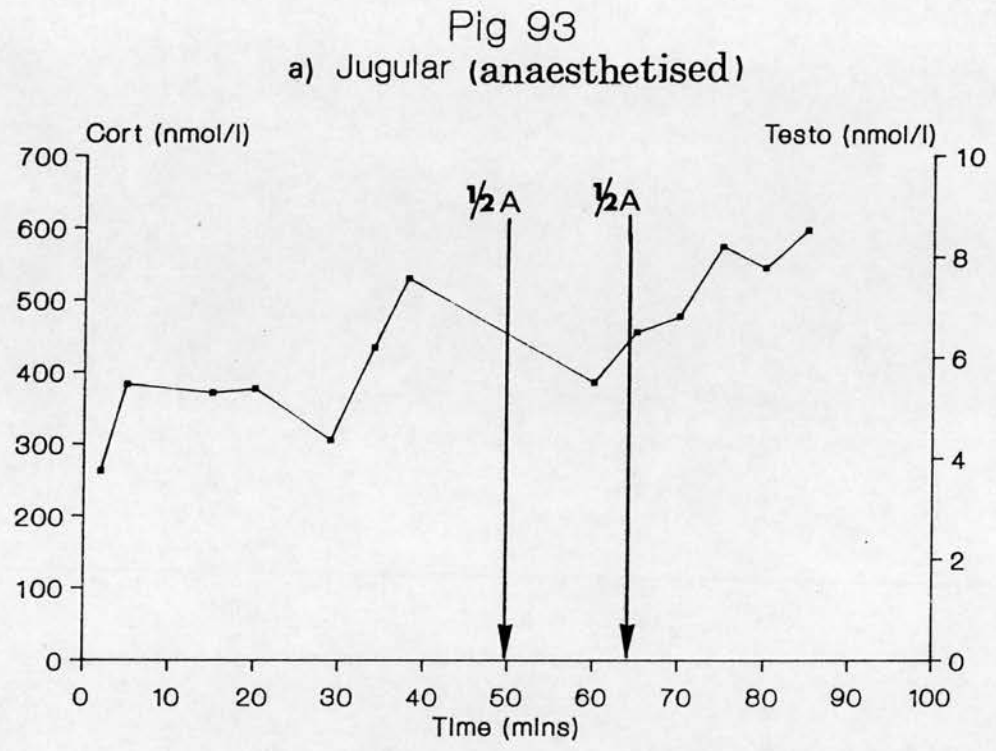


Figure 11.6. Group 3, ACTH challenge, anaesthetised animal.



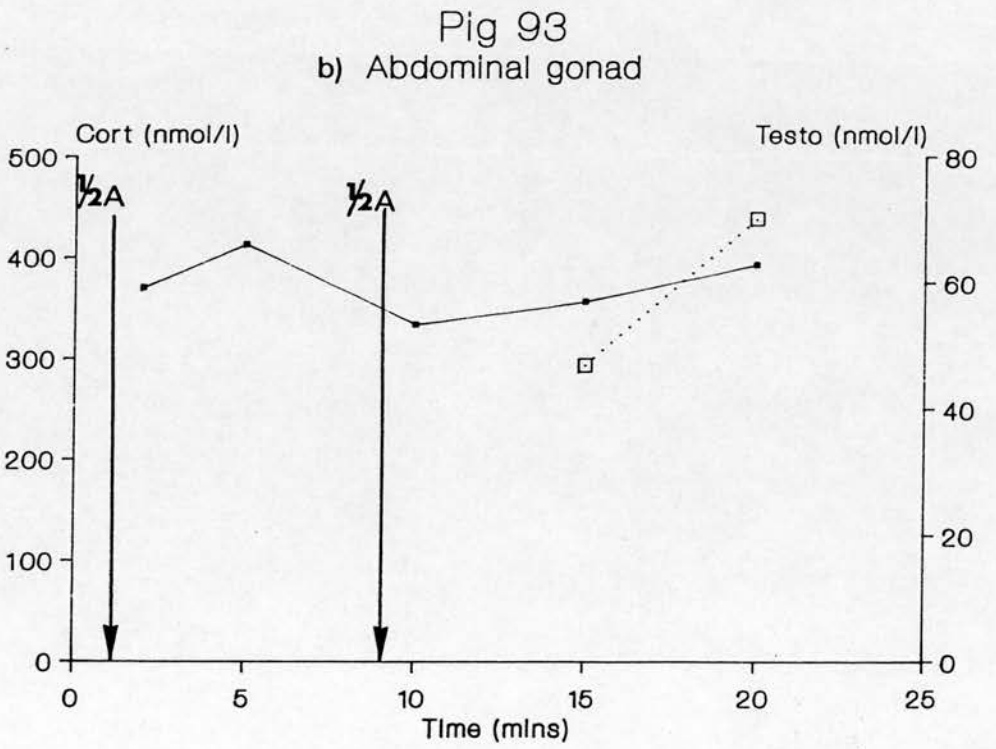
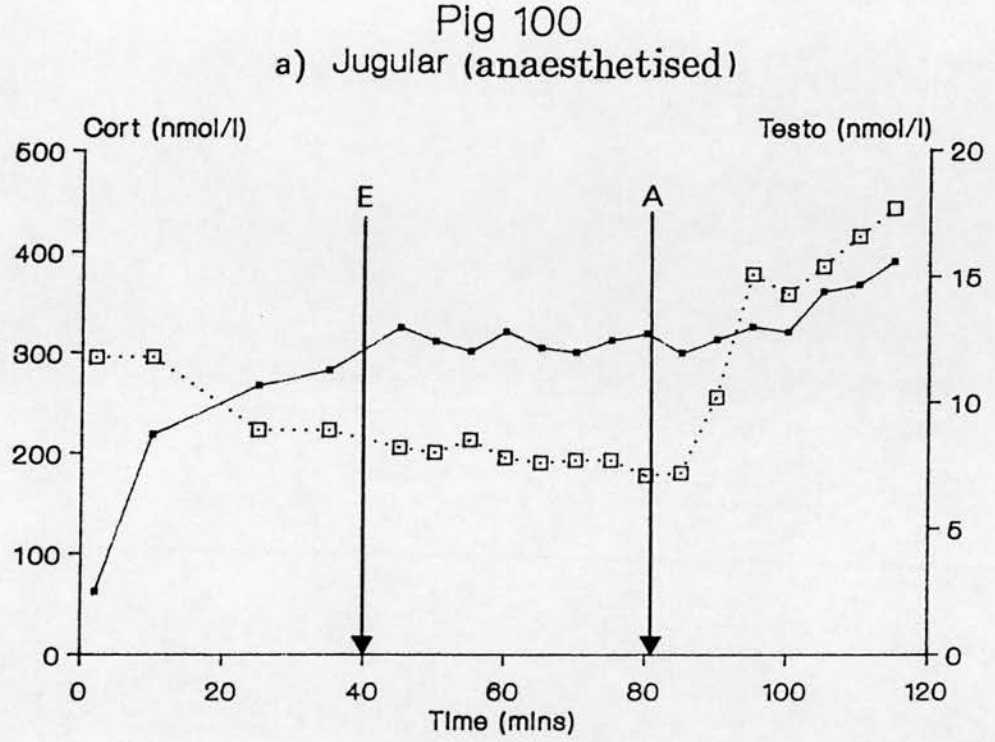


Figure 11.7. Group 3, ACTH challenge, anaesthetised animal. Testosterone concentrations in jugular venous blood (a) below assay sensitivity. Two challenges of ACTH (1/2 ml).



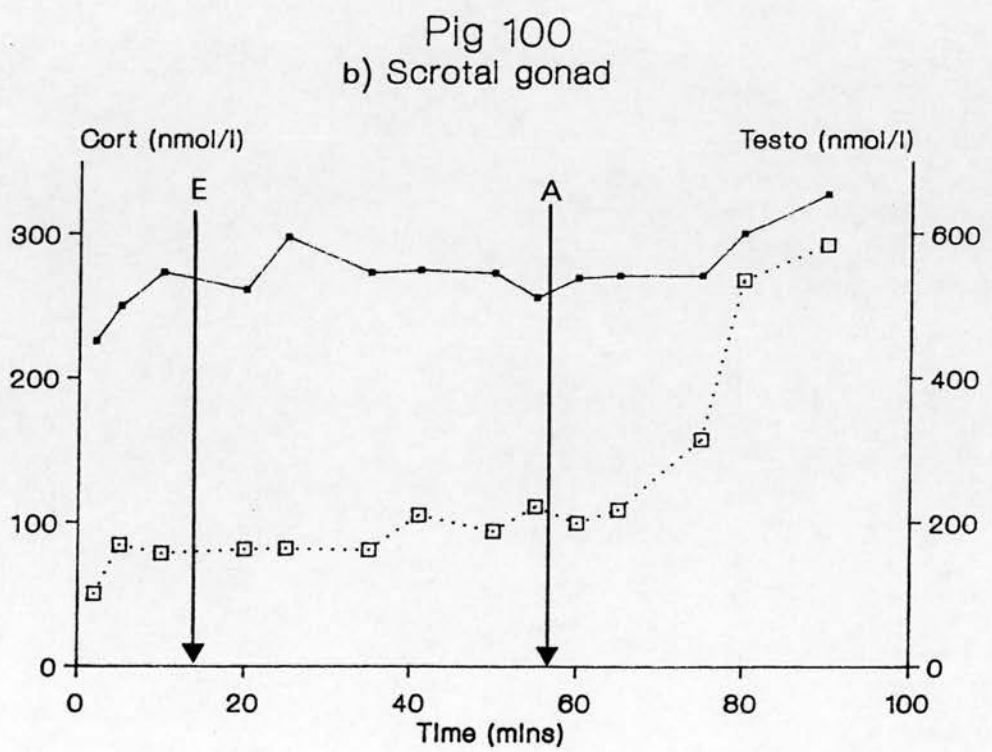


Figure 11.8. Group 3, ACTH challenge, anaesthetised animal. Concentrations of cortisol and testosterone before and after an injection of Eagle's medium followed by ACTH.

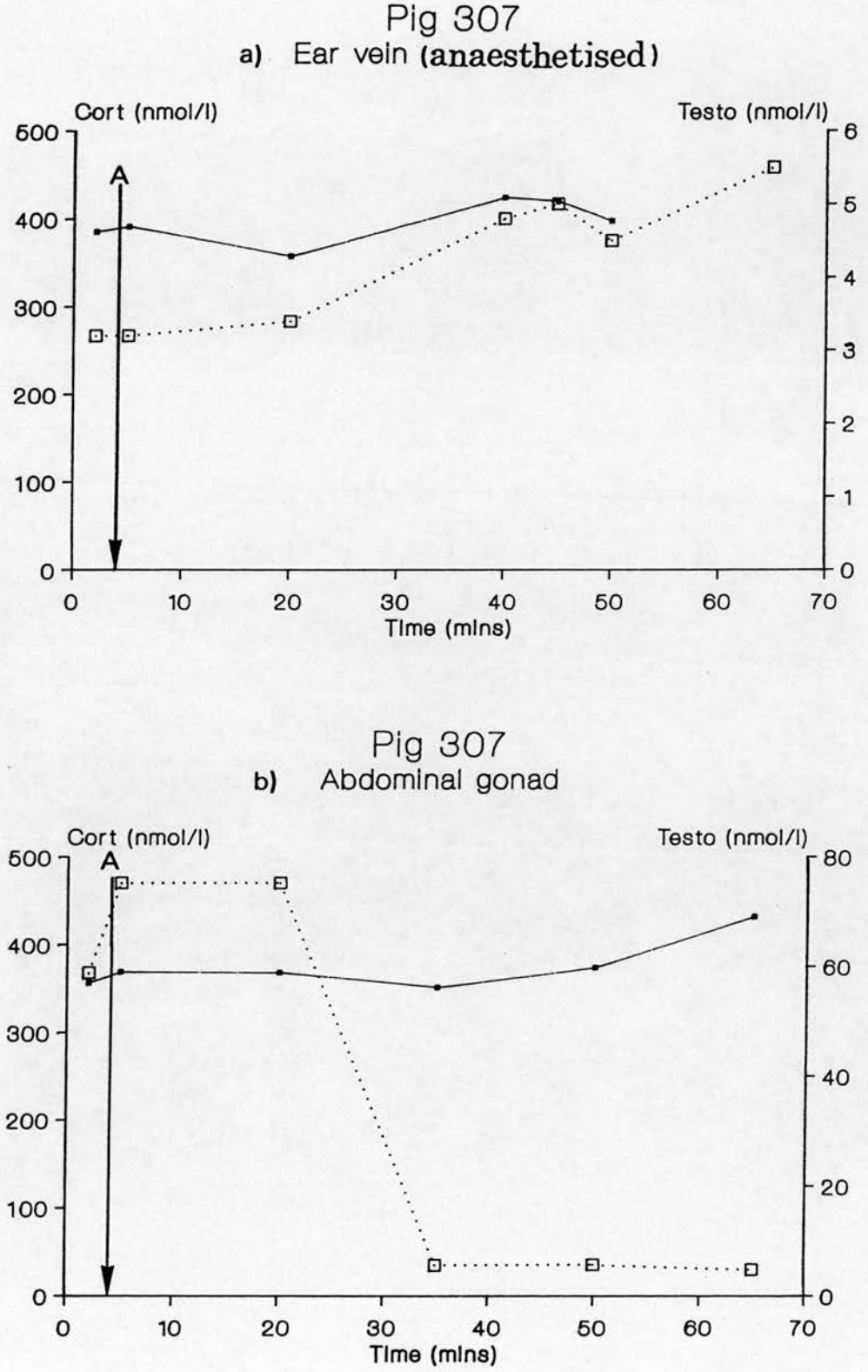


Figure 11.9. Group 3, ACTH challenge, anaesthetised animal.

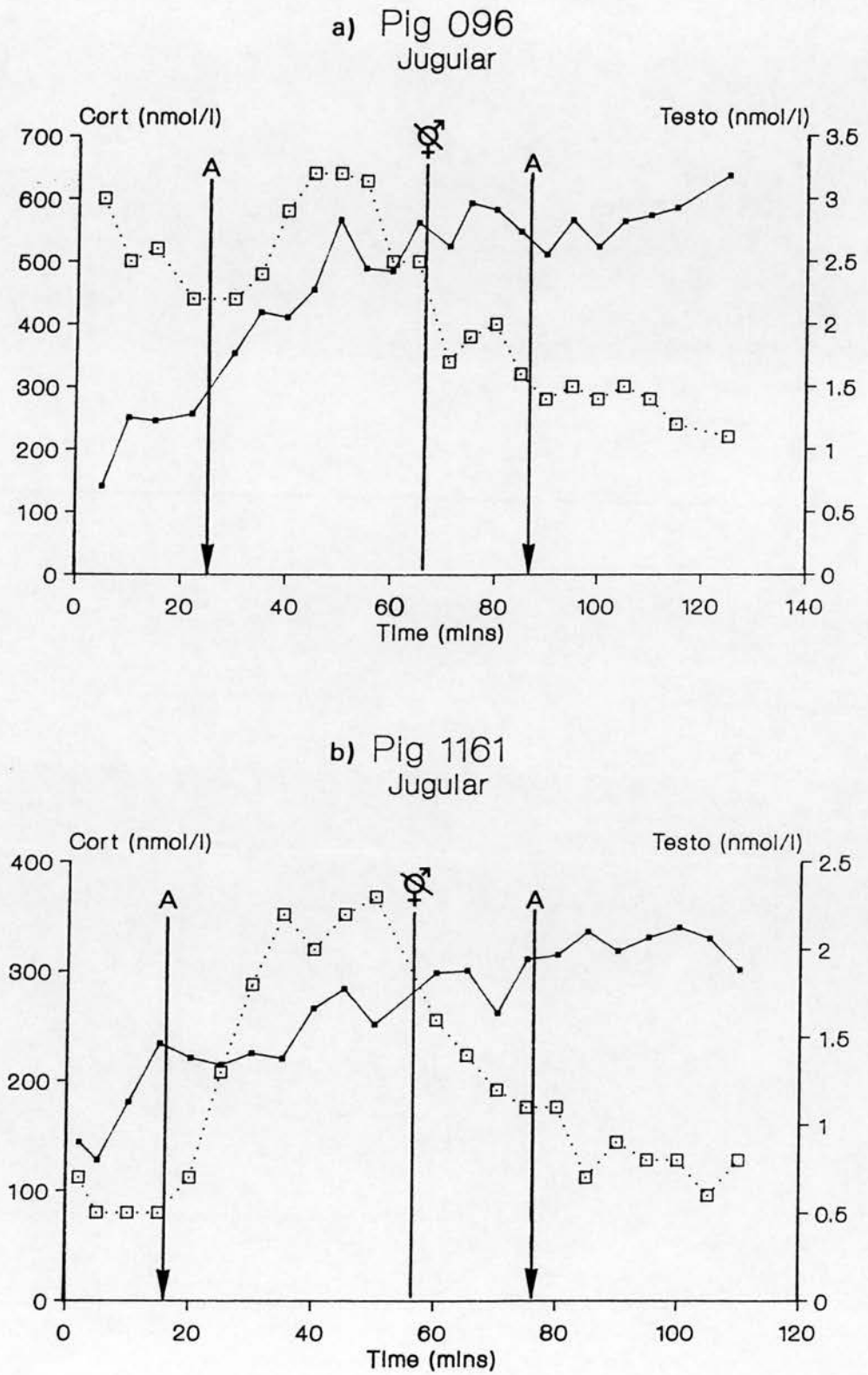


Figure 11.10. Group 4, ACTH challenge before and after gonadectomy.

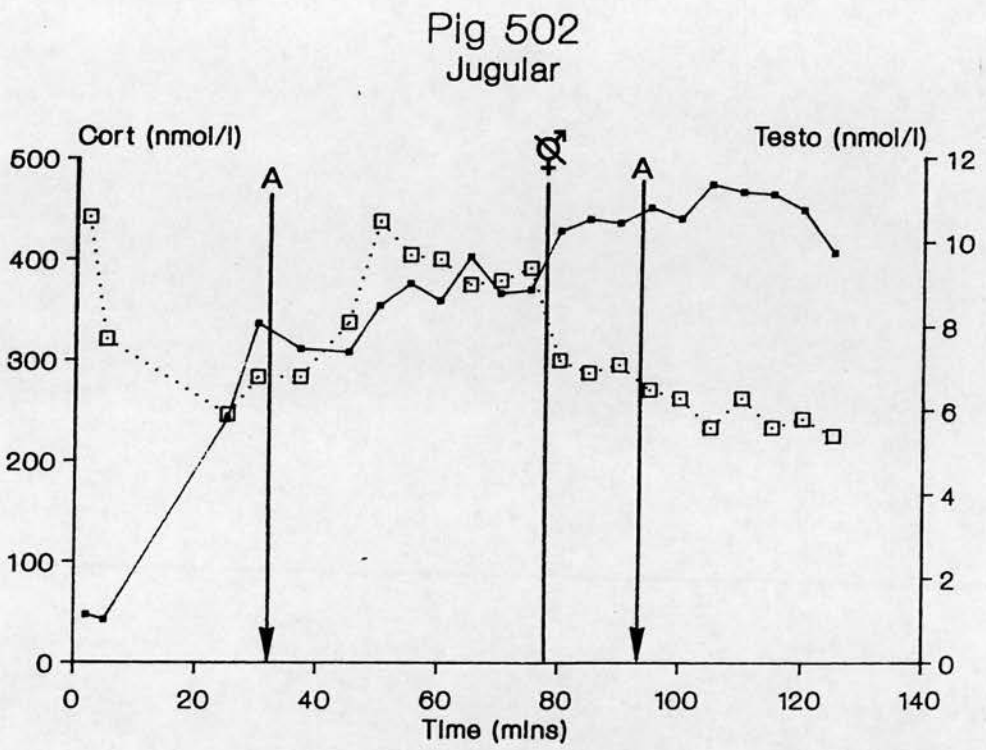
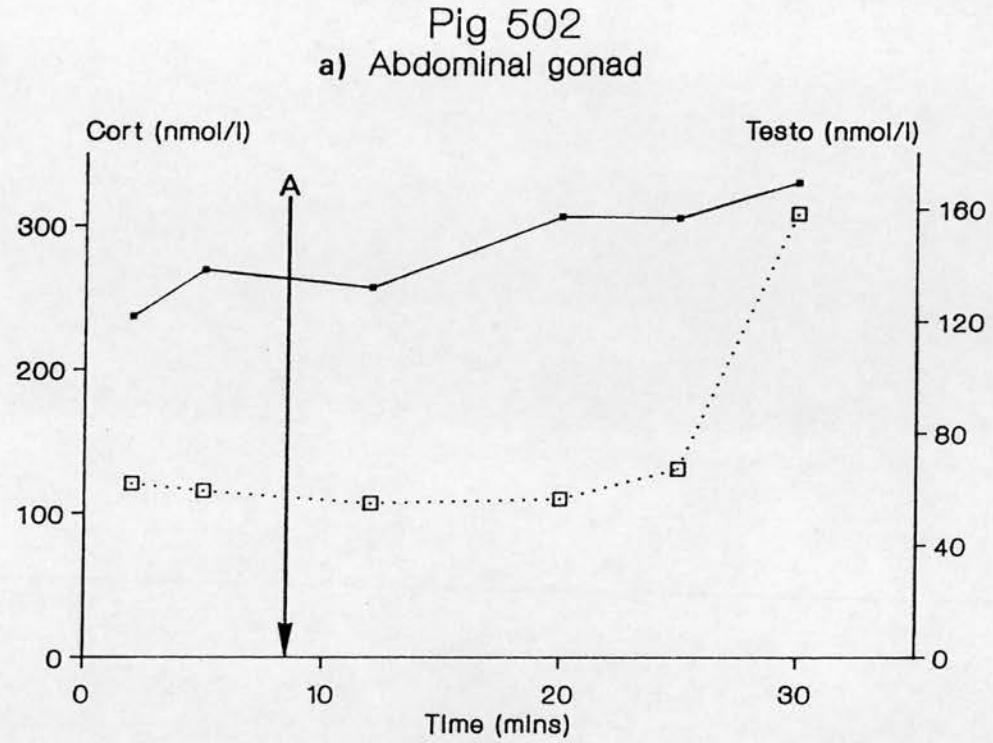


Figure 11.11. Group 4, ACTH challenge before and after gonadectomy.



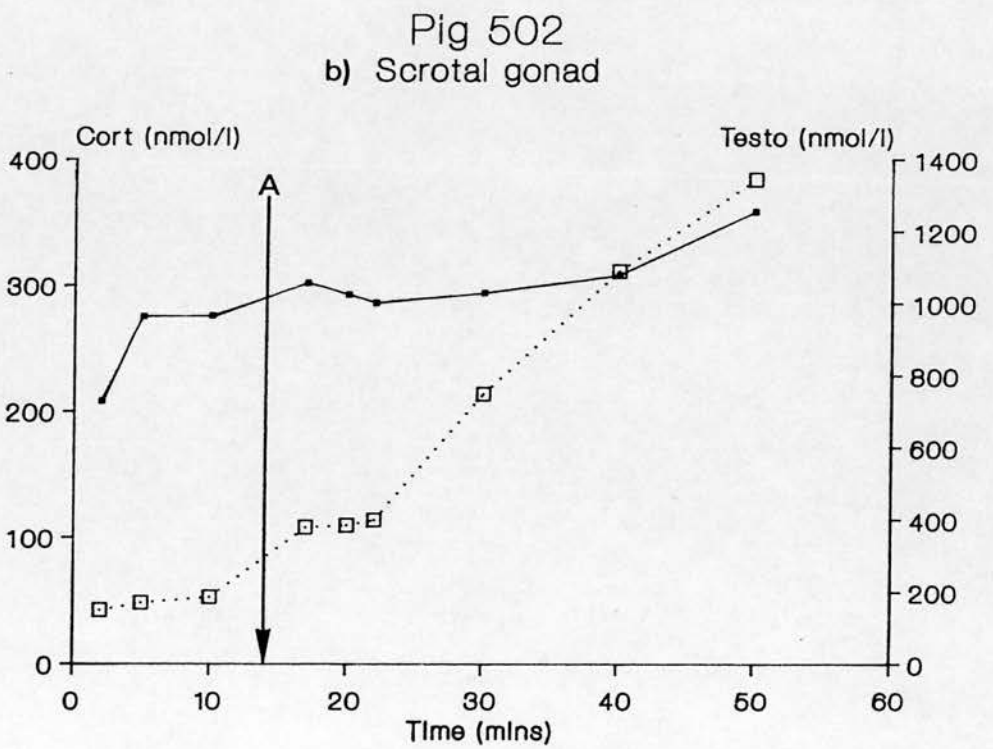
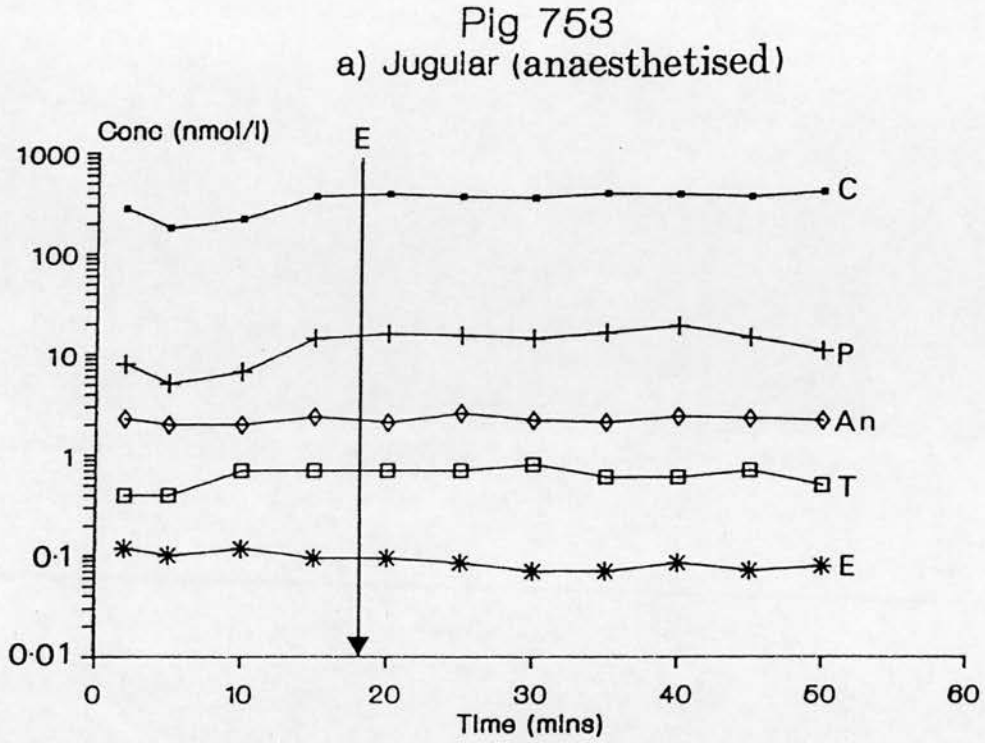


Figure 11.12. Group 4, ACTH challenge before and after gonadectomy.



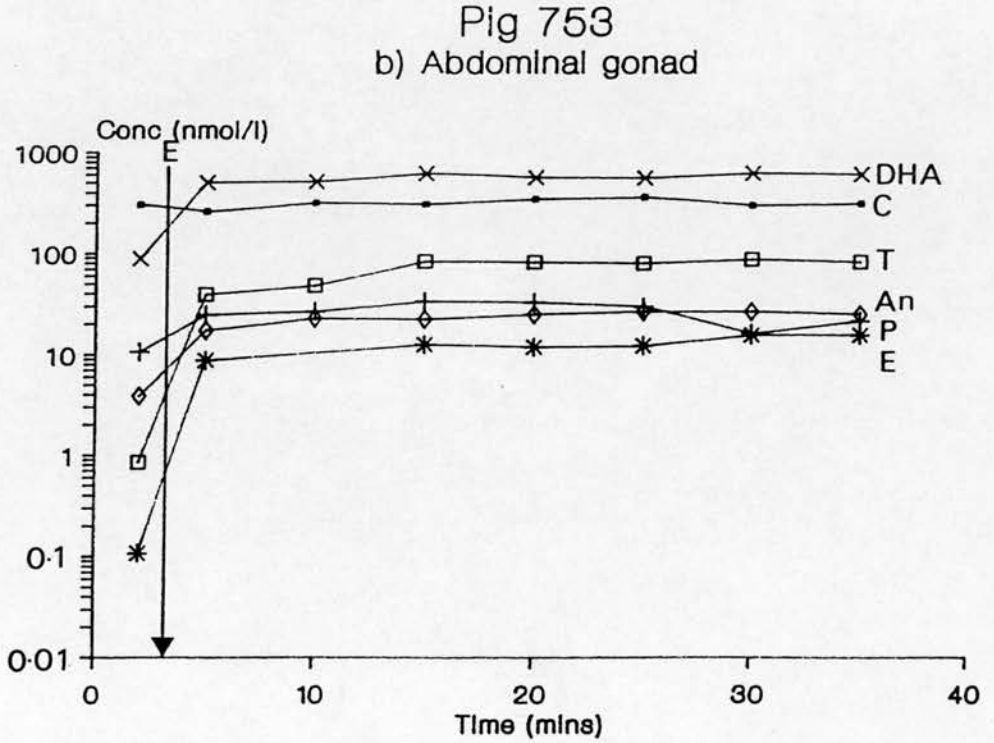


Figure 11.13. Group 1, control. Concentrations of androstenedione (An), cortisol (C), dehydroepiandrosterone (DHA), oestradiol (E), progesterone (P) and testosterone (T) in pig 753 in, a) jugular venous blood and b) abdominal gonadal venous blood before and after an injection of Eagle's medium (arrow). Concentrations of DHA in (a) below assay sensitivity.

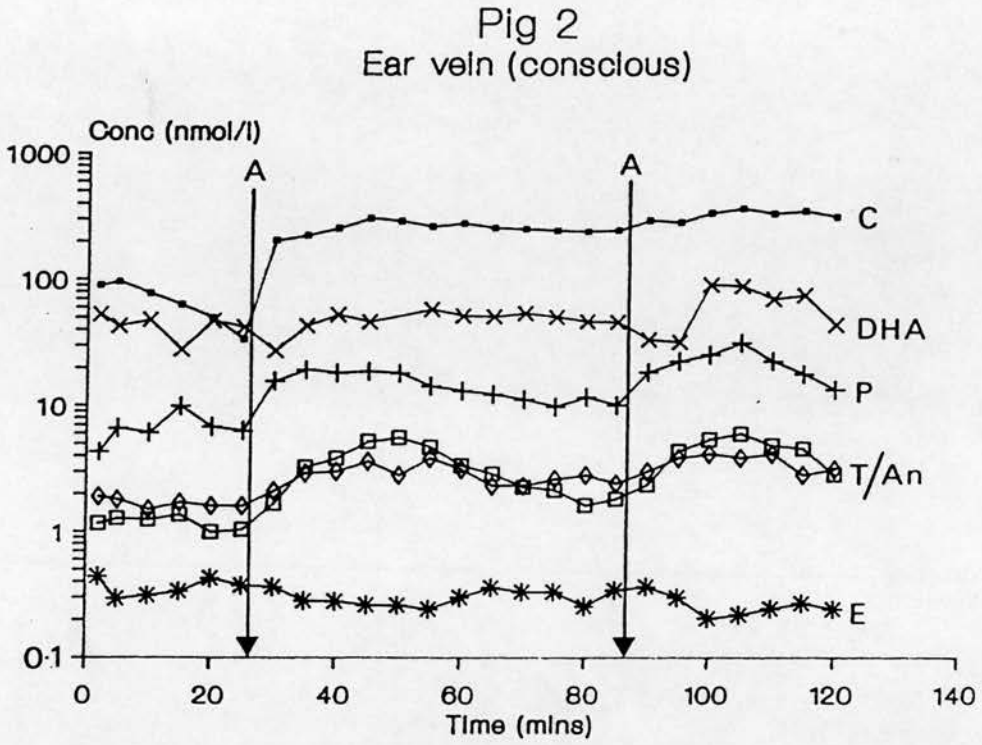
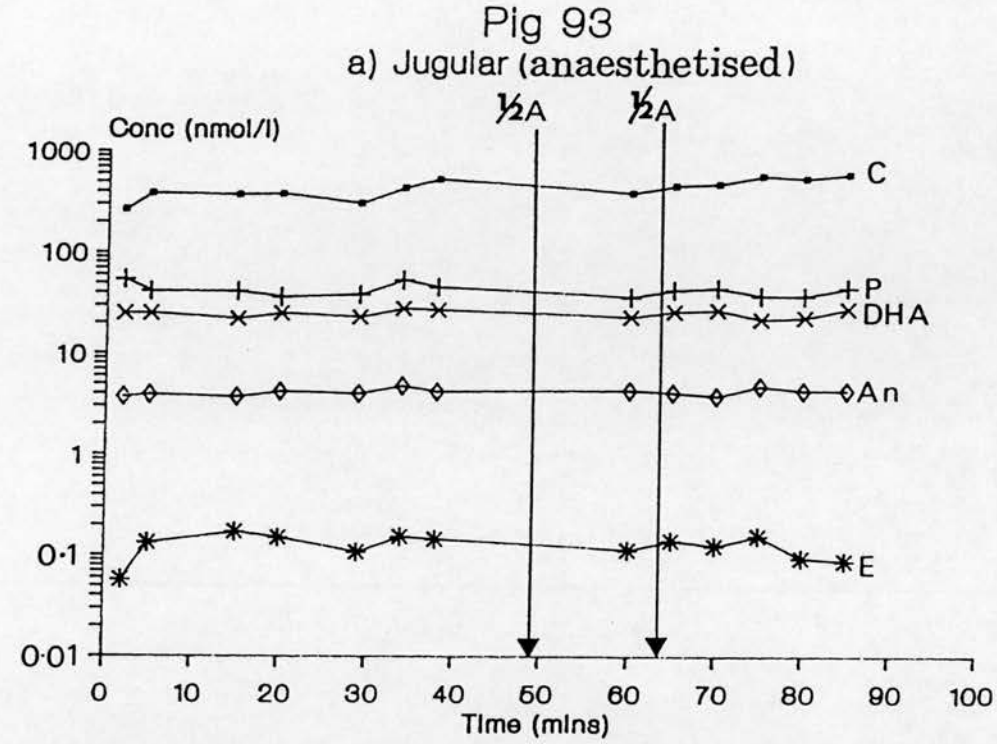


Figure 11.14. Group 2, ACTH challenge, conscious animal.



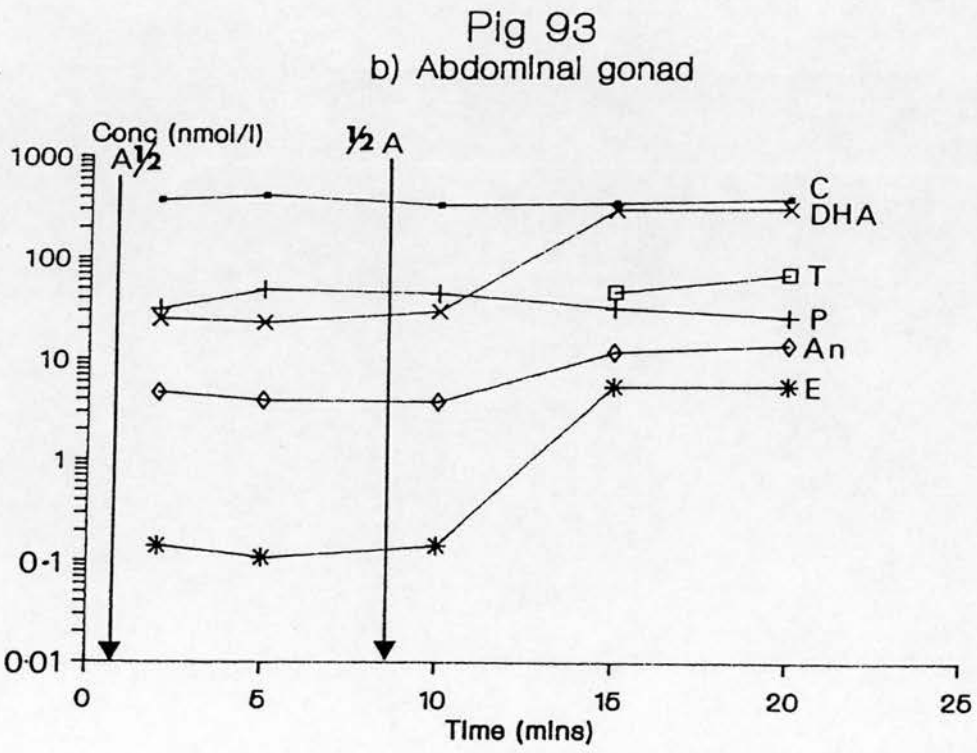


Figure 11.15. Group 3, ACTH challenge, anaesthetised animal.

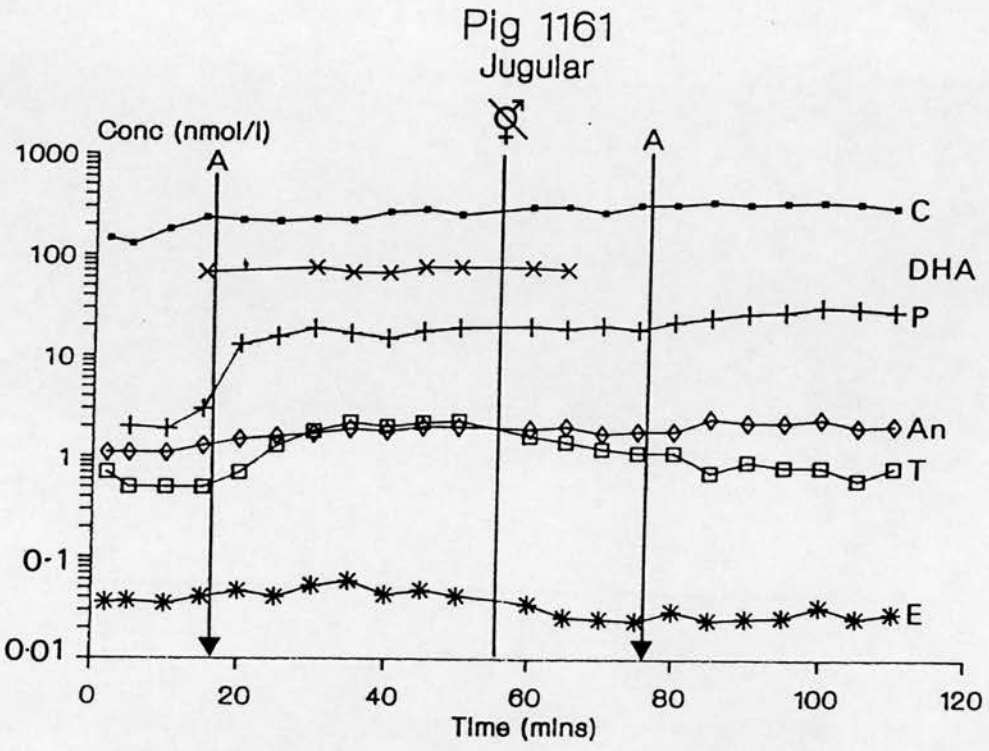


Figure 11.16. Group 4, ACTH challenge before and after gonadectomy.

11.4 Discussion

a) The effect of surgery on blood cortisol concentrations

Animals in group 1 were anaesthetised and operated on for the insertion of catheters, but did not receive any ACTH challenge. Those in group two had recovered from surgery and were challenged with ACTH. The rise in blood cortisol levels seen in the group 1 animals (to 404.5 and 522.1 nmol/l) therefore indicates to what extent surgery was responsible. The mean of the maximum cortisol concentrations recorded in jugular plasma of group 2 animals (370.8 nmol/l) reflects the influence of an ACTH challenge in the absence of surgical stress. The finding that maximum cortisol concentrations recorded in jugular plasma from animals (intersex or normal) subjected to surgery and an ACTH challenge (group 3) were no higher than those in group 1 animals, implies that cortisol secretion is at a maximum during surgery.

b) The gonadal response to an ACTH challenge

The peripheral and gonadal venous blood concentrations of cortisol in the normal gilts following an ACTH challenge were higher than those recorded in the intersex animals (group 3), inferring that the intersex gonad is not secreting unusually high levels of cortisol in response to an ACTH challenge. Jugular plasma cortisol concentrations found in normal boars challenged with ACTH were within the range reported in this study *eg.* 324 nmol/l (Liptrap and Raeside 1975), 545 nmol/l (Pitzel *et al.*, 1980), both recorded within 2 hours of the challenge. In order to show that it is the gonad and not the adrenal gland which is responding to the ACTH, cortisol concentrations in the gonadectomised animals must be considered. In all three animals (group 4) cortisol concentrations continued to rise following the second Synacthen injection, implying that the intersex gonad was not the source of cortisol. The finding that the mean plasma cortisol concentration in normal gilts (group 3) was above that for the intersexes also indicates that the intersex gonad was not a source of unexpectedly high cortisol concentrations.

The testosterone concentrations in jugular venous blood in the gonadectomised animals indicate that this hormone is secreted in response to the ACTH challenge. This response *i.e.* stimulation of gonadal testosterone secretion, has been observed in normal boars by several workers (Liptrap and Raeside, 1975; Hahmeier *et al.*, 1980; Juniewicz and Johnson, 1981). Pitzel *et al.* (1979) found that, following castration, testosterone levels did not rise to the same extent as seen in entire animals, but peak levels of testosterone were only slightly depressed by adrenalectomy in ACTH challenged animals. Juniewicz and Johnson (1984) concluded that raised cortisol concentrations are not the cause of gonadal testosterone secretion since injections of cortisol failed to produce such a rise in testosterone. Suggestions that adrenal progesterone may act as a precursor for testosterone arose from the demonstration of raised progesterone concentrations following ACTH administration (Juniewicz and Johnson, 1981), supported by the present experiment. However, this suggestion was refuted by the same authors (Juniewicz and Johnson, 1984) when they failed to induce an increase in testosterone secretion by administering progesterone to boars. Juniewicz and Johnson (1984) also demonstrated that the increase in testosterone was independent of LH secretion.

In conclusion, this experiment has failed - from an endocrine viewpoint - to demonstrate the presence of functional adrenal tissue in the gonads of intersex pigs. In response to an ACTH challenge, or to surgical stress, cortisol concentrations were raised in normal gilts and in intersex animals. Removal of the gonads failed to reduce cortisol secretion in the intersex animals; by contrast, gonadal secretion of testosterone in response to an ACTH challenge was demonstrated. Work on normal boars indicates that this response is not mediated via LH, cortisol or progesterone. It appears that testicular tissue in normal boars, and in intersex pigs, possesses ACTH receptors, resulting in stimulation of testosterone secretion following an ACTH challenge. The results do not give any indication as to the aetiology of the intersex condition, but do demonstrate that the testicular tissue in the intersex pig is functioning, as regards response to ACTH stimulation, as a testis would in a normal boar.

CHAPTER 12 THE H-Y ANTIGEN STATUS OF TWO INTERSEX PIGS

12.1 Introduction

Despite evidence that H-Y antigen may not be the mammalian testis determining factor (McLaren *et al.*, 1984; Simpson *et al.*, 1987), there is still support for the proposal that this sex specific antigen is involved in testicular differentiation and/or function in mammals (Burgoyne *et al.*, 1986; Wiberg, in press). With few exceptions, the majority of male mammals type H-Y positive and females type negative. The availability of XX animals possessing testicular tissue therefore offers the opportunity to extend understanding of the H-Y antigen status of intersex mammals.

The method chosen to assess the H-Y status of an individual is important since different techniques have been found to produce conflicting results. Since H-Y antigen was originally defined by means of graft transplantation within an inbred strain of mice (Eichwald and Silmser, 1955; Billingham and Silvers, 1960), this method was chosen to determine the status of two intersex pigs.

12.2 Materials and Methods

The method used was based on that described by Wiberg (1985). Female C57BL/6 (B6) mice are immunised with cells from male, female or intersex pigs and are then grafted with B6 male skin grafts. H-Y antigen positive cells are able to induce accelerated rejection of syngeneic male grafts.

Experimental animals

C57BL/6 (B6) male and virgin female mice were obtained from Charles River GmbH (Sulzfeld, FRG). Females were 67 +/-5 days old on the day of

priming and 99 +/-5 days old at the time of transplantation with skin grafts from males of the same age.

Peripheral blood (20 ml) was collected into sterile, heparinised tubes from pigs 4, 7, one normal gilt and one mature boar. Leucocytes were recovered by means of Ficoll-Paque (Pharmacia Inc) separation media.

Immunisation

10 female mice per group were primed by intraperitoneal injection with $7.5x10^6$ white blood cells (WBCs) (in 0.5 ml of PBS) from the following pigs:- a normal boar, a gilt, intersex 4 or intersex 7. A control group was injected with 0.5 ml PBS alone The groups were then coded.

Transplantation

Primed and unprimed females were transplanted with four syngeneic male trunk skin grafts approximately one month after priming. The female mice were bandaged for nine days, after which grafts were assessed and scored daily. Grafting techniques and appraisal of graft rejection were performed according to the method of Harnasch (1979).

Median survival times (MSTs) of grafts and standard deviations were determined (Litchfield, 1949). The groups were decoded and statistical significance of graft survival between groups was calculated by means of the Wilcoxon-Mann-Whitney *U*-test (Sachs, 1982).

12.3 Results

Table 12.1 gives the results of graft rejection times on the B6 females and the median survival times (+/- se) of the grafts. Results of statistical analysis of differences between graft survival times for the various groups are given in Table 12.2. Figure 12.1 expresses the results in graphic form.

Comparison of graft rejection times (Table 12.2) shows that graft rejection was significantly faster ($p < 0.01$) by male-primed mice than unprimed, female or intersex 7-primed mice. The difference between male-primed and intersex 4-primed mice was significant at the 2% level ($p < 0.02$). Graft rejection times on female-primed mice did not differ significantly from those on intersex-primed mice.

Table 12.1. Rejection times of syngeneic male grafts by B6 females primed with intraperitoneal injections of pig white blood cells of different sexual phenotypic and sex chromosomal origin

B6 females primed with	Sex chromosomes of priming cells	N*	Rejection times of grafts (no. x day of rejection)	MST+ (days)	S.D.+ (days)
Unprimed		40	3x14, 1x15, 3x16, 1x17, 2x18, 4x19, 10x21, 5x23, 5x >29, 6xTF‡	18.8	1.2
Female	XX	40	2x13, 1x14, 4x15, 3x16, 2x17, 2x18, 3x19, 3x21, 5x22, 4x23, 1x26, 2x >29, 4xTF, 4xD§	18.0	1.2
Male	XY	40	1x12, 3x14, 7x15, 5x16, 1x17, 3x18, 6x19, 1x21, 1x23, 12xD	15.5	1.1
Intersex **4**	XX	40	7x14, 5x15, 1x16, 3x17, 2x18, 1x19, 10x21, 2x22, 6x26, 3x >29	18.0	1.3
Intersex **7**	XX	40	2x14, 1x15, 3x16, 1x17, 5x18, 1x19, 4x21, 6x22, 6x23, 5x26, 1x >29, 1xTF, 4xD	19.5	1.2

* N, number of grafts transplanted. The number of females used is N/4.

+ MST, median survival times of grafts; S.D., standard deviations of MSTs, determined by the method of Litchfield (1949).

‡ TF, technical failure. Grafts were lost or loosened with the removal of bandages, and were not further evaluated.

§ D, grafts represented by mice which died on the **day** of transplantation.

Table 12.2 *P* values * from comparison in pairs of graft rejection processes in the groups of unprimed B6 females, and the B6 females primed with pig male and female WBCs prior to receiving syngeneic male grafts.

μA		μB	
	UNPRIMED	FEMALE	MALE
MALE	<0.001	<0.01	
FEMALE	<0.03		
INTERSEX 4		>0.5	<0.02
INTERSEX 7		>0.1	<0.001

* Two-sided *P* values of the null hypothesis (H_o : μA = μB) tested, using the Wilcoxon-Mann-Whitney U test with tied ranks, as described by Sachs (1982).

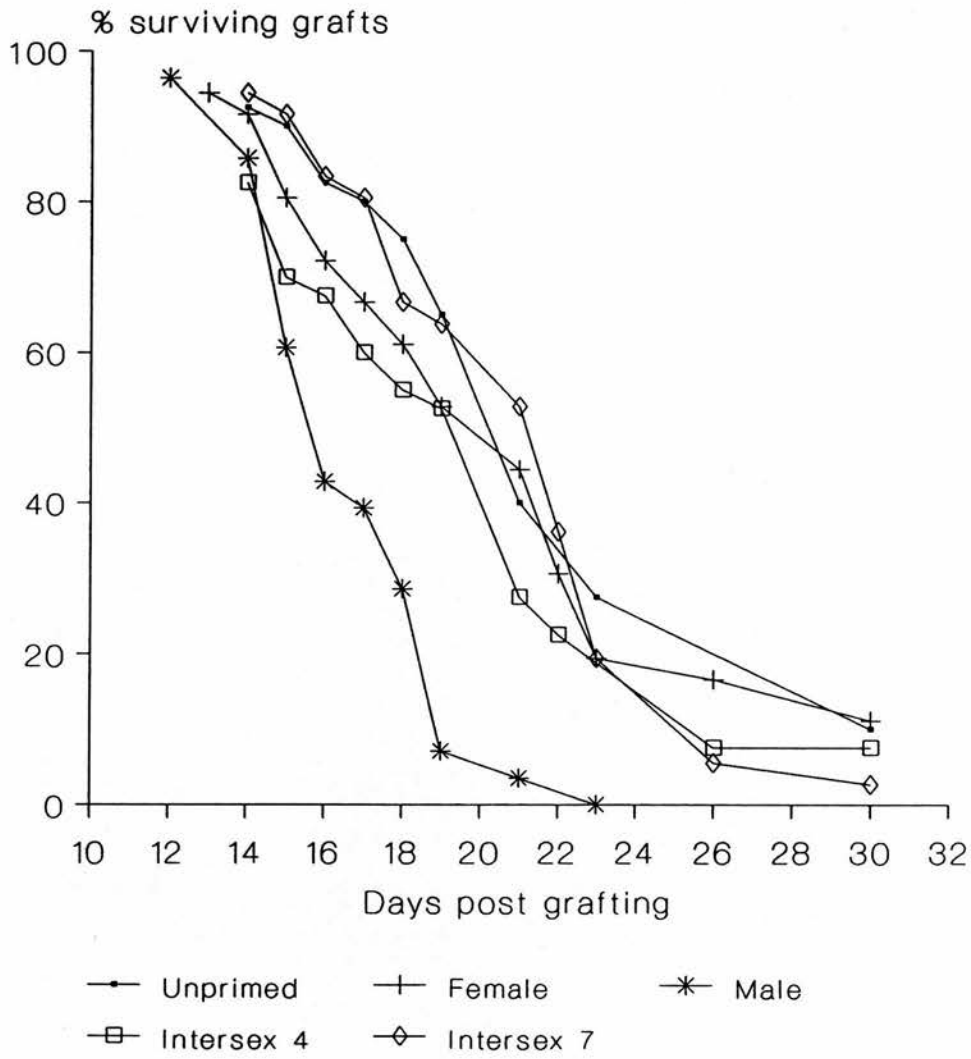


Figure 12.1. Graft survival times of syngeneic male trunk skin grafts on B6 female mice primed with white blood cells from a normal female, normal male or an intersex pig, or injected with PBS alone (unprimed).

12.4 Discussion

The accelerated graft rejection demonstrated by the mice primed with WBCs from the boar is evidence of their immunization against H-Y antigen. Since the graft rejection process on male primed mice was significantly different from that of female primed mice, the boar (of proven fertility) can be classified as H-Y positive, and the gilt and both intersex pigs classified as negative, despite the presence of testicular tissue in the intersex animals.

Wolf *et al.* (1980b) found that SDM (serologically detected male antigen) levels for XO women were mid-way between those for XX and XY individuals, and suggested that a threshold SDM level may exist, above which testes form. However, the intersex pigs tested here must have had H-Y titres of much less than half those of the boar since Billingham *et al.* (1965) showed that priming with as few as $1x10^6$ male cells will initiate a male primed graft rejection response. In the present experiment, $7.5x10^6$ cells were used for priming but the graft rejection response still indicated that the pigs were H-Y antigen negative.

XX human males have been found to type H-Y negative using the cytotoxic T cell test (Goulmy *et al.*, 1983) whereas true hermaphrodites (XX) type SDM positive (Waibel *et al.*, 1987). Sex reversed mice (XX*Sxr*) usually type positive (Simpson *et al.*, 1981) with the exception of a line, designated XX*Sxr'*, which type negative (McLaren *et al.*, 1984; Simpson *et al.*, 1986). Intersex goats (XX) type SDM positive, as do sex reversed, and true hermaphrodite (XX) cocker spaniels (Wachtel, 1983). This study of intersex pigs supports the findings of Goulmy *et al.* (1983) and McLaren *et al.* (1984), indicating that testicular development is possible in the absence of H-Y antigen whether tested by the cytotoxic T cell test (mice and men) or the skin graft transplantation assay (pigs).

Regarding the chromosomal location of the H-Y gene, if *Tdy* (the testis determining gene) is present in the intersex pigs, then *Hy* (the H-Y antigen gene) and *Tdy* are not the same gene. Wiberg (1988) suggests that the H-Y structural gene is autosomal (X- and Y- linked genes determining its expression), and that the presence of two X chromosomes overrides any X- linked gene responsible for H-Y expression. Since both

intersex pigs tested were XX, and both were H-Yt antigen negative, the H-Yt structural gene could be autosomal, or may be located on an X chromosome, but, due to the presence of two X chromosomes, the antigen is not expressed. Without knowledge of the chromosomal location of structural and determining genes involved in H-Yt antigen expression, firm conclusions cannot be drawn. The present experiment does, however, provide proof that testicular differentiation is not dependent on H-Yt antigen.

DISCUSSION

The physiology of intersexuality in pigs

Of fifteen intersex pigs studied, nine were found to possess two testes, no ovarian tissue being apparent; in one animal (number 5), the gonads were not identified. Considering the extent of testicular tissue found in these animals, it is hardly surprising that the genital morphology and sexual behaviour are affected, The fact that the animals are not completely sex reversed indicates that testicular function in an XX individual is not equivalent to that of an XY animal, or that target tissues are in some way protected from a raised circulatory testosterone concentration. Peripheral testosterone concentrations were found to be lower than those in normal boars (1-3 nmol/l in the intersexes compared with 3-10 nmol/l, Ford and Schanbacher,1977; Colenbrander *et al.*, 1978), but concentrations in gonadal tissue have been found to be comparable (Hunter *et al.*, 1985). Urinary steroid metabolites in intersex pigs have also been found to be similar to those of the boar (Cook *et al.*, 1987). The low peripheral testosterone concentrations therefore indicate rapid metabolism of gonadal steroids.

The extent of masculinization of the external genitalia, and of the brain, does not appear to be related to the amount of testicular tissue present (see Chapters 9 and 10) since animals with one testis were as easily identified by their masculinized external genitalia as those with two testes. The exceptions were animals possessing a scrotal gonad, a scrotal gonad was always a testis, hence external morphology was an indication of gonadal type.

None of the intersex pigs involved in the behavioural study (Chapter 9) showed a "female" response to the boar and, in general, appeared to possess more male behavioural traits than female. This behaviour, and the male response, in LH secretion, to an oestradiol challenge (Chapter 10) indicate early exposure of the brain to testosterone. The effect is apparently quantitative, since some animals showed signs of previous

ovulations (animals 4 and 93), and intersex pigs have been known to become pregnant (Scofield *et al.*, 1969). Brain insensitivity to oestradiol may explain the lack of behavioural response to raised plasma concentrations of this steroid, as well as the inability to ovulate. In the normal mature female, a raised plasma oestrogen concentration is the gonadal biochemical signal responsible for initiating oestrous behaviour. It may be that prenatal exposure of the intersex brain to testosterone means that this behavioural response is never possible, whatever the concentration of oestrogen reaching the brain. On the other hand, oestrous behaviour can be induced in boars by administration of oestradiol (Ford and Schanbacher, 1977), indicating that prolonged exposure to oestradiol may negate the prenatal effects of testosterone. It is not known whether the same is true for the induction of ovulation in an intersex pig.

Ovulation is apparently more likely to occur from an ovary than from an ovotestis (Gerneke, 1967), although pig 4 had luteal tissue in an ovotestis. The low ovulation rate observed from ovotestes may be due to a local effect of testicular tissue on ovarian FSH/LH receptors. Hunter *et al.* (1985) could not induce ovulation from ovotesticular tissue with a PMSG challenge. The lack of pituitary response to an oestradiol challenge (as measured by the luteinizing hormone response to an oestradiol benzoate injection) also indicates an effect of testosterone at the hypothalamic/pituitary level.

The mechanism involved in descent of the testes to the scrotum is believed to be testosterone dependent, the growth of the gubernaculum depending on fetal secretion of testosterone (Wensing and Colenbrander, 1973). The gubernaculum is a mesenchymal thickening running from the caudal part of the testis, over the mesonephros towards the inguinal canal, and ending in a knob-like expansion in the scrotum (Wensing, 1968). The abdominal position of the testes in intersex pigs could be due to a lack of testosterone secretion during fetal development. However, O *et al.* (1988) observed a dimorphism in the gubernaculum between male and female marsupials, before gonadal differentiation was evident, suggesting that gubernacular development is genetically determined prior to any influence of testosterone. This may also be true in eutherian mammals, such that raised levels of testosterone in intersex pigs are more

effective at inducing testicular descent in the XY than in the XX fetus. Examination of young fetuses would be required to establish whether sexual dimorphism in the gubernaculum precedes gonadal differentiation.

Histological study of ovotestes showed that the ovarian and testicular portions of the gonad were always well separated, usually by a thick layer of connective tissue. Some workers have noted that ovarian tissue occurs next to the gonadal stalk, but this was not observed in the present study. Ovarian tissue could be polar or next to the stalk, although it was always confined to discrete areas and never dispersed throughout the gonad. Follicles were sparse, indicating either that the presence of testicular tissue affects follicular development, or that the cause of the condition also affects germ cell survival such that follicle numbers are reduced. Alternatively, germ cell survival could in some way influence gonadal development, a proposal discussed in more detail in the next section.

Testicular tissue in intersex pigs is never fertile. It is possible that germ cells are present in the seminiferous tubules in the fetus, but that these XX germ cells do not survive in a testicular environment. Oocytes are found in the testes of XX*Sxr* mice, but only in young animals, the oocytes degenerating after approximately day 15 after birth (McLaren, 1980). In the testes of a six day old intersex pig (which was not karyotyped) dividing cells were observed which may have been germ cells. These apparently do not survive as no such cells were seen in older animals.

The aetiology of intersexuality in pigs

Whilst not all animals used in this study were karyotyped, evidence from previous workers, and from the animals which were typed, indicates that the majority of cases are XX. There are few exceptions reported, and those which do turn out to be XX/XY mosaics, have been identified by analysing 25 chromosome spreads per animal (Bosma *et al.*, 1975). Lack of evidence of chimaerism, the identification of intersex fetuses flanked *in*

utero by two females, and the normal development of the uterus in intersex pigs are all observations which suggest that placental anastomosis between male and female fetuses is not the cause of intersexuality.

There is evidence to suppose that the intersex condition is heritable and therefore has a genetic cause (Sittman, 1973; Sittman *et al.*, 1980; Laus *et al.*, 1984). Studies on the nature of inheritance indicate that an autosomal gene (or genes) is responsible (Sittman, 1973; Laus *et al.*, 1984). The problem with such an explanation is that gonadal development is asymmetrical (a testis being more likely to form on the right hand side) whereas a genetic mutation/translocation would be present in every cell of the body. The same may be said, however, of the *Sxr* translocation in sex reversed mice. These male mice have an XX karyotype, yet develop testes (which are sterile) due to the presence of a portion of the Y chromosome (including the testis determining region *Tdy*) on the pseudoautosomal region of the paternal X chromosome. When this X*Sxr* chromosome is combined with another spontaneously arising mutation, T(X;16)16H (or T16H) female, male (sterile) or intersex offspring result. This is because the T16H mutation leads to preferential inactivation of the X*Sxr* chromosome but the inactivation does not always spread to the translocated *Sxr* region. The result is a mosaic of *Sxr* active and *Sxr* inactive cells, translated into gonadal development as ovarian and testicular tissue. In the absence of the T16H translocation, inactivation of *Sxr* would be less likely since it would now depend not only on the extent of "spreading" of inactivation along the X*Sxr* chromosome, but also on random rather than non-random inactivation of the chromosome (McLaren, 1983). Thus female XX*Sxr* mice are less common than T16H/X*Sxr* females. If spreading of inactivation into the *Sxr* region within a T16H/X*Sxr* mouse were random, one might expect to find ovaries and testes randomly distributed amongst the mice. In fact, such mice are more likely to develop testes on the left hand side than on the right (Ward *et al.*, 1987). X-inactivation occurs early in embryonic development, possibly when only 21 cells are present (Nesbitt, 1971), so that the genetic composition of the adult mosaic depends on the number of cells in which inactivation has spread into *Sxr* at this early stage, Thus asymmetrical gonadal development can be caused by a systemic genetic defect, the

distribution of ovarian and testicular tissue being determined by the extent of X-inactivation. Ovotestes in T16H/X*Sxr* mice are rare, probably because fewer than 50% of XY cells (or *Sxr*-active cells) are required in a gonad for a normal testis to develop (Mclaren, 1983).

Comparing the T16H/X*Sxr* mouse with the intersex pig, it is possible that the testis determining factor is located on an X chromosome in the pigs, and that non-random inactivation of the translocated X chromosome leads to preferential testicular development on the right hand side. If a crossover model similar to that proposed by Burgoyne (1982) is to be invoked in the aetiology of intersexuality in pigs, then a Y chromosome with a duplicate *TDF* (testis determining) region would have to be uncommon (*i.e.* the carrier male would be a mosaic of affected and normal Y chromosomes) such that XX intersexes occurred at a low frequency. In the mouse, a carrier male is one in which every Y chromosome carries the additional *Tdy* portion on the Y chromosome distal tip, and these carrier males produce one sex reversed offspring (XX*Sxr*) for every three normal offspring, *i.e.* a sex ratio of 3 males:1 female. If such a mechanism were operating in the pig, then one would expect to see a similar imbalance in sex ratio.

If intersex pigs do indeed carry a testis determining portion of the Y chromosome, this would explain why testicular tissue in these animals is devoid of germ cells. Singh *et al.* (1987) and Burgoyne *et al.* (1988) have shown that Sertoli cells in XX/XY mouse chimaeras are XY whereas follicle cells may be XX or XY (McLaren, 1988). In the intersex pig, Sertoli cells are therefore likely to be those in which the testis determining gene(s) is expressed, producing an environment incompatible with the survival of XX germ cells. That the germ cells are XX is assumed from the observation that intersex pigs may produce healthy oocytes. Burgoyne *et al.* (1988) also suggest that anti-Müllerian hormone produced by fetal Sertoli cells brings about testicular differentiation throughout the gonad, a proposal supported by the findings of Vigier *et al.* (1987) who showed that anti-Müllerian hormone could induce testicular development in a fetal rat ovary. In this context, it is interesting to note that anti-Müllerian hormone production in intersex pigs is poor, judging by the presence of Fallopian tubes and uteri adjacent to testes.

An alternative explanation for the paucity of germ cells in the testes, would be that these cells do play an important role in differentiation. The evidence that gonadal differentiation can occur in the absence of germ cells is based mainly on work in the chick, not in mammals. The ovary, in mammals, is believed to develop in the absence of some testis determining factor. However, if it is assumed that colonisation by germ cells determines ovarian development, and that, in the absence of sufficient numbers of germ cells reaching the gonadal anlage, a testicular-like structure forms, several features of the intersex gonad can be explained; 1) sterility of the testes, 2) the paucity in primordial follicles in the ovarian tissue of intersex pigs, 3) asymmetrical gonadal development. The position of the mesonephros (forming the adrenal glands) is known to be asymmetrical in the embryo and could well affect colonisation of the gonadal ridges by germ cells such that the right hand side is less well colonised and a testis results. A heritable component may be introduced by assuming that survival, or successful migration of germ cells is genetically determined. Evidence from the mouse indicates that gonadal differentiation may proceed even if germ cell numbers reaching the gonadal ridge are reduced (as in the mutations *Steel* and *White spotting*) (McLaren, 1985), but this does not mean that the same would be true if no germ cells were present. In the male, the presence of the Y chromosome would result in testicular differentiation despite sufficient germ cell colonisation. Alternatively, timing of colonisation may be important, such that, in an XX individual a delay in arrival of the germ cells results in testicular differentiation. This might arise if migration of the germ cells was inhibited.

Many references to intersexuality in pigs suggest that the presence of H-Y antigen would explain testicular development in these animals. Tests for H-Y transplantation antigen on two intersex pigs showed the animals to type H-Yt negative, and it can be assumed that raised H-Y antigen titres are not responsible for the condition. It may be worth testing for SDM (serologically detected male antigen) as it seems that the two antigens have different genetic determinants and may have different roles (Wiberg, 1987). The tests performed on the two intersex pigs were carried out using blood and not gonadal tissue, so that a local effect may be operating, resulting in the gonads typing H-Y positive whilst

other organs type negative. This would indeed have to be so in those individuals possessing different gonadal types; if H-Y antigen is involved in testicular differentiation it would be unlikely that an ovary would type H-Y positive.

The asymmetry of development of testicular tissue poses a problem in the aetiology of the condition. A systemic factor would seem to be precluded by the presence of different gonadal types *eg.* an ovary and testis, two ovotestes or an ovary or testis with a contralateral ovotestis. The adrenal glands have therefore been implicated in the aetiology of testicular differentiation since; a) these organs develop in close proximity to the fetal gonad, b) are known to secrete androgens and c) show asymmetry in the structure of their blood supply and in their position in the adult animal. However, an ACTH challenge did not elicit an unusual endocrine response from the gonads of intersex pigs and, on this basis, failed to provide evidence of the presence of adrenal tissue in the gonad. Histological examination of gonadal tissue from these animals revealed hilar cells, but no areas of adrenocortical tissue. "Adrenal rests" are found in the ovaries of several species but do not result in testicular development. It may be that, in the intersex pig, adrenal tissue incorporated into the developing gonad does not respond to ACTH in the manner expected and hence is not identified by means of an ACTH challenge. However, for adrenal tissue to cause testicular development, it would also have to be assumed that hormonal secretion was responsible for gonadal sex reversal, a supposition not born out by previous attempts to reverse gonadal sex, in mammals, by hormone administration.

CONCLUSIONS

There have been several studies of the intersex condition in pigs, the majority of which have found the animals to have an XX chromosome constitution despite the presence of secretory, but sterile, testicular tissue. Observations made in the present study confirm that testicular tissue is never fertile, and is more likely to be found in the right gonad than on the left hand side.

Previously, brief descriptions of male behavioural traits in intersex animals have been made. This thesis describes detailed observations on the behaviour and, also relating to brain sex, on the luteinizing hormone response to an oestradiol benzoate challenge. Results from both experiments indicate that exposure of the brain to testosterone, pre- and postnatally, has profound effects on brain development. The lack of hypothalamic/pituitary response to raised oestradiol concentrations may explain why many intersex pigs do not ovulate, despite the presence of ovarian tissue. Even if ovulation does occur, behavioural oestrus (*i.e.* receptivity to the male) is necessary for mating to take place. Observations of the sexual behaviour in these animals indicate that they are often aggressive towards the male, showing behavioural traits associated with mature boars rather than with normal gilts.

H-Y antigen has been proposed as being the testis determinant in mammals since males, but not females, in a wide range of species, type H-Y positive. However, there are no reports of the determination of the H-Y antigen status in pigs. The transplantation assay was chosen as the method for H-Y determination of intersex pigs, as *in vitro* tests may detect an antigen different to that first defined by transplantation methods. H-Y transplantation antigen assay on two intersex animals (using a boar and gilt as controls) showed them to be negative, indicating that this putative testis determining antigen was not necessary for testicular differentiation. In mice, there is evidence that testes can develop in H-Y negative individuals. The results obtained from the intersex pigs, supported this finding.

The adrenal glands have been implicated in the development of testicular tissue in XX individuals. To test for the presence of adrenal tissue in

the intersex gonad, the gonadal endocrine response to an ACTH challenge was measured in intersex pigs. Cortisol concentrations in jugular plasma were raised following the ACTH challenge. However, this rise in cortisol concentration was seen in the entire or gonadectomised intersex, indicating that gonadal secretion was not responsible. If adrenal tissue was present in the gonad, it did not respond with an increase in cortisol secretion when challenged with ACTH. Testicular tissue in the normal boar is known to respond to ACTH with increased testosterone secretion, a response also elicited by challenging testicular tissue in the intersex pig. Testicular tissue in intersex pigs, and in boars, must therefore possess receptors for ACTH. The detection of an endocrine response, to an ACTH challenge, is therefore a result of the tissue type rather than an indication of the occurrence of unusual cell-types within the gonad.

Given that intersex pigs have an XX karyotype and are not XX/XY mosaics, it is possible that a portion of the Y chromosome carrying *TDF* is present on the X chromosome but is not detected by microscopic examination. Non-random inactivation could then lead to expression of *TDF* in the right gonad more often than the left. The addition of the testis determining sequence to the paternal X chromosome (during male meiosis) would, however, result in a Y chromosome lacking *TDF*, in turn leading to XY females and a change in the sex ratio of offspring of the affected male. There is no evidence that this is so, and an alternative suggestion is made relating to the aetiology of intersexuality, without recourse to incorporation of Y chromosome material.

The germ cells were at one time thought to be the determinants of gonadal sex, colonisation of the gonadal ridge by the germ cells, migrating from their extragonadal site, signalling development as an ovary or a testis. This supposition lost favour following experiments on chicks, involving destruction of the germ cells. It was found that initial differentiation of the gonad was not impaired, and the conclusion drawn was that germ cells did not play a role in determining the sex of the gonad. However, observations of gonadal development did not continue beyond the early embryonic stages, nor were they undertaken in a range of species, and some doubt was expressed as to the effectiveness of the

treatment used to kill the germ cells. It is therefore suggested that the germ cells may play a role in gonadal differentiation.

It is proposed that, in an XX individual, insufficient colonisation of the gonadal anlage, by primordial germ cells, leads to differentiation of the gonad into a testis-like structure. Asymmetrical gonadal development would then be explained in terms of numbers of germ cells reaching the left and right gonadal anlagen. The presence of two X chromosomes would only lead to ovarian development if a "critical" population of germ cells reached the gonadal anlage by the time of gonadal differentiation. In the the presence of a Y chromosome, testicular differentiation would occur whatever the population of germ cells colonising the gonadal ridge. Such a proposal challenges the supposition that ovarian development occurs "by default", *i.e.* if there is no Y chromosome present. It is proposed that this is only true if there are sufficient numbers of germ cells within the gonad at the time of differentiation; if not, then the undifferentiated gonad becomes a testis. Investigation of this proposal, in the pig, would require the selective destruction of primordial germ cells, before their migration was complete, followed by histological examination of the resulting gonad. Alternatively, prediction of the occurrence of an intersex fetus would enable early study of gonads and assessment of their colonisation by germ cells. Prediction of the arrival of an intersex fetus would possibly be the major problem in work of this kind.

Whilst the study of animals in which the usual developmental pathway has not been adhered to is, in itself, of interest, "exceptions to the rule" also provide an opportunity to re-assess assumptions made about "normal" development. For example, the view that the presence of a Y chromosome is essential for testicular development, is challenged by the identification of XX hermaphrodites. Despite the recent cloning of the region of the Y chromosome thought to be responsible for testicular determination, and the prospects this offers for the manipulation of gender, the exact process of transformation of an undifferentiated gonad into an ovary or a testis is not known. Considering, however, the widespread interest in the subjects of sexual determination and differentiation, discussion promises to continue for many years to come.

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APPENDIX

Contents:-

Figures A1 to A8, showing results of assays for steroids in peripheral blood samples taken from intersex pigs, following an ACTH challenge (Chapter 11).

Published papers are included in the back folder.

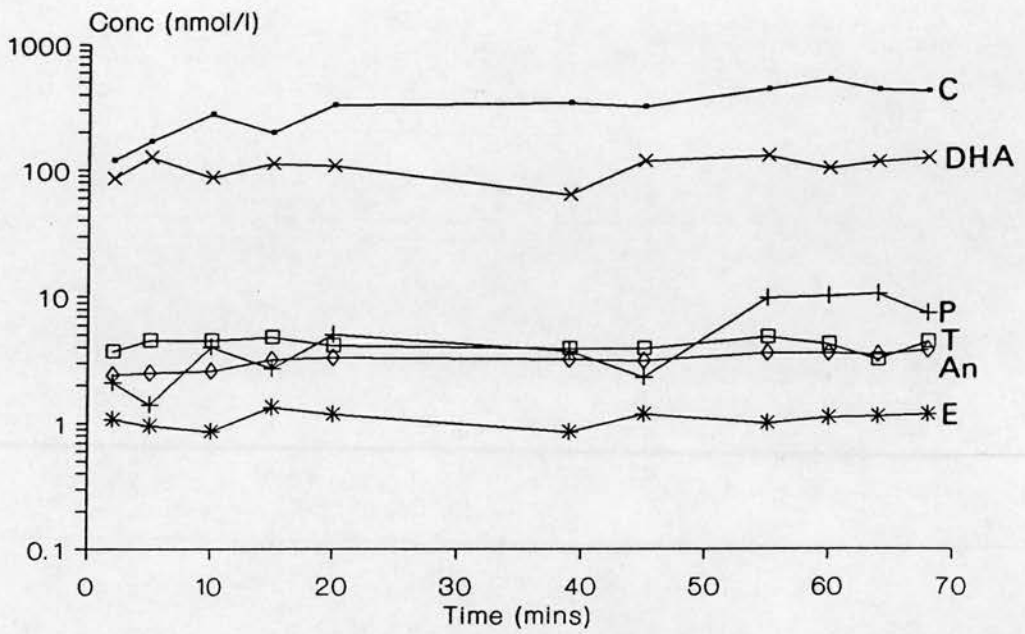
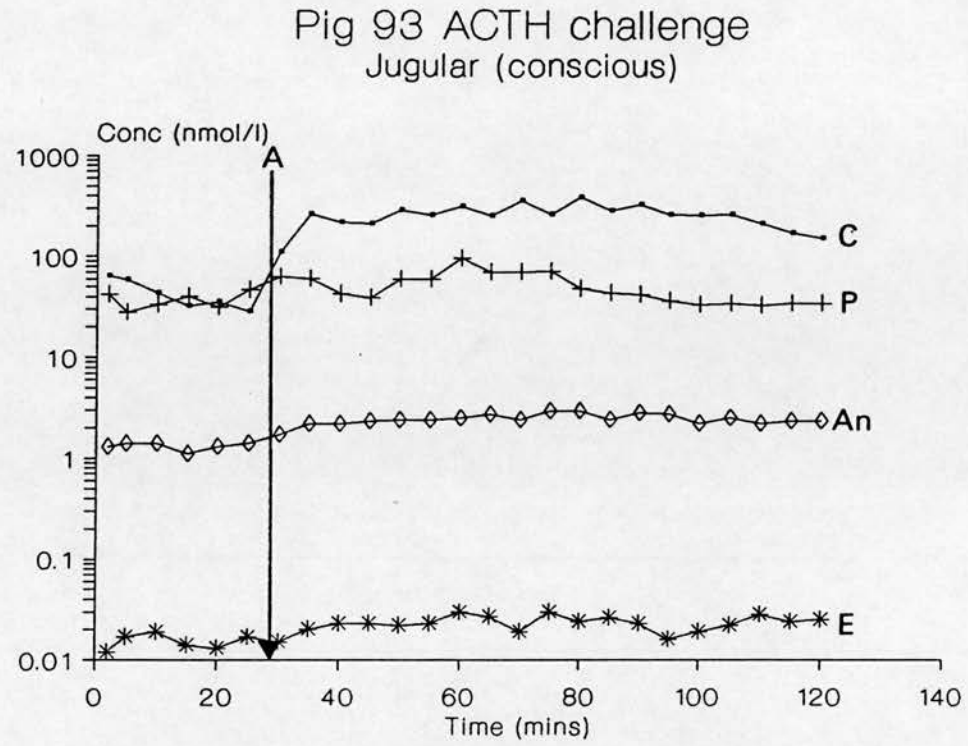


Figure A.1.

Figures A.1. to A.8.

Concentrations of androstenedione (An), cortisol (C), dehydroepiandrosterone (DHA), oestradiol (E), progesterone (P) and testosterone (T) in intersex pigs. Control animals = no ACTH challenge given, conscious animals = challenged when conscious, anaesthetised = challenged under anaesthetic, gonadectomised = challenged with ACTH before and after gonadectomy.


Pig 93 ACTH challenge
Jugular (conscious)
Conc (nmol/l)
1000
100
10
1
0.1
0.01
A
C
P
An
E
0
20
40
60
80
100
120
140
Time (mins)

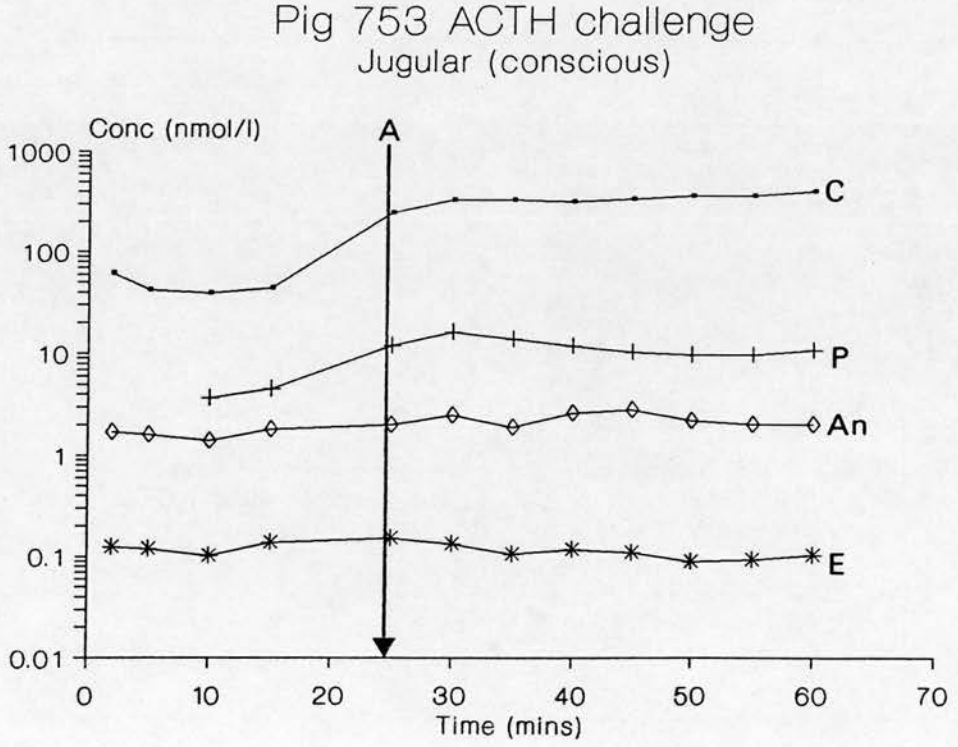
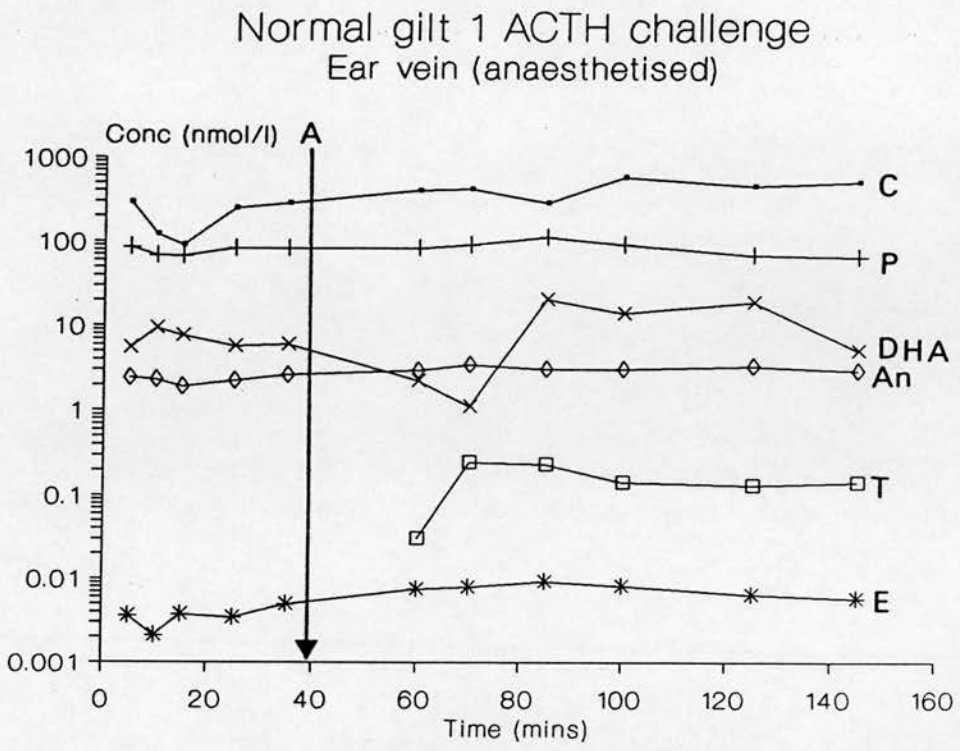

Pig 753 ACTH challenge
Jugular (conscious)
Conc (nmol/l)
1000
100
10
1
0.1
0.01
A
C
P
An
E
0
10
20
30
40
50
60
70
Time (mins)

Figure A.2.


Normal gilt 1 ACTH challenge
Ear vein (anaesthetised)
Conc (nmol/l)
A
1000
100
10
1
0.1
0.01
0.001
C
P
DHA
An
T
E
0
20
40
60
80
100
120
140
160
Time (mins)

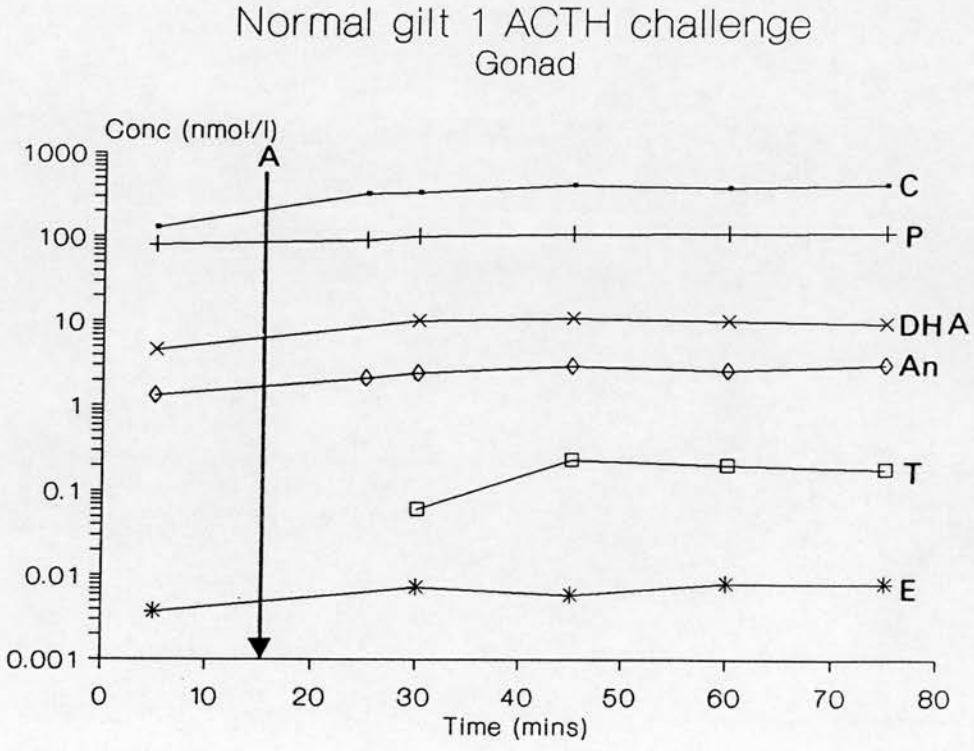
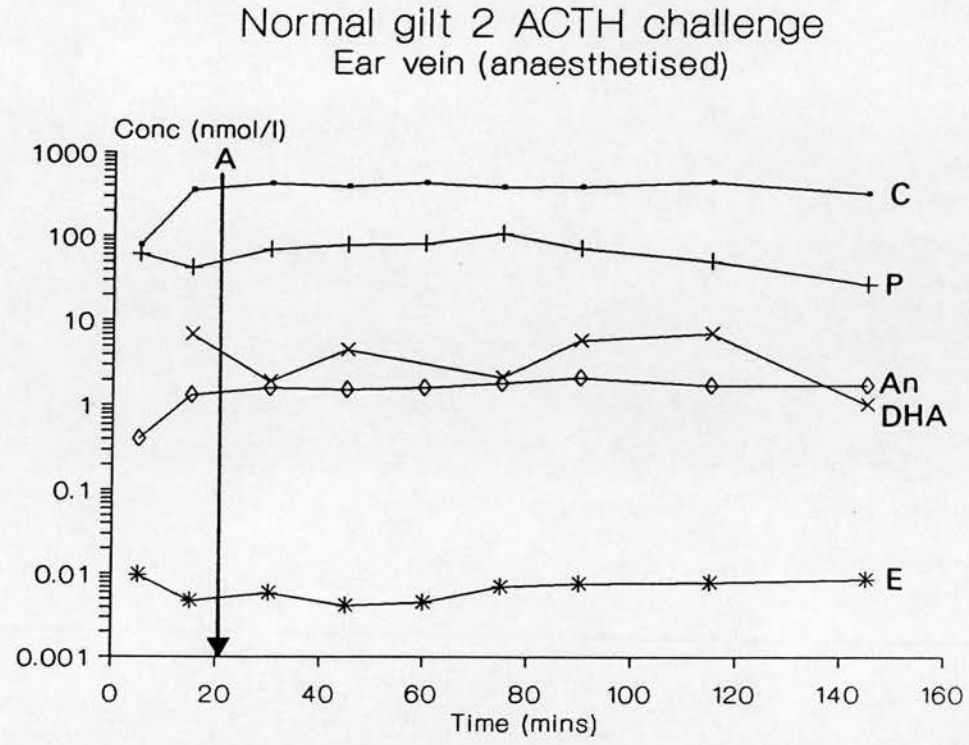

Normal gilt 1 ACTH challenge
Gonad
Conc (nmol/l)
A
1000
100
10
1
0.1
0.01
0.001
C
P
DHA
An
T
E
0
10
20
30
40
50
60
70
80
Time (mins)

Figure A.3.


Normal gilt 2 ACTH challenge
Ear vein (anaesthetised)
Conc (nmol/l)
1000
100
10
1
0.1
0.01
0.001
A
C
P
An
DHA
E
0
20
40
60
80
100
120
140
160
Time (mins)

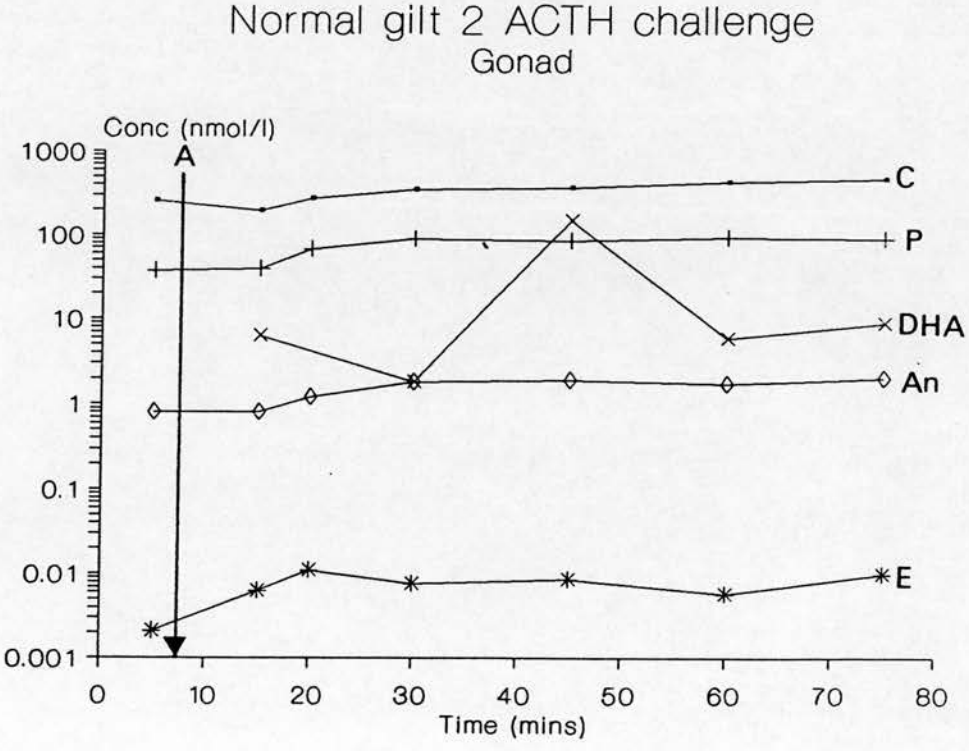
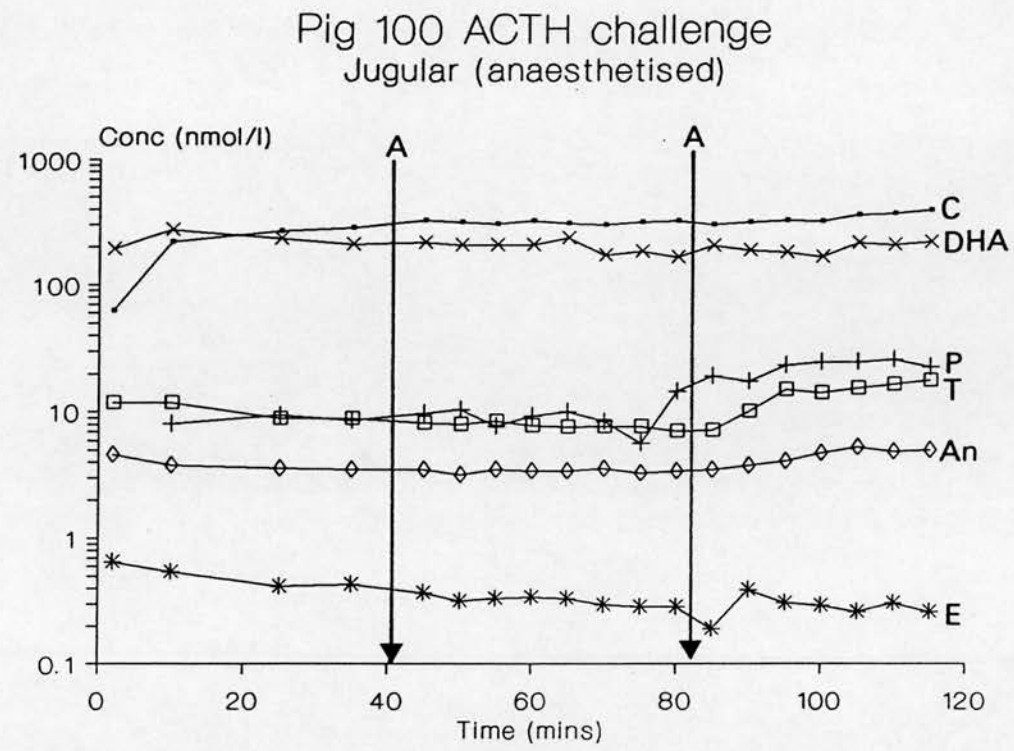

Normal gilt 2 ACTH challenge
Gonad
Conc (nmol/l)
1000
100
10
1
0.1
0.01
0.001
A
C
P
DHA
An
E
0
10
20
30
40
50
60
70
80
Time (mins)

Figure A.4.


Pig 100 ACTH challenge
Jugular (anaesthetised)
Conc (nmol/l)
1000
100
10
1
0.1
A
A
C
DHA
P
T
An
E
0
20
40
60
80
100
120
Time (mins)

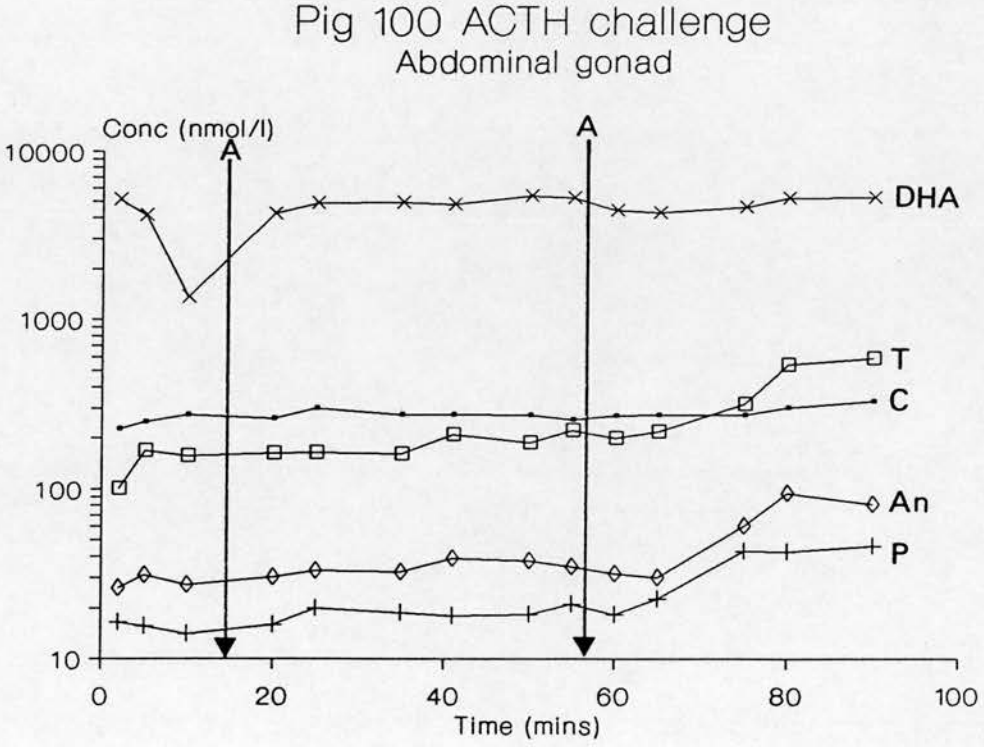
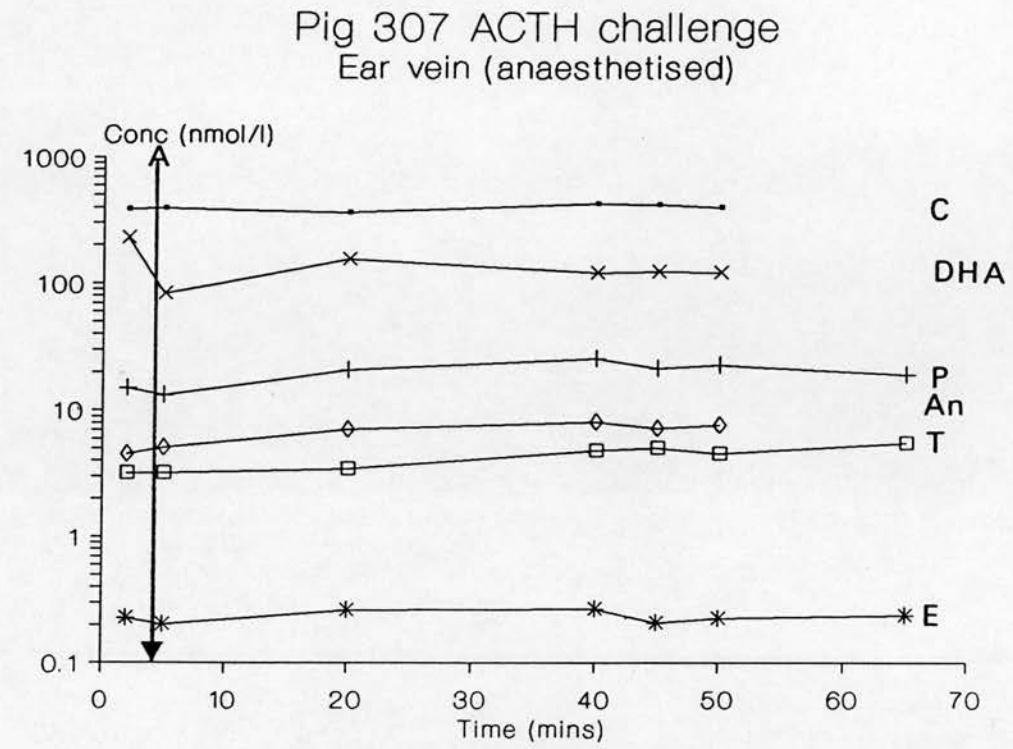

Pig 100 ACTH challenge
Abdominal gonad
Conc (nmol/l)
10000
1000
100
10
A
A
DHA
T
C
An
P
0
20
40
60
80
100
Time (mins)

Figure A.5.


Pig 307 ACTH challenge
Ear vein (anaesthetised)
Conc (nmol/l)
A
1000
100
10
1
0.1
C
DHA
P
An
T
E
0
10
20
30
40
50
60
70
Time (mins)

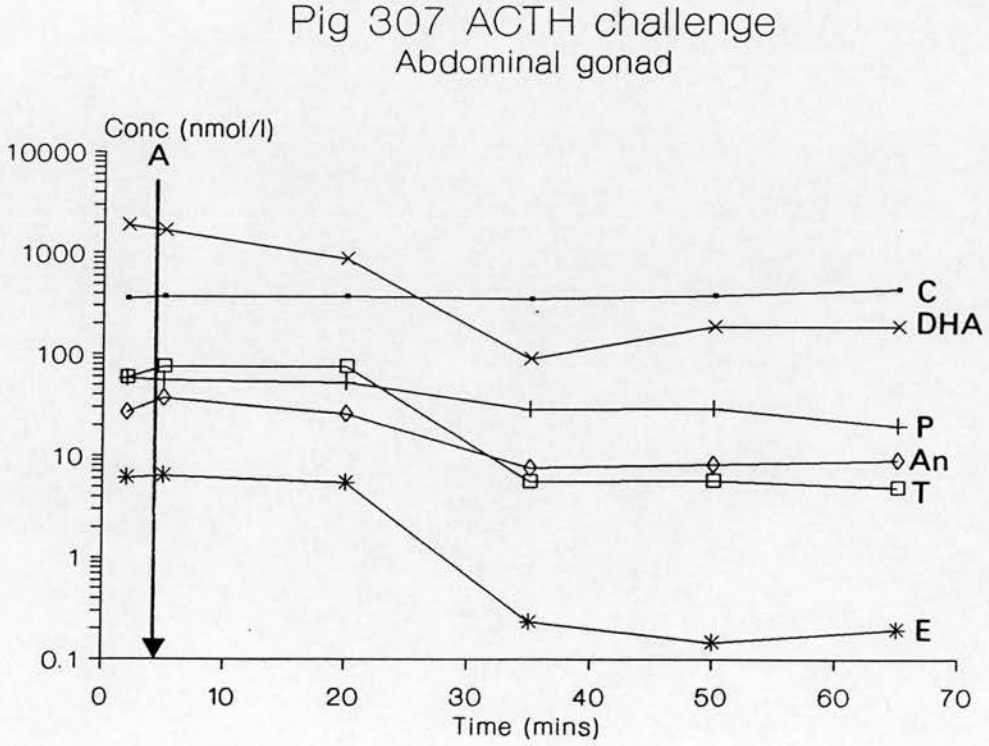
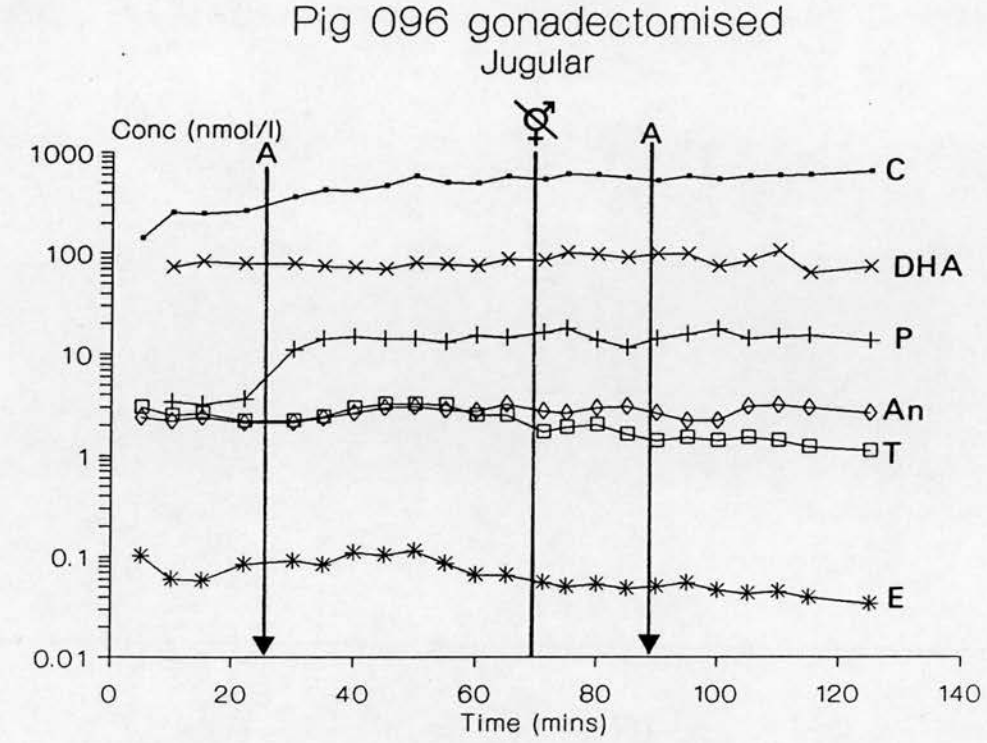

Pig 307 ACTH challenge
Abdominal gonad
Conc (nmol/l)
A
10000
1000
100
10
1
0.1
C
DHA
P
An
T
E
0
10
20
30
40
50
60
70
Time (mins)

Figure A.6.


Pig 096 gonadectomised
Jugular
Conc (nmol/l)
1000
100
10
1
0.1
0.01
A
A
C
DHA
P
An
T
E
0
20
40
60
80
100
120
140
Time (mins)

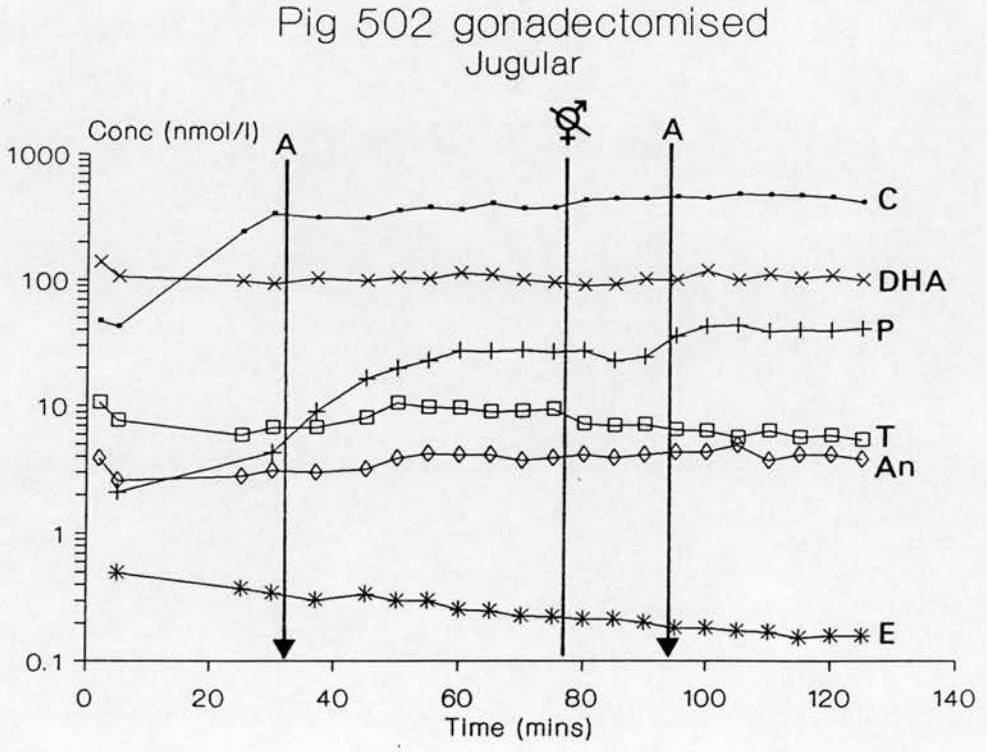
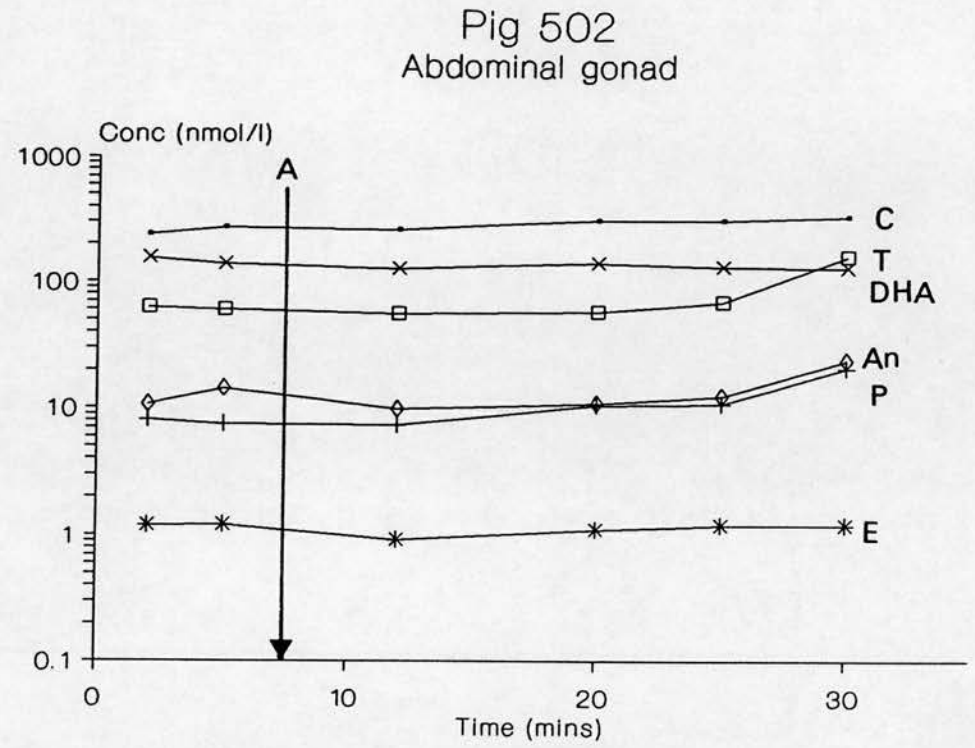

Pig 502 gonadectomised
Jugular
Conc (nmol/l)
1000
100
10
1
0.1
A
A
C
DHA
P
T
An
E
0
20
40
60
80
100
120
140
Time (mins)

Figure A.7.


Pig 502
Abdominal gonad
Conc (nmol/l)
1000
100
10
1
0.1
A
C
T
DHA
An
P
E
0
10
20
30
Time (mins)

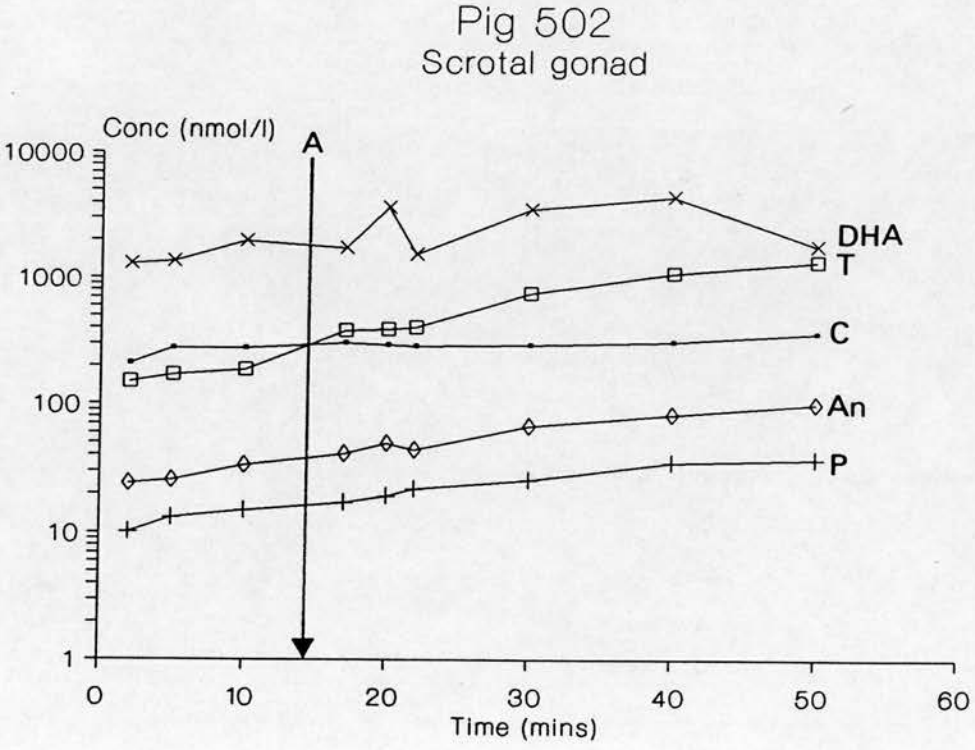

Pig 502
Scrotal gonad
Conc (nmol/l)
10000
1000
100
10
1
A
DHA
T
C
An
P
0
10
20
30
40
50
60
Time (mins)

Figure A.8.

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AND
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H-Y transplantation antigen status of two intersex pigs

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The term H-Y antigen was introduced in 1960 to describe an apparently male specific histocompatability antigen detected by skin graft transplantation, and later by serological methods. The observation that in mammals, testicular development was associated with expression of H-Y antigen, led to proposals that this was the Y linked "testis determining factor", although this is now disputed. Different laboratory methods may also define separate antigens. To ascertain the transplantation antigen status of intersex pigs, two animals were tested using the mouse skin graft assay. Intersex pigs are genetically XX, but the presence of testicular tissue in the form of scrotal or abdominal testes, or ovotestes, causes varying degrees of masculinization of the genital tract. The testicular tissue is composed of interstitial cells, seminiferous tubules and Sertoli cells, but germ cells are not observed. White blood cells from the two intersex pigs and from one male and one female control animal were used to sensitize female mice of an inbred strain. Skin grafts from male mice of the same strain were transplanted onto the females and the graft rejection times compared. Median survival times (days) were 18.0 and 19.5 for the two intersex pigs, 18.0 for female primed and 15.5 for male primed mice. Grafts on female and intersex primed mice survived significantly longer than those on male primed mice ($p<0.01$). These results show that intersex pigs type H-Y negative using the transplantation assay, and hence that testicular development is possible despite the absence of H-Y transplantation antigen. The results support the notion that H-Y (transplantation) antigen is not the testis determining factor in mammals.

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Pig News and Information

Intersexuality in pigs: some current views

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Introduction

According to Greek mythology, Hermaphroditus was the son of Hermes and Aphrodite, who merged with the nymph Salmacis to form one body with male and female characteristics. Organisms possessing the reproductive organs of both sexes are thus termed hermaphrodites.

Among plants and invertebrates, hermaphroditism is common and self-fertilization may occur; in mammals, sexual dimorphism is the norm and classification as male or female on the basis of phenotype is, in most cases, simple. For anyone involved in animal production this is important since an animal's sex determines whether it is bought, when it is sold and how it is handled, housed and fed. Even in our own species, the manner in which we behave towards someone is partially determined by their phenotypic sex, and one of the first questions asked about a new baby is 'is it a boy or a girl?'.

The occurrence of a farm animal of apparently mixed gender is therefore perplexing, challenging our previous assumptions about the immutability of the sexes and prompting us to think again about sex differentiation and development.

Intersex pigs

The term 'intersex' is usually applied to any animal in which the diagnosis of sex is confused. Individuals may be true hermaphrodites (possessing ovarian and testicular tissue) or pseudohermaphrodites if the gonads are of one sex but the reproductive tract shows characteristics of the opposite sex. Male pseudohermaphrodites have testes but largely female external genitalia whereas a female pseudohermaphrodite has ovaries and a masculinized tract.

Intersex pigs (sometimes known as 'wilgils') may be identified by external features such as an upturned vulva, enlarged (sometimes protruding) clitoris, a skinfold or prepuce behind the umbilicus and, in some cases, external testes and vulva seen in the same animal (see Fig. 1). Masculine patterns of behaviour may also be observed such as mounting of oestrus gilts or aggression (with excessive salivation) in the presence of a boar.

Surgical investigation usually reveals a well developed uterus and, according to Breeuwsma (1970) and Bäckström & Henricson (1971), most intersex pigs have two (usually abdominal) testes, i.e. they are male pseudohermaphrodites. On the other hand, up to 40% of intersexes studied by Breeuwsma were true hermaphrodites possessing an ovary and a testis or at least one ovotestis. In the cases where an intersex pig possesses one normal ovary, it is almost always found on the left hand side (Hunter *et al.* 1985) and may be functional, so that intersex pigs can become pregnant if the oviduct is patent (Scofield *et al.* 1969). The testicular tissue, however, is not productive and germ cells have never been observed in this tissue in intersex pigs.

Genetic analysis of cells (usually peripheral blood leucocytes) almost always reveals a 38XX, i.e. normal female, chromosome constitution. Karyotyping of ovarian and testicular cells from an intersex pig have revealed the female XX complement (Cavazos, Cook & Hunter 1985, unpublished) and since XX germ cells cannot survive in a testicular environment (Short 1982), it is perhaps not surprising that germ cells have not been observed in the testicular tissue.

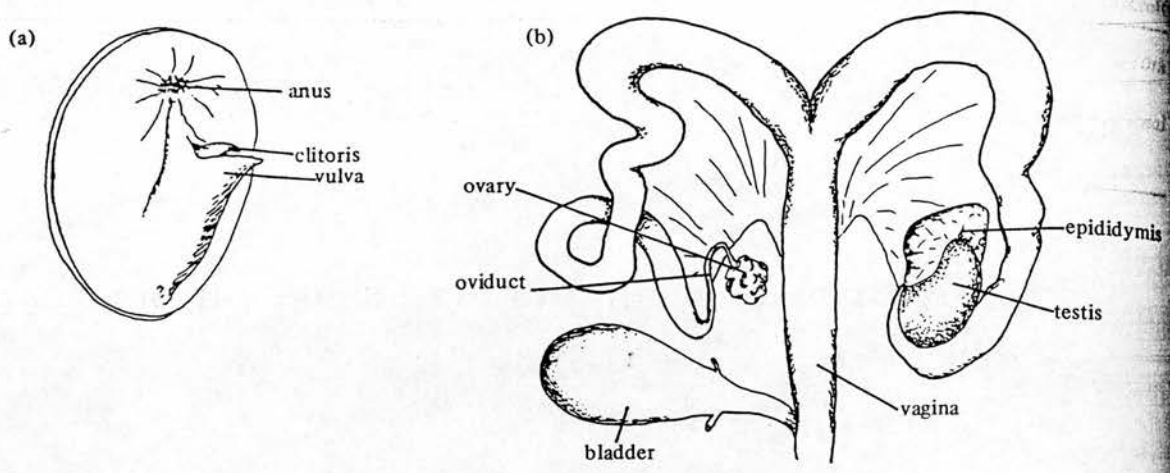

Fig. 1 (a) External genitalia and (b) reproductive tract and gonads of an intersex pig (dorsal view).

The incidence of intersexuality in pigs. Implications in production

Estimates for the incidence of intersexuality in populations of domestic pigs range from 0.2% in Sweden (Bäckström & Henricson 1971) to 10% on some islands of Vanuatu. On the latter, according to Baker (1928), intersex pigs were of great value for slaughter on occasions when a chief ascended to a higher rank, and because of the animals' importance, the islanders apparently attempted to breed intersexes from sows which had already produced them, and from females born in the same litter as intersexes.

In Europe, Breeuwsma's estimate (1970) of an incidence of 0.5% among commercial pig herds is probably the most accurate, but may only apply to the Netherlands. On certain farms the incidence could be much higher. Breeuwsma found 2.01% of pigs on one farm were affected; Hunter *et al.* (1982) estimated that 1-2% of gilts in a closed pig breeding herd in Scotland were intersexes.

Whilst there is no evidence that growth rates of intersexes are below average, their meat may be rejected due to 'boar taint' caused by testicular hormones (Booth & Polge 1976). Most intersexes are infertile and therefore cannot be used as herd replacements, and the few fertile intersexes which do occur (and go undetected) may be one source of small litters in a pig herd (Scofield *et al.* 1969). These factors, plus losses due to stress caused by the behavioural anomalies of intersexes, mean the condition may be responsible for a higher loss in production than its reported frequency of occurrence suggest.

Comparisons with intersexuality in other farm species

Among farm species, goats show a high incidence of intersexuality. Eaton & Simmons (1939) reported that 11.1% of a Saanen herd were affected and Biggers & McFeely (1966) quoted 5.8% to 14.9% across different breeds. Externally, the animals ranged from 'almost normal' males to 'almost normal' females, but internal investigations rarely revealed any ovarian tissue - only abdominal or scrotal testes. Unlike the situation in pigs, very few if any intersex goats are true hermaphrodites. The chromosome constitution is usually 60XX, i.e. a normal female karyotype.

The fact that most, if not all, intersex goats are hornless led Hamerton *et al.* (1969) to propose a mechanism by which the autosomal dominant gene 'Polled' may control a gene on the X chromosome leading to testes formation in genetic females. Such a mechanism has never been proved, but the observed link between the single gene trait of polledness and intersexuality, and the symmetrical appearance of two testes in the affected animals adds weight to the theory of a genetically controlled condition. This is not the case in pig intersexes where the high incidence of true hermaphroditism and the frequent asymmetry of gonadal development makes a genetic explanation more difficult.

Intersexuality in cattle and sheep is almost always associated with an XX/XY chromosome constitution caused by vascular anastomosis between placentae of male and female foetuses. The male foetus develops normally but the reproductive tract (and sometimes the gonads) of the female are masculinized. Freemartinism (as it is known) is reported to occur in approximately 92% of bovine females born of a twin pregnancy with a male foetus and at a much lower incidence in sheep (Marcum 1974).

This freemartin syndrome has been suggested as a cause of intersexuality in pigs, but there are several important differences between the condition in pigs and cattle. For example:

(1) The uterus in freemartins is always affected, with normal development never occurring, whereas intersex pigs may possess a patent uterus.
(2)Blood chimaerism, a sure indication of vascular anastomosis, is rarely found in intersex pigs; most have a 38XX chromosome constitution.
(3)An intersex pig foetus may be flanked *in utero* by female foetuses, thus excluding the possibility of vascular anastomosis as an explanation (Breeuwsma, 1970).

Possible causes of intersexuality in pigs

Since the vast majority of intersex pigs is presumably not the result of a male foetus influencing the normal development of a female foetus (freemartinism), other explanations must be found.

Genetic causes

Whilst analysis of different tissues nearly always reveals a 38XX karyotype, this does not exclude the possibility of undetected XY cells, or of a portion of Y chromosome being translocated onto an X chromosome or onto an autosome. Human XX males and XX male mice (XXSxr males) are known to carry a portion of Y chromosome on one of the X chromosomes (Evans *et al.* 1979, McLaren 1983). From the literature, it appears that intersexuality in pigs is a hereditary characteristic, reports often referring to boars which produce unusually high incidences of affected offspring. Laus *et al.* (1984) suggest a method of inheritance involving one autosomal mutation and Sittman *et al.* (1980), analyzing Breeuwsma's data (1970), favour inheritance by few, rather than many, autosomal genes.

The main problem in accepting a genetic cause of intersexuality in pigs is the asymmetry in gonadal development in these animals. In other species where a genetic cause has been established or seems probable, e.g. sex reversed mice and intersex goats, both gonads, with few exceptions, develop as testes. The high incidence of true hermaphroditism in pigs and the asymmetrical development of ovarian and testicular tissue make explanation by genetic factors difficult without involving quite subtle theories of local inactivation of genes.

Hormonal causes

It is widely accepted that, in placental mammals, gonadal sex cannot be changed in an embryo by administering hormones to the pregnant dam. Sex steroids have an effect on the reproductive tract and external genitalia such that administration of appropriate doses of androgens to pregnant mammals, at a certain stage of gestation, will produce masculinized female offspring. In opossums, testicular differentiation can be prevented by postnatal treatment with oestradiol benzoate (Fadem & Tesoriero 1986) but gonadal differentiation has never been reversed.

In some eutherian species, oestrogen-binding proteins in the foetus may protect it from the detrimental effects of maternal and placental oestrogens (Wilson *et al.* 1981) and presumably render externally administered oestrogen ineffective as well. The immutability of mammalian gonadal sex thus precludes suggestions that hormonal aberrations in the sow can affect gonadal development in her litter. In hermaphrodite animals, the problem is explaining the presence of ovarian and testicular tissue in an apparently genetically normal female, since masculinization of the reproductive tract can be seen as a natural consequence of gonadal secretions.

The adrenal glands

Several authors (Gerneke 1967, Breeuwsma 1970, Hunter *et al.* 1985) have postulated that adreno-cortical secretions (which include androgens) may affect gonadal development. The adrenals and gonads develop in close juxtaposition, and it is always the prenatal cranial pole of the gonad which becomes testicular in an ovotestis; after rotation of the gonad, the cranial pole becomes the postnatal caudal pole. In a condition well documented in man, the adrenogenital syndrome, continuous ACTH stimulation causes adreno-cortical hypertrophy resulting in increased production of androgens and hence in virilization. Gerneke (1967) found evidence of adrenal hypertrophy in intersex pigs, suggesting excessive androgen secretion, but the author pointed out important differences between the condition in man and that in intersex pigs. In human females, it is the urogenital sinus and genital tubercle which are affected and not, as seen in pigs, the gonads. Also, the condition is known to affect male and female human foetuses but intersex pigs are genetically female; lastly, hypertrophy of the adrenals is evident in affected humans but this is apparently not so in most intersex pigs (Breeuwsma 1970). Since numerous attempts to alter gonadal sex in mammals by administration of hormones have so far failed (Short 1982), it seems unlikely that adrenal steroids can change an ovary to a testis in the developing foetus.

Hunter *et al.* (1985) have suggested that incorporation of adreno-cortical tissue in the developing gonad may upset the process of differentiation, and in the ovaries of juvenile rabbits Mori & Matsumoto (1974) claimed to have found adreno-cortical tissue, although Byskov (1975) suggested

that cells were the Hilar rete gland of the ovary and not adrenal cells. Whether or not adrenal cells are found in the gonads of pig foetuses and whether they can affect differentiation and development is not known.

Crowding *in utero*

Evidence has emerged from Breeuwsma's study (1970) that intersex pigs are more likely to occur in large litters - as the litter size in the herd increased, so did the proportion of intersex animals. Breeuwsma's explanation is that 'crowding' of blastocysts brings about brief contact between males and females allowing diffusion of androgens into the female, and that this, with additional adrenal influences, causes masculinization of a gonad. Intra-uterine position in mice and rats does affect sexual development such that female foetuses between two males have, in adult life, longer and more irregular oestrous cycles, a greater ano-genital distance and are sexually less attractive to males than a female which developed between two female foetuses. This apparently occurs without any vascular anastomosis between the placentae (Vom Saal 1983). However, gonadal sex is not affected by intra-uterine position in these mice and rats, and if, in pig blastocysts, androgens were to diffuse from male to female, as Breeuwsma suggests, we must again assume a hormonal influence on gonadal differentiation.

The pineal gland

In all eight intersex pigs studied by Gerneke (1967), hypertrophy of the pineal gland was observed but no explanation was given for this condition. In those animals which exhibit seasonal breeding, the pineal gland is involved in synchronization of breeding activity with changes in day length. Whilst pigs, through domestication, have lost this seasonal trait (the wild boar is a seasonal breeder), it does not necessarily mean that the pineal gland is redundant. Pineal tumours in children delay sexual development and pinealectomy in cats causes an increase in gonadal weight (Swenson 1970), indicating that the pineal gland may have a role in gonadal function, and hence possibly in gonadal development in intersex pigs.

Gonadal growth rates and asymmetry

In human hermaphrodites, as in intersex pigs, testes are more often found on the right hand side and ovaries on the left (Mittwoch & Mahadevaiah 1980). By studying growth rates of human foetal gonads, these authors found that testes exceed ovaries in rate of growth and, in both sexes, right gonads are larger than those on the left. This led to the proposal that, because of different growth rates, an undifferentiated gonad develops as an ovary or as a testis; the presence of a Y chromosome results in growth above the 'base rate' required for ovarian development, hence a testis forms. In the XX hermaphrodite, additional growth necessary for testicular development ensues from a source other than the Y chromosome and this growth is more likely to affect the right gonad which grows faster than the left. The authors suggest that a threshold mechanism operates such that a gonad which exceeds this threshold (of size, number of cell divisions, etc.) becomes a testis. This would account for lateral asymmetry in hermaphrodites since the faster growing right gonad would be more likely to reach the threshold than the left gonad by the time that histological differentiation begins. Mittwoch & Mahadevaiah do not, however, offer an explanation as to why one gonad should grow faster than the other.

To result in an ovotestis, regions within the gonad would have to grow at different rates so that one part reached the threshold and became testicular whilst the rest did not do so and developed as ovarian tissue. The predominance of testicular tissue at the (prenatal) cranial pole of ovotestes in hermaphrodite pigs would suggest faster growth at this end of the undifferentiated gonad, and since testicular tissue differentiates before ovarian tissue, this would presumably be so for the ovotestis.

The observations that the growth rate of the testis exceeds that of the ovary (in mammals) at the time of sexual differentiation and that the right gonad (which in true hermaphrodites is more likely to contain testicular tissue) is larger than the left suggests a causal relationship between enhanced growth rate and sexual differentiation. However, there is no evidence that such a relationship exists and Mittwoch & Mahadevaiah's findings (1980) do not prove causality.

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Animal Breeding Abstracts

Intersexuality in Domestic Pigs: A Guide to Mechanisms of Gonadal Differentiation?

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I. Abstract

After briefly summarising current views on sex determination and differentiation in mammals, the condition of intersexuality in XX domestic pigs has been described, and the responses to diverse experimental treatments analysed in the light of findings in normal females. The paper then concentrates on the aetiology of ovotestis formation. Explanations based on placental fusion and migration of cells between neighbouring male and female foetuses were not favoured, nor were those based on translocation of a portion of the Y-chromosome onto an X-chromosome or even onto an autosome, since systemic (i.e. bilateral) rather than unilateral influences would be anticipated. Nonetheless, based on the premise of an asynchrony in the rate of development of right and left gonads, and a variable time of inactivation of the paternally-derived X-chromosome in animals carrying a translocated portion of the Y-chromosome, a range of degrees of ovotestis formation could be envisaged. Other lines of explanation invoke a masculinising influence of the embryonic adrenal cortex upon the neighbouring gonad, either indirectly by means of secretions or directly via incorporation of adrenocortical cells. Finally, and currently favoured by the authors, is the possibility of failure of primordial germ cells to reach and colonise the embryonic genital ridges in adequate numbers. An important inference from this interpretation would be that the primordial germ cells (diploid cells) exert a significant influence on the pattern of differentiation of the gonad.

II. Introduction

Anomalous sexual development in farm animals has attracted attention for many years, and quite detailed reports exist for diverse conditions in cattle (Marcum, 1974), sheep (Bruere and Macnab, 1968), goats (Hamerton *et al.*, 1969) and pigs (Baker, 1925; Brambell, 1929; Scofield, Cooper and Lamming, 1969; Breeuwsma, 1970). In a majority of these reports, the emphasis has been descriptive rather than analytical although, as long ago as 1779, John Hunter was attempting to understand the aetiology of freemartinism in cattle. More recent studies have progressed from morphological and histological observations to karyotyping and banding studies in a search for possible genetic lesions underlying the aetiology of intersexuality.

An important step forward in domestic farm animals was the recognition of a relatively high incidence of intersexuality in the University of Edinburgh's pig breeding herd at Easter Howgate, Midlothian, enabling detailed examination of the gonads and genital tract of such animals at laparotomy before commencing experimental studies (Hunter, Baker and Cook, 1982; Hunter, Cook and Baker, 1985). Observations in these animals constitute a background to this paper, in which intersex animals have been regarded as genetic females with at least one ovotestis or testis-like

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structure; in more extreme circumstances, both gonads may be testis-like structures, and one or both may assume a scrotal location. There are macroscopically visible disturbances to the Müllerian duct system involving the Fallopian tube(s). Proliferation of a cranial portion of the Wolffian duct as an epididymis is also characteristic.

Before discussing specific details of intersexuality in these experimental animals, a concise reminder of widely accepted views on sex determination and differentiation in placental mammals may be helpful. This is presented in a simple and straightforward manner, and avoids questions of interpretation that come to the fore later in the paper.

III. Sex Determination and Differentiation

The genetic sex of a mammalian embryo is determined by the sex chromosome constitution of the spermatozoon that fertilises the egg. Due to the symmetry of events during spermatogenesis, the two meiotic divisions arising from each spermatogonium give rise to two X-bearing and two Y-bearing spermatozoa. Equal numbers of these cells are presumed to be present in the ejaculate, and likewise presumed to have a comparable chance of effecting fertilization. Penetration and activation of the egg by an X-bearing sperm initiates formation of a female embryo, whereas penetration by a Y-bearing sperm prompts formation of a male. According to the Lyon hypothesis, one of the X-chromosomes in a female embryo becomes inactivated to avoid an overdosage of X-linked genes (Lyon, 1961, 1974). The inactivated chromosome can be revealed as heterochromatin — a pycnotic lump in the nucleus of somatic cells. The precise time of X-inactivation remains uncertain for the large farm animals.

1. Differentiation of gonads

The manner whereby male or female gonads are formed is not fully understood, but the germ cell line is first identified not in the presumptive gonads — that is among the somatic cells of the genital folds or ridges — but rather in a portion of one of the extra-embryonic membranes, the yolk sac endoderm. At a little over three weeks after fertilization in the pig, primordial germ cells can be distinguished migrating from the yolk sac to the region of the hind gut, from where they pass through the dorsal gut mesentery to colonise bilateral folds of tissue, the so-called genital ridges (see Patten, 1948). Passage of germ cells through the embryonic tissues is thought to be principally by means of amoeboid movement rather than by passive transport in the proliferating vascular system. In the latter part of their migration, the germ cells move from the developing kidney (mesonephros) into the adjoining gonadal tissue.

Once within the genital ridges, the primordial germ cells become concentrated in a cortical or medullary location according to the genetic sex of the embryo. If XX, that is female, the germ cells remain in the cortical region, and the gonads develop as ovaries. If male, the primordial germ cells pass to the medulla, and the gonads develop as testes. A significant phase of tissue reorganisation underlies primordial germ cell location and gonadogenesis. Despite the emphasis on cell migration in gonadal formation, cortical or medullary dominance is said to be established before colonisation with germ cells, for gonadal tissue apparently commences to differentiate according to its own genetic constitution. This dogma is called into question in a later part of this paper.

A massive multiplication of germ cells is completed before birth in female foetuses, for example producing 6-7 million cells in the human (Baker, 1963, 1972), and several hundred thousand in cattle (Erickson, 1966). This multiplication is followed by a phase of very extensive atresia. Thus, the greatest number of oocytes ever to be present in the ovaries is found before the time of birth, whilst wastage by atresia represents the fate of the overwhelming majority of oocytes. Whether the residual population has in some manner been selected for fitness remains an unanswered (and unanswerable ?) problem, as does the associated question of whether those oocytes that avoid atresia have equal developmental potential.

In the male, by contrast, the gonads do not develop an active, sperm-producing epithelium until puberty, at which time the testes of a majority of placental mammals have attained a scrotal location. Spermatogenesis does not proceed at abdominal temperatures — indeed prolonged cryptorchidism leads to destruction of the germinal epithelium, and hence sterility. Except in seasonal breeders or when pathological conditions intervene, production of spermatozoa is continuous and profligate from puberty onwards, though this does not necessarily infer a willingness to mate. Formation of gametes (spermatozoa) by the seminiferous epithelium requires a complex form of physical and biochemical support from specific somatic cells termed Sertoli cells. Arguments can be mounted for a two-way form of programming, or conversation, between the germ cells and Sertoli cells (see below).

2. Development of the duct system

Full differentiation of the reproductive tract requires the formation of functional gonads. Just as the gonad is initially plastic and can be programmed to form an ovary or a testis-like structure, so the duct systems can be preferentially programmed to give male or female genital tracts, for the very

young embryo possesses both Wolffian and Müllerian ducts. If the embryo is genetically male and has formed testes, then the paired Wolffian ducts are stimulated to develop, whereas the Müllerian ducts regress and are invariably vestigial by the time of birth. Wolffian duct development is largely under the influence of gonadal androgens, whereas Müllerian duct regression is prompted by a Sertoli cell protein termed Müllerian inhibiting substance (MIS) or anti-Müllerian hormone (AMH) (Josso *et al.*, 1979; Vigier *et al.*, 1984); this protein is now known to have close affinities with a follicular fluid (granulosa cell) protein called inhibin. In genetically female embryos, the Müllerian ducts are permitted to develop in the absence of androgen and AMH. Thus, femaleness may be regarded as a permissive state expressing itself in the absence of a Y-chromosome and the dominant condition of maleness.

IV. Studies of Intersex Pigs

Precise data on the numbers of intersex pigs revealed at laparotomy are not available, but at least 10 per year have been identified at surgery in Edinburgh since 1978. The following general observations are therefore based on more than 100 animals. Full details of intersex pigs described in the specific experimental studies that follow are given in Hunter *et al.* (1982, 1985) or in subsequent abstracts.

1. External features

Bearing in mind that intersex animals in the Edinburgh herd were all characterised as genetically female (i.e. XX sex chromosomes), the prominent tusks and frothy saliva observed in these animals provide immediate clues to the nature of gonadal endocrine activity. The well-developed clitoris that was always displayed on reflecting the lips of the up-turned vulva similarly infers androgenic stimulation. Somewhat less tangible observations were the extremely coarse hair and the pungent odour associated with the skin and saliva, both reminiscent of mature males.

Scrotal development was conspicuous in about 15% of the intersex animals, and at least one gonad could be palpated therein, invariably on the right-hand side of the scrotal sac (one exception). Furthermore, animals with scrotal development showed a penile sheath, and penile musculature could be detected by palpation or surgery. During mid-ventral laparotomy to expose the reproductive organs, the texture of the skin — as monitored with the scalpel blade — was consistently tougher than in gilts of comparable age from the same herd.

The behaviour of these animals has been described in some detail elsewhere (Hunter *et al.*, 1982, 1985; Chalmers, 1988). A spectrum of sexual activity was noted, ranging from characteristic female traits to the highly aggressive behaviour seen in mature boars.

2. Morphology of gonads and genital tract

As inferred above, a range of gonadal types was noted in intersex animals; these are illustrated in colour in the papers of Hunter *et al.* (1982, 1985). The most frequent condition was an ovary on the left side and an ovotestis on the right. In such animals, the ovary usually presented evidence of cyclic activity, bearing follicles of preovulatory diameter or mature corpora lutea. Some ovaries showed no evidence of such mature structures, in which case Graafian follicles did not exceed 4-5 mm in diameter. The ovotestis in these animals ranged in proportions from an approximately equal (by volume) distribution of ovarian and testicular tissue to greater than 90% of testicular tissue. Indeed, in some animals, macroscopic evidence of ovarian tissue could not be found in the right gonad. In these situations, the gonad was spherical and measured 6-7 cm in diameter.

A more striking condition of intersexuality was the appearance of both gonads as ovotestes. In such instances, there was no evidence for cyclic activity in the (variable) portion of ovarian tissue. Finally, in this gradation, there were those instances in which both gonads were testis-like structures, no trace of ovarian tissue being revealed grossly or upon histological examination. Irrespective of whether the gonad was abdominal or scrotal, the testicular tissue was found to be composed of seminiferous tubules and extensive interstitial tissue, but germ cells were never detected in the tubules — only Sertoli-like cells. It is worth emphasising that these presumptive Sertoli cells produced a poor staining reaction when compared with tissue from mature boars (Hunter *et al.*, 1982).

Turning to the genital tract, a patent bicornuate uterus was observed in all animals. In those showing evidence of cyclic ovarian activity, the dimensions of uterine tissues were comparable with those in normal females but, in the presence of bilateral ovotestes, the uterus was usually of immature appearance. In the latter situation, the cervix was also poorly developed and its patency was in doubt. Uterine fluid was frequently abundant in animals bearing ovotestes, and at autopsy this was noted to resemble pyometritis, though sometimes with a yellow watery component. Urine may have refluxed into the uterus in these instances (assuming cervical patency). Although the uterus was clearly abdominal in most animals, portions of one uterine horn had passed through the inguinal canal to enter the scrotum in two animals with a scrotal gonad. The observation was made during surgery for cannulation of the gonadal vein.

Anomalies were found in the cranial portion of the reproductive tract in all animals. The Fallopian tube adjoining an ovotestis was always underdeveloped, especially the isthmic portion, which was sometimes scarcely visible. However, the ampulla and especially the fimbriated infundibulum never approached the size characteristic of mature animals, and the gonad therefore could not be enveloped by the fimbriated extremity. By contrast, the proximal segment of the Wolffian duct had developed into a conspicuous and well-vascularised epididymis adjoining all ovotestes. In animals in which testicular tissue was prominent, the epididymis was distended by a viscous fluid, but histological section of course revealed the absence of sperm cells in the duct lumen (see Hunter *et al.*, 1982).

With the above paragraphs as background, various experimental observations can now be presented before discussing the aetiology of intersex animals.

3. Chromosome constitution

Karyotype studies of blood leukocytes were used to establish that intersex animals were genetically female. A modification of the whole blood microtechnique was used, and air-dried chromosome spreads (15-30 per animal) and subsequent banding techniques followed the method of Buckland, Fletcher and Chandley (1976). Whilst there was no ambiguity in the finding of two X-chromosomes in all animals, nor was there any evidence of the small metacentric Y-chromosome, the standard diploid number of 38 chromosomes was not revealed in all spreads. In one animal examined by Hunter *et al.* (1985), only 35 and 36 chromosomes were counted in two of the spreads. These low counts were associated with a breakage of cells on the microscope slide, and loss of chromosomes from the metaphase plate. The banding techniques failed to produce evidence for a translocated portion of the Y-chromosome.

Attempts were repeatedly made to culture testicular tissue from the ovotestis of a series of animals, but cells from only one animal revealed metaphase spreads: the testicular tissue had two X-chromosomes. As to further studies of the genetic constitution of intersex animals, molecular probing for Y-related DNA sequences would now seem the most incisive way forward.

4. Gonadal response to injected gonadotropins

In animals with two ovotestes and no signs of cyclic activity nor Graafian follicles larger than 1-2 mm diameter, challenges were given with placental gonadotropins in an attempt to stimulate follicular growth (Hunter *et al.*, 1982). However, a single subcutaneous injection of 1000 IU PMSG failed to provoke detectable responses in terms of follicular growth or oestrogenic changes in the genital tract. In a further intersex animal, with a small ovary on the left and a large ovotestis on the right, ovarian tissue in the right gonad failed to respond visibly to a similar dose of PMSG. The vesicular follicles had remained at a diameter of approximately 1 mm when examined one week later (Hunter *et al.*, 1985).

Bearing in mind that these Graafian follicles must have undergone enlargement since antral formation, inferring the presence of receptors for endogenous gonadotropins, it is difficult to explain the apparent lack of response to PMSG. Androgen or protein secretions from the ovotestis may have inhibited expression of the receptors for gonadotropins in the follicular tissues. If this were so, then the failure of unilateral ovariectomy to prompt growth of follicles in the ovotestis becomes understandable (Hunter *et al.*, 1985).

5. Hypothalamo-pituitary response to oestradiol challenge

Intersex animals showing cyclic ovarian activity in at least one of the gonads are presumed to have undergone a preovulatory gonadotropin surge: in other words, their 'brain sex' could be regarded as female (see Short, 1982). On the other hand, it is unclear whether acyclic animals with ovotestes have the ability to release a surge of gonadotropic hormones. Accordingly, such animals were given an acute oestradiol challenge, following which secretion of luteinising hormone (LH) into peripheral blood was monitored (Chalmers *et al.*, 1987). Although there was some variability in results, none of five animals showed a positive feedback response of LH comparable to that reported in cyclic females (see Elsaesser and Foxcroft, 1978). Thus, the brain in these animals was presumed to have undergone a variable degree of masculinisation, for the hypothalamus did not possess the ability to initiate a full preovulatory LH surge in response to the positive feedback influence of oestradiol.

6. Gonadal response to ACTH challenge

Because of the condition recorded occasionally in humans of islets of adrenocortical tissue within the gonads forming virilising tumours (Novak and Woodruff, 1967), the question arose as to whether adrenocortical tissue might similarly be found in the gonads of intersex pigs. One means of testing this proposition would be to challenge the animal with a standard clinical dose of ACTH (Synacthen), and then examine the response in gonadal venous blood. In an intersex pig with abdominal and scrotal gonads, intravenous ACTH increased cortisol and stimulated gonadal steroid

secretion by up to tenfold for testosterone, androstenedione, oestradiol and dehydroepiandrostenedione, whereas the responses in peripheral blood were attenuated (Cavazos *et al.*, 1987). Since the intersex gonad is responding to ACTH stimulation, a conventional interpretation would be that cells within the gonad must possess receptors for ACTH; testicular tissue in normal boars appears to have such receptors (Juniewicz and Johnson, 1984). Thus, endocrine evidence is accumulating for incorporation of adrenocortical or adrenocortical-like cells in the gonads of intersex pigs. The relevance of this observation is discussed below.

V. Aetiology of Intersexuality

Tentative explanations for the condition of intersexuality in pigs have focused on either (1) some degree of vascular anastomosis between fused placentae, with transplacental migration of cells from a neighbouring male embryo or (2) specific genetic errors, such as the presence of XY cells indicating formation of a chimaera, or translocation of a portion of the Y-chromosome onto an X-chromosome or even onto an autosome. However, placental fusion is a rare event in this species, for overlap of neighbouring allantochorionic sacs invariably leads to necrosis of the tips, thereby precluding fusion. In fact, none of these lines of explanation seems appropriate in situations in which the intersexual condition is found unilaterally, i.e. when one gonad is an ovotestis whilst the other is an apparently normal ovary, for systemic influences would have been expected to follow. Even so, a more complex form of interpreting varying gonadal abnormalities is offered in the last section of the paper. As to possible masculinising influences of unusual titres of H-Y antigen, again this explanation cannot easily be invoked when the condition appears unilaterally. Moreover, transplantation assays for H-Y antigen activity in the blood of intersex pigs have proved negative (Chalmers, Wiberg and Hunter, 1988).

1. Inadequate colonisation by germ cells

Ovotestis formation and the variable ratio of ovarian to testicular-like tissue might have a completely different aetiology, stemming from the failure of an adequate colonisation of the presumptive gonads by primordial germ cells. In genetically-female intersex pigs, such an hypothesis would predict an erroneous formation or migration of primordial germ cells resulting in a variable but incomplete colonisation of one or both genital ridges. This, in turn, would find expression in a variable development of cortical and medullary components of the presumptive gonad(s), either unilaterally or bilaterally. It might be expected, moreover, that with a limited colonisation of the genital ridges by germ cells in an XX animal subsequently found to have developed an ovotestis, the ovarian portion would adjoin the gonadal stalk or pedicle and the testicular portion would be devoid of germ cells. This has invariably been the case in the present observations, except of course in animals in which ovarian tissue was not detected at all.

In XX intersex animals possessing one or two ovotestes, inferences to be drawn if the above hypothesis is correct are as follows.

(*a*) The primordial germ cells may have a direct influence on differentiation of the gonad, and the extent of such colonisation — at least by XX germ cells in intersex animals — determines the relative proportions of cortex and medulla, that is the ratio of ovarian to testicular-like tissue. Even in genetic females, as judged from the current studies, there may be a predisposition for the gonads to become testis-like, except and until colonised by XX germ cells.

This suggestion is not unreasonable when it is realised that the primordial germ cells (diploid cells) must in some way be able to express themselves if they are to migrate from their extra-embryonic site in the yolk sac to the genital ridges of the presumptive gonads. If the primordial germ cells can therefore 'talk' to the systems that influence their migration, they could presumably also 'talk' to the gonadal tissues to influence the nature of cortical and medullary differentiation. This accords with the well-known follicular degeneration if and when the oocyte dies *in situ*. Hence, the mammalian gonad may be waiting for a germinal source of programming to enable its full and final differentiation. A number of delicate experimental models could be used to test this hypothesis.

(*b*) In the absence of any detectable ovarian tissue, suppression of Müllerian duct development and promotion of Wolffian duct derivatives can be quite limited in extent; as noted earlier, a well-formed bicornuate uterus is invariably observed in intersex pigs. The presence of testicular-like tissue in genetic females would therefore appear insufficient to masculinise most of the duct system. For the latter to occur, an adequate production of Müllerian-inhibiting substance by the Sertoli cells may require the influence of germ cells in the seminiferous tubules. Sertoli cells in the ovotestes of intersex pigs have been referred to as 'Sertoli-like cells' due to their poorly staining appearance (Hunter *et al.*, 1982). Hence, the argument that a full spectrum of Sertoli cell function may require stimulation from adjacent germ cells, just as normal spermatogenesis requires the intimate support of fully-competent Sertoli-cells. Such mutual dependence between somatic and germ cells has its female counterpart in the oocyte-granulosa cell relationship.

Outstanding questions arising from the present hypothesis are the following.

(1) Is the primary defect leading to ovotestis formation simply elaboration of an insufficient number of primordial germ cells?
(2) Are there errors in the migration or survival of primordial germ cells leading to an insufficient or unilateral colonisation of the presumptive gonads, perhaps with wayward cells passing to the embryonic adrenal glands or being enveloped by mesonephric tissues?

If the answer to (2) is yes, might a lack of chemotactic factors be implicated? Because certain boars are known to produce a relatively high incidence of the intersex condition in their offspring, it should be possible using histological techniques to study the extent of the germ cell population and the pattern of its migration from the yolk sac, and to compare these events with those in embryos generated by boars lacking this hereditary feature. Such a study is currently under way in our laboratory, focusing on passage of germ cells to embryonic adrenal tissue. This phenomenon of ectopic germinal elements has already been noted in the mouse (Zamboni and Upadhyay, 1983), even though morphological abnormalities in the reproductive system were not reported.

2. Translocation and gonadal asynchrony

Despite the above arguments concerning the germ cells, an open mind should still be maintained as to the involvement of a translocated portion of the Y-chromosome onto one of the X-chromosomes in the aetiology of the intersex condition. If development of the two gonads were asynchronous and the time of inactivation of the paternal X-chromosome were variable in these XX animals, but late enough in development to involve gonadogenesis, then it would be possible for a translocated portion of the Y-chromosome to prescribe a variable degree of testicular formation before losing influence during the inactivation process. This proposition is illustrated in Figure 1. However, this line of reasoning would be compromised by the report that pig embryos first reveal sex chromatin at the stage of about 50 cells (Lyon, 1974) — unless Y-derived DNA entrains processes that continue after inactivation.

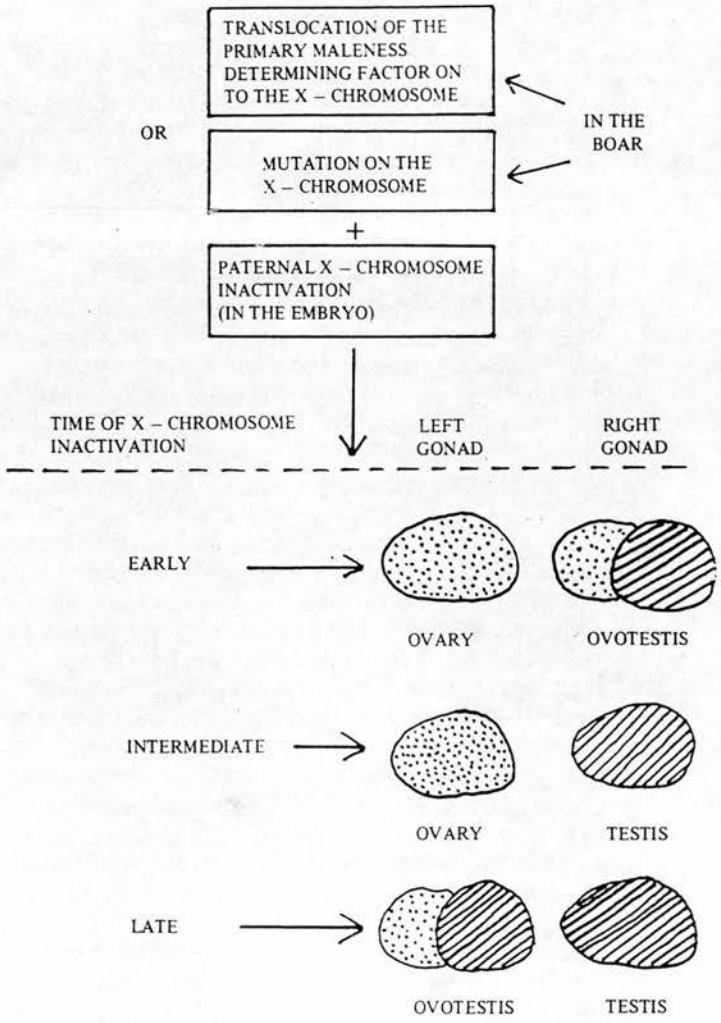

Fig. 1. The model suggests the manner whereby varying gonadal constitutions could arise from translocation of male-determining sequences onto an X-chromosome, or by an appropriate mutation on an X-chromosome, in the boar followed by inactivation of the paternal X-chromosome at different times in the embryo. A presumption in this model is that the right-hand gonad develops at a different rate than the left.

VI. Conclusion

In practical terms, instances of intersexuality represent a source of direct loss in the breeding herd, and such animals may also promote losses due to their aggressive behaviour. To a biologist, however, their greatest importance may be because they emphasise the lack of fundamental knowledge on the topic of gonadal formation. Since intersex animals seem to place a major questionmark over textbook dogma on sex determination and differentiation in mammals, they undoubtedly warrant much more extensive study. It is salutary to realise that even in 1988, there is still not a full appreciation of how males become males and females become females. This incomplete understanding presents a surprising contrast when viewed alongside projects aimed at controlling or predetermining the sex of mammalian embryos.

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