FERTILITY IN THE MALE EQUINE

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF PLATES

SUMMARY

The original aim of this study was to investigate whether hormone estimation could be used to diagnose the presence of cryptorchid testes in male equines. The initial study concluded that testosterone assay of blood samples taken before and after human chorionic gonadotrophin (HCG) administration, was the most reliable testosterone based test. However, this test was not ideal. Because of an overlap in the ranges of testosterone concentration in samples taken from stallions and geldings, diagnosis could not be reached if both the pre and post HCG samples contained testosterone concentrations in this overlap range. Therefore, this HCG stimulation test would occasionally fail to provide a diagnosis of whether testicular tissue was present, or not, in cases of badly behaved animals that appeared to be geldings.

Moreover this test could not be used to differentiate whether an animal with one scrotal testis was a unilateral cryptorchid stallion, or a hemicastrate stallion. The use of the HCG stimulation test in these cases would merely prove that the scrotal testis was present.

Because of these problems an alternative approach involving hormone estimation was sought. A subsequent study investigated whether the estimation of FSH in blood samples from animals suspected of possessing testes, could provide

a more reliable diagnosis than the HCG stimulation test.

This required the preparation of an assay for the estimation of FSH in blood samples.

The levels of FSH in the peripheral circulation of stallions were found to be more stable than the levels of testosterone observed previously. Therefore, unlike testosterone, FSH levels in single blood samples could be taken as representative of the average daily concentration in that animal. Unfortunately, the results presented in this thesis suggested that, under some circumstances, blood samples from stallions and geldings could contain similar concentrations of FSH. This appeared to result from a trend towards higher levels of FSH in older stallions, and a trend towards lower levels of FSH in older geldings. In addition, levels of FSH in a young cryptorchid stallion with no scrotal testis and one abdominal testis were similar to those in true geldings. Therefore FSH estimation could not be used to differentiate between those animals with stallion like behaviour that possessed testes and those that did not possess testes.

In addition, unilateral cryptorchids produced blood samples with levels of FSH similar to those found in normal young stallions. This was thought to result from compensatory hypersecretion of inhibin by the scrotal testis present in these cases. These results suggested that FSH estimation could not be used to differentiate the hemicastrate stallion from the unilateral cryptorchid

stallion.

This initial study of cryptorchidism led to an interest in the association between hormone levels and fertility and infertility in stallions. A stallion with a single, undeveloped, abdominal testis, had peripheral circulating levels of testosterone similar to those found in normal stallions. This suggested that Leydig cell function was not affected by local changes in spermatogenesis. However, the results of the estimation of FSH in blood samples taken from this stallion with one abdominal testis, which showed no evidence of spermatogenesis, demonstrated that peripheral FSH concentrations may be affected by germinal epithelial dysfunction. In this case germinal epithelial arrest was accompanied by elevated peripheral levels of FSH. This could be explained by an inhibin feedback loop hypothesis. In addition, an older stallion that consistently ejaculated fewer spermatozoa during semen collection, also produced blood samples with higher levels of FSH. Both these results suggested that alterations to normal seminiferous epithelial function may result in an elevation of peripheral FSH levels.

Vasectomy of a stallion resulted in no changes in the peripheral concentration of FSH. This suggested that infertility in a stallion exhibiting azoospermia, and normal levels of FSH, could be diagnosed as a case of obstructive disease of the excurrent ducts. Infertility

associated with elevated peripheral FSH levels could be diagnosed as resulting from damage of the germinal epithelium of the seminiferous tubules.

CHAPTER ONE

GENERAL INTRODUCTION

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1.1. INTRODUCTION

The successful stallion is one who passes his genetic material on to the next generation. In the feral state the stallion only covers the four to five mares in his own small family group (Klingel, 1982). These mares foal during the early spring which allows the foals to take advantage of the spring and summer grass. To maintain a 365-day interval between foalings the stallion must get his mares in foal during their first oestrus after parturition, known as the foal heat, or at the subsequent oestrus. The life of a feral stallion is therefore characterised by many months of sexual inactivity, when his only function is to maintain the integrity of his family group and prevent his being superceded. This is interrupted by a period of intense sexual activity in the spring when he must be able to identify his mares that are in oestrus. He must then be able to mount these mares, up to six times a day, and ejaculate sufficient viable, motile spermatozoa through the cervix and into the uterus for conception to occur (Catcott and Smithcors, 1972).

With domestication the role of the stallion has changed. Now a stallion may be asked to cover mares over a prolonged artificial season that has been arbitarily arranged to begin in early February. Instead of 4-5 mares

he is now expected to cover 40-50 mares in a season.

Although the horse has been domesticated for over 2000 years selection has been based upon athletic prowess and, or, physical conformation rather than reproductive performance. As a result the horse has retained much of its feral type reproductive physiology and behaviour, but is faced with an artificial breeding situation to which it is adapting.

1.2. THE NORMAL STALLION

To fulfil his function a stallion must; (a) produce sufficient normal spermatozoa, (b) identify which of his mares are receptive and about to ovulate and, (c) mount these mares, gain intromission and ejaculate spermatozoa through the cervix and into the uterus.

1.2.1. Sperm production

Spermatozoa are produced by the testes and mature to functional competence within the epididymis (Johnson, Amann and Pickett, 1980).

The testes, epididymis and the ductus deferens together constitute the scrotal contents.

1.2.1.1. The scrotum

The scrotum is located between the inner thighs of the stallion and consists of two pouches of pliable, oily, sparsely haired skin, separated medially by a fibrous raphe. As the contents and the pouch are both lined by smooth serous membranes the scrotal contents move freely within each scrotal sac. This enables contraction of a sheet of muscle, beneath the scrotal skin, to move the testes and their attached tissues up against the body wall in times of danger or extreme cold. Relaxation of this "dartos" muscle occurs in the calm animal and in warmer environments, lowering the testes between the legs to aid heat loss.

1.2.1.2. The testes

Each testis lies within the scrotum with its long

axis lying craniocaudally. The testes of the stallion are "pessary" shaped and have a resilient consistency. Within breeds of horses testes size varies with age and body weight (Thompson, Pickett and Squires, 1979b). In-vivo scrotal width also changes with testes position, as the dartos muscle contracts or relaxes (Gebauer et al 1974b). Cox (1982b) has shown that, after birth, a colt's testes change little in size until he reaches 16 months of age, after which they increase rapidly in size until the stallion is mature at 2 to 3 years of age. They then continue to grow slowly as the adult gains body weight. In the adult thoroughbred they measure approximately 105mm long and 55mm across and weigh 160g (Pickett, Voss and Squires, 1981a).

In the stallion each testis consists of a mass of granular parenchyma surrounded by a tough fibrous tunic, the tunica albuginea. Over 60% of the parenchyma of the testis of a normal stallion is made up of seminiferous tubules (Pickett, Voss and Squires, 1981a). Each seminiferous tubule is formed from sheets of cells joined by "tight" junctions. These junctions are impermeable to large molecules and to some ions (Amann, 1981). This cell layer constitutes a "blood-testis barrier", separating the cells within the tubules from the rest of the body. The cells that form this barrier are the "Sertoli" cells which are responsible for controlling the environment within the tubules.

Within the tubules the germ cells that are

responsible for the generation of spermatozoa divide frequently to form generations of ordinary germ cells, or spermatogonia as they are known. The mechanism of initiation of spermatogenesis in a particular segment of tubule is as yet unknown. The first stage of this process is when certain of these germ cells divide by mitosis to form a different type of germ cell, viz the primary spermatocytes. The primary spermatocytes then divide by meiosis to form two haploid secondary spermatocytes. These quickly divide again, each producing two spermatids. The spermatids do not divide but undergo a slow metamorphosis into spermatozoa. Spermiogenesis involves a close association with the "nursing", nutritive Sertoli cells, with each spermatid docking into a fold of Sertoli cell cytoplasm (Pickett, Voss and Squires, 1981a).

The whole process from primary spermatocyte to spermatozoan takes 35-39 days (Swierstra, Pickett and Gebauer, 1975a). Waves of spermatogenesis pass down each tubule, starting at a point furthest from the straight collecting ducts which anastomose with the efferent ducts that drain the seminiferous tubules. In the epididymides, these ducts converge to form a single epididymal duct.

The seminiferous tubules are supported by a structural and nutritive connective tissue. Within this connective tissue are groups of the pale cuboidal cells first identified by Leydig in 1850. These "Leydig" cells synthesise and secrete steroid hormones into the surrounding interstitial fluid (Ryan and Raeside, 1984). This

interstitial fluid drains into capillaries and so enters the general circulation.

1.2.1.3. The epididymides

Each epididymis forms a 1cm diameter cylinder that runs along the length of the dorsal border of each testis, and is attached to the testis at both ends. Each epididymis can be roughly divided into three parts; (a) the cranial end, the head of the epididymis containing the efferent ducts that converge to form the epididymal duct proper, (b) the middle part covering most of the dorsal border of the testis and, (c) the caudal part, the tail of the epididymis. The tail of the epididymis can easily be palpated in the stallion as a walnut sized protrusion at the caudal end of the testis.

The cells lining the cranial part of the epididymal duct pump intratubular fluid out of the epididymal duct, into the epididymal interstitial tissue (Crabo, 1965). At this stage the spermatozoa are immotile and so this pumping action is responsible for drawing the spermatozoa from the efferent ducts and the seminiferous tubules into the head of the epididymis and forward into the body of the epididymis. This pumping action has also been shown to concentrate the spermatozoa, the hormones and the proteins present within the epididymal duct fluid (Crabo, 1965).

During their passage through the body of the epididymis the spermatozoa lose the last remnant of their spermatogonial cytoplasm which is seen microscopically as a

cytoplasmic droplet. As this is shed the spermatozoa entering the tail of the epididymis become motile and fertile (Johnson, Amann and Pickett, 1980).

In the stallion, the tail of the epididymis is the major storage area for the mature spermatozoa and over 60% of the stored spermatozoa are stored there (Pickett, Voss and Squires, 1981a). Overuse of a stallion may result in a depletion of the number of spermatozoa stored within the epididymal tail, but does not affect the number of spermatozoa present within the head and body of the epididymis. The time taken for the passage of the spermatozoa through the epididymis varies with frequency of ejaculation, but usually takes 8-11 days (Swierstra, Pickett and Gebauer, 1975a). The total time from the begining of spermatogenesis to ejaculation is reported as 49 days (Swierstra, Pickett and Gebauer, 1975a) or 55 days (Pickett, Voss and Squires, 1981a).

Reproductive success for a normal stallion demands that these spermatozoa are ejaculated into the uterus of a receptive mare (Catcott and Smithcors, 1972). To achieve this the stallion must identify which mares are receptive and this requires a normal male sex drive or libido.

1.2.2. Stallion libido

The adult stallion must identify which of his mares are in oestrus and desire to mate with them. The complex behavioural interaction between mare and stallion, with the stallion's sexual advances being vigorously rejected or placidly tolerated, is known as teasing. During

teasing it is the absence of rejection that is most important in arousing the stallion (Pickett, Squires and Voss, 1981b). Having identified the receptive mare the stallion must have the drive, or libido, to mount and gain intromission. For many stallions libido is the factor that limits the number of mares that he can mate per day (Pickett, Squires and Voss, 1981b). The mechanisms that maintain normal libido in the stallion will be described later in this introduction.

1.2.3. Copulation

For a successful mating the stallion must pass viable spermatozoa to mares he has identified as receptive. This requires; (a) a transport system to carry the mature spermatozoa from the tail of the epididymis to the uterus of the mare, and (b) a fluid medium to carry and protect the spermatozoa during their journey. The transport system consists of the tubular tract, which is made up of the ductus deferens, the urethra and the penile urethra. The vehicle for the transport of the sperm rich epididymal secretion is the fluid secreted by the accessory sex glands.

1.2.3.1. The tubular tract

The ductus deferens is a continuation of the epididymal duct that passes from the tail of the epididymis, alongside the body of the epididymis and upwards into the abdomen within the spermatic cord. This cord also contains the spermatic artery and vein, the deferent nerve, artery and vein, and the cremaster muscle.

After passing through the inguinal canal the vas deferens joins the pelvic urethra lying on the floor of the pelvis. Before anastomosis with the pelvic urethra the ductus wall widens to form the cigar shaped ampulla. The distal portion of the extrapelvic or penile part of the urethra lies within the shaft of the penis of the stallion. The penile urethra is surrounded by a spongy connective tissue, the corpus spongiosum. The penile part of the urethra within the shaft of the penis is also covered dorsally by a network of vascular spaces, the corpus cavernosum.

When the stallion becomes sexually aroused the normal tonic parasympathetic stimulation of the penis subsides and sympathetic stimulation predominates. Electrical stimulation of the postganglionic sympathetic fibres in the hypogastric nerve "saturates" the smooth muscle of the tubular tract, accessory sex glands and blood vessels, with Noradrenaline and Adrenaline (Rasbech, 1975). Simultaneous contraction of the ischiocavernosus muscles pulls the penis up against the rigid ischial arch, obstructing venous outflow from the penis. Simultaneous relaxation of the smooth muscle of the Helicine arteries aids the flow of blood into the vascular spaces of the penis, and since the contraction of the ischiocavernosus muscle obstructs venous drainage from the penis, the penis fills with blood. To augment this engorgement the bulbospongiosus muscles, running between the urethra and the corpus cavernosum, start to contract, pumping blood into the cavernous spaces surrounding the urethra (Beckett et al

1975).

Externally, the first sign that a stallion is about to gain an erection is when the prepucial orifice drops and becomes obvious against the line of the ventral thorax. The penis then drops vertically downward, due to sympathetic stimulation and relaxation of the retractor penis muscle. As engorgement proceeds it becomes turgid and erect.

1.2.3.2. Accessory sex glands

These glands are responsible for secreting fluids which protect, dilute, and carry the spermatozoa from their storage site in the tail of the epididymis to the urethral process. In the stallion the accessory sex glands are the vesicular glands, the prostate gland, the urethral glands and the bulbourethral glands. The secretions of these glands are expelled into the urethra during ejaculation. The two vesicular glands are the largest and most cranial of the glands lying as long pear-shaped structures over the base of the ampullae of the deferent ducts. The smaller bilobed prostate lies immediately caudal to the vesicular glands. Nearer the root of the penis lie the smaller bulbourethral glands. The urethral glands are less discrete glands, scattered along the length of the intrapelvic urethra. All the accessory sex glands are surrounded by a thick smooth-muscle wall. During erection, sympathetic stimulation of the smooth muscle of the urethral and prostate glands causes contraction of these muscle fibres and the ejection of their secretion into the urethral

lumen. This forms the first secretion produced upon erection, and is a clear saline solution which flushes out the urethra prior to ejaculation (Mann, 1975). This is refered to as the first, or presperm, fraction of the ejaculate of the stallion. Once the stallion achieves intromission stimulation of the glans penis by the vagina stimulates the sensory fibres in the pudendal nerve and triggers the ejaculation reflex (Rasbech, 1975). Intense stimulation of the ischiocavernosus muscles is followed by waves of compression which flow distally along the penis (Beckett et al 1975). Simultaneous activity of muscle surrounding the vesicular glands, and the bulbourethral glands, forces the secretion of these glands into the urethra, where it mixes with spermatozoa from the tail of the epididymis. These accessory gland secretions dilute the epididymal secretion and prevent the oxidation and peroxidation of the spermatozoa (Mann, 1975).

The mechanism by which the spermatozoa in the tail of the epididymis are transported to, and along, the ductus deferens in this species remains unclear. Seminal transport along the urethra is thought to result from waves of compression, that pass along the corpus spongiosum of the penis during ejaculation (Beckett et al 1975). These contractions eject the semen with considerable force, in a sequence of "jets". With each of the successive 5 - 10 jets the amount of epididymal contribution to the semen diminishes by 50%, with 76% of the spermatozoa being ejaculated in the first 3 jets (Kosiniak, 1975). The jets

containing the epididymal secretion are referred to as the sperm rich fraction of the ejaculate.

The final part of the normal ejaculation sequence is the ejection of another secretion of the vesicular glands. This fraction has a tacky, "gel-like" consistency and is rich in carbohydrate. This may be a source of lactic acid in the ejaculate (Gebauer et al 1976). It is also an antiperoxidant, complexes calcium ions and buffers the ejaculate. In this it way may help to reduce the development of abnormalities in the spermatozoa (Mann, 1975, and Klug, et al 1979). In certain circumstances some stallions may not include this fraction in an ejaculate.

To operate successfully this reproductive mechanism requires the integration of a large number of complex systems. To achieve conception all these systems must work together in the correct sequence, and this requires central coordinating centres. These centres lie in the floor of the third ventricle of the brain stem and control reproductive functions via two communication systems. These are; (a) the nervous system, that responds rapidly to visual, olfactory, tactile and auditory signals to induce the immediate manifestations of erection and ejaculation, and (b) the neurohumoral system that controls those functions that require a more prolonged regulation, such as the maintenance of libido, spermatogenesis and accessory sex gland function.

1.2.4. The Neural control of stallion reproduction

The sympathetic nerve supply to the tubular genital tract controls immediate responses to sexual stimuli. Rath et al (1984) and Klug et al (1982), have demonstrated that within the tubular tract of the male equine, sympathetic constrictoric impulses run along alpha nerve fibres, and dilatoric impulses run along beta nerve fibres. Therefore, if spermatogenesis is proceding normally, the administration to stallions of beta blocking drugs, such as Bunitrolol, and alpha stimulating drugs, such as Noradrenalin Hydrochloride, cause an increase in the sperm cell concentration of the ejaculate and an increased sperm output. Sympathetic pathways may also control some of the immediate manifestations of libido, since these same drugs also reduce the number of mounts per ejaculation, and increase the libido of both normal and impotent stallions.

However, the work described in this thesis is associated with the hormonal control of reproduction in the normal and abnormal male equine, and not neural control mechanisms.

1.2.5. The endocrine control of stallion reproduction

The majority of the work investigating the role of hormones in the control of reproduction in the equine has been carried out in the mare. Fortunately, work in other species has shown that the structures of the hormones involved in this control are common to both the male and female animals. The physical mechanism by which these hormones are secreted into the general circulation are also common to both sexes although secretion patterns and the functions of these hormones does differ between the sexes.

Early work investigating the origin of reproductive hormones in the female equine was carried out by Day (1940), who demonstrated that the onset of cyclical reproductive activity in the mare could be hastened by the administration of multiple injections of horse pituitary extracts. This suggested that hormones originating from the pituitary could act systemically and control reproductive function in these animals. Using modern biochemical techniques the proteins responsible for these actions have now been isolated, and shown to be identical to the protein hormones that control equally important and diverse functions in the stallion (Wesson and Ginther, 1980). These hormones are; Gonadotrophin Releasing Hormone (GnRH), Luteinising Hormone (LH), and Follicle Stimulating Hormone (FSH).

1.2.5.1. Gonadotrophin Releasing Hormone

Experiments in other species have demonstrated a

delicate plexus of veins, the Hypophyseal Portal System, connecting the hypothalamus and the pituitary (Green and Harris, 1947). A hormone passing along this delicate venous shunt, and which stimulated LH release from the pituitary, was subsequently identified and named gonadotrophin releasing hormone (GnRH) (McCann, Taleisnik and Friedman, 1960). GnRH is not species specific and is a short peptide with a low molecular weight. In ovariectomised mares GnRH is present throughout the hypothalamus, but is especially concentrated in the median eminence and arcuate nucleus ventrally and medially, and the preoptic area, suprachiasmatic nucleus and anterior hypothalamic area rostrally (Strauss et al 1979). The site of storage of GnRH in the stallion has not been studied but it is expected that similar sites to those located in the mare will be involved. This GnRH passes down the hypophyseal portal system to the pituitary where it binds to the gonadotroph cells of the anterior pituitary. Administration of GnRH systemically to the recently castrated male horse results in a rise in systemic levels of both LH and FSH. This suggests that the binding of GnRH to gonadotroph cells stimulates LH and FSH release and synthesis (Thompson et al 1979a; Burns and Douglas, 1984a).

1.2.5.2. Luteinising Hormone

Equine luteinising Hormone (LH) is a polymorphic glycoprotein with a constant amino acid composition, but varying sialic acid content (Irvine, 1979). During

synthesis sialic acid is the last part of the molecule to be added and so LH with various amounts of sialic acid present may be released from the pituitary upon stimulation by GnRH (Hagen and McNeilly, 1975). The amount of sialic acid present determines the biopotency of the LH and the rate at which it is degraded by the liver (Irvine, 1979; Vaitukaitus and Ross, 1971). Whatever their molecular form the LH molecules all have the same function when released into the systemic circulation. Thompson et al (1979a) have reported that the concentration of steroid hormones in the peripheral circulation regulates the secretion of LH by the pituitary. Castration of an adult stallion was followed by a rise in peripheral LH levels. However when gonadal steroids were administered to male equines 30 days after castration LH concentrations fell to precastration levels. This suggests that the steroid hormones secreted by the interstitial tissue of the testes inhibit LH secretion in the normal stallion. This inhibition may result from either inhibition of LH synthesis or from inhibition of LH release. As the concentration of LH in the pituitaries of animals treated with testosterone following castration was lower than that in untreated animals, it would appear that LH synthesis was inhibited following testosterone administration. Conversely the concentration of LH in the pituitaries of animals treated with oestradiol following castration was much higher than in the untreated animals, and this suggests that either oestradiol stimulates LH synthesis, or that oestradiol inhibits LH release, leading

to a stockpiling of LH within the pituitary (Thompson et al 1979a).

These changes in LH secretion may result from altered pituitary function alone or, from changes in hypothalamic control of the pituitary via GnRH. When GnRH was administered to animals that had received testosterone after castration, there was very little stimulation of LH release. This suggests that the inhibition of pituitary LH synthesis by testosterone, is the result of a reduction in the sensitivity of the pituitary to GnRH (Thompson et al 1979).

However, when animals treated with oestradiol following castration received GnRH, they responded by producing higher levels of LH than were produced by control castrates receiving GnRH in the same way. This probably reflects the increased amount of stored LH in the pituitaries of these oestradiol treated animals.

This limited data concerning the control of LH in the male animal suggests that, in the normal stallion LH secretion is inhibited at the pituitary level by steroids secreted by the interstitial cells of the testis. Testosterone is thought to inhibit the response of the pituitary to GnRH secretion and thus inhibit LH synthesis, and oestradiol is thought to inhibit LH release per se.

LH is secreted in a pulsatile fashion in many species (Ellis and Desjardins, 1982), but its pattern of secretion in the stallion has not been determined. A seasonal trend in stallion LH levels has been reported,

with a marked rise in the spring (Burns et al 1984b; Harris, Irvine and Evans, 1983; Johnson and Thompson, 1983). The castration of mature stallions abolishes their seasonal pattern of LH secretion (Irvine and Alexander, 1982). It would appear therefore, that the testicular hormones modify LH secretion, with a negative feedback in the autumn and a positive feedback in the spring. Further research is required to clarify this picture.

Other factors may also affect peripheral LH concentrations, and may disrupt the resting pattern of LH secretion. Irvine, Alexander and Hughes (1984) have demonstrated that stallion plasma LH concentrations rise following copulation. This is thought to result from anticipatory changes in either GnRH release, or sensitivity to GnRH, following tactile and olfactory stimulation.

The main target cell for LH in the stallion is the Leydig cell of the interstitial tissue of the testis. Stimulation of the Leydig cell, by LH binding to receptors on the cell membrane, results in increased steroid hormone synthesis and release (Ryan and Raeside, 1984; Licht et al 1979). The most important of the steroid hormones released is testosterone, and the biopotency of equine LH is determined by measuring the testosterone production of cultured rat Leydig cells, following the administration of LH (Licht et al 1979).

In the stallion the Leydig cell receptors to which the LH binds are specific for LH. However if certain binding blockers were not present, the actual high affinity

binding area of the receptors of these Leydig cells would bind FSH or thyroid stimulating hormone (TSH), as well (Combarnous and Henge, 1981). This type of binding specificity and the polymorphism of equine LH are two complications unique to study of this gonadotrophin in this species. Binding of the LH to the Leydig cells stimulates steroidogenesis by triggering membrane bound enzymes to release cyclic AMP. This cAMP then phosphorylates and activates the multitude of enzymes involved in steroid synthesis (in: Stryer, 1975).

1.2.5.3. Steroid hormones

The seven unconjugated steroid hormones found in significant concentrations within the peripheral circulation of the stallion are; (a) the androgens; testosterone, androstenedione, dihydrotestosterone, delta-5-androstenediol, and dehydroepiandrosterone, listed in order of decreasing plasma concentration, and (b) the oestrogens, of which only oestradiol 17-beta and oestrone are present in stallion plasma in significant quantity (Ganjam, 1979).

Systemic GnRH administration results in the release of LH from the pituitary, and this leads to a rise in plasma steroid concentration, especially testosterone concentration. This rise usually appears within 30 minutes of the administration of GnRH (Burns and Douglas, 1984a; Thompson et al 1979a). However, rises in LH are not always accompanied by rises in testosterone. For example, the copulation response of increased LH release is not mirrored

by changes in peripheral testosterone concentration (Irvine, Alexander and Hughes, 1984).

Pulsatile changes in testosterone concentration, similar to changes in LH concentration seen in other species, have been observed in the stallion (Ganjam, 1979; Cox and Williams, 1975a). However, other evidence suggests that the pattern of secretion of testosterone in the stallion is a smooth biphasic diurnal pattern with no erratic episodes or pulses (Cox et al 1973; Carson and Thompson, 1979). The results of the study by Ganjam (1979), which claims that random pulsatile changes do occur, represent the cumulative results from seven stallions. The other reports documenting diurnal patterns were based on less data taken from fewer stallions.

A seasonal change in testosterone concentration has been reported (Ganjam and Kenney, 1975; Harris, Irvine and Evans, 1983; Johnson and Thompson, 1983; Burns et al 1984b). Although LH secretion may control testosterone release, these seasonal changes in testosterone concentration in the stallion do not follow seasonal changes in LH concentration, as might be expected. Testosterone concentrations reach a peak in May, and then fall gradually over the summer to reach trough levels in December. However, the seasonal LH peak follows after the testosterone peak in the spring, and LH levels appear to rise as testosterone levels fall in the autumn (Harris, Irvine and Evans, 1983; Johnson and Thompson, 1983; Burns et al 1984b). This may indicate that testosterone secretion

does not depend solely upon LH levels. Exposing stallions to 16 hours of light from 1st December causes an elevation in peripheral circulating levels of dehydroepiandrosterone, androstenedione, testosterone and oestrone, compared with control animals receiving normal daylength (Burns et al 1984b). Therefore seasonal changes in daylight length could be responsible for the seasonal pattern of testosterone secretion. Such light treatments have a variable effects upon LH levels, and this may account for the disparity between the seasonal patterns of the secretion of LH and testosterone described by Burns et al (1984b) and Burns et al (1982).

Sexual stimulation of the stallion has been shown to stimulate LH release (Irvine, Alexander and Hughes, 1984; Harris, Irvine and Evans, 1983). Some workers have reported an increase in testosterone secretion in response to sexual stimulation (Ganjam and Kenney, 1975). Others report no such increased secretion (Irvine, Alexander and Hughes, 1984; Harris, Irvine and Evans, 1983; Cox and Williams, 1975a). Such differences in LH and testosterone response, as yet not fully understood, may represent another possible disparity between LH release and testosterone secretion.

The steroid hormones, especially testosterone, are responsible for maintaining the male reproductive tubular tract (Thompson et al 1980), semen production, stallion libido (Turner and Kirkpatrick, 1982), and stallion conformation. Castration removes the major source of

testosterone from the stallion, viz the Leydig cells of the interstitial tissue of the testes (Crowe et al 1977). Testosterone and oestradiol levels fall rapidly during the first 6 hours following castration(Thompson et al 1979a; Ganjam and Kenney, 1975). During the subsequent 2-3 months the ability to ejaculate diminishes, followed by the loss of desire to mount and subsequently the ability to maintain an erection (Thompson et al 1980). These changes can be prevented by the administration of exogenous gonadal steroids. Testosterone replacement therapy restores both libido and ability to ejaculate. Oestradiol therapy restores libido only, but to a greater extent than the administration of testosterone alone achieves (Thompson et al 1980). Since testosterone is readily converted to oestradiol by peripheral aromatases these results suggest that oestradiol is responsible for the maintenance of libido in the stallion. Further evidence supporting this view comes from a study of impotent stallions (Wallach, Pickett and Nett, 1983). Impotent stallions, with very poor libido, were found to possess normal levels of testosterone but lower than normal levels of LH and oestradiol.

The administration of testosterone alone to geldings restores the ability to produce an ejaculate. This would suggest that testosterone is responsible for the maintenance of accessory sex gland function in the normal stallion. Furthermore, in the absence of gonadal steroids the accessory glands atrophy (Thompson et al 1980).

Gonadal steroids are also involved in the control

of the secretion of LH. Administration of testosterone to the normal stallion inhibits the release of LH and FSH, and so reduces spermatozoal production, spermatozoal output, semen quality, and testes size, by inhibiting spermatogenesis (Squires et al 1981; Carson and Thompson, 1979). These effects are reversible and the sudden cessation of testosterone treatment rapidly removes the inhibition of gonadotrophin release, and a rebound effect with high gonadotrophin levels and increased spermatogenesis may follow (Carson and Thompson, 1979).

Much higher concentrations of gonadal steroids are present within the interstitial fluid, surrounding the seminiferous tubules, than are present in the peripheral circulation (Setchel and Cox, 1982). Therefore, changes in local concentrations of hormones may not be reflected by changes in peripheral concentrations. Thus, investigation of levels of gonadal steroids in the circulating plasma is probably not the most accurate method of demonstrating the local role that gonadal steroids play in regulating reproductive functions, especially spermatogenesis.

1.2.5.4. Follicle Stimulating Hormone

The same releasing hormone (GnRH) is involved in the release of both LH and FSH in the normal stallion (Thompson et al 1979a; Burns and Douglas, 1984a), and the administration of synthetic GnRH results in FSH release as well as LH release. The specificity of action of FSH is determined by the beta subunit of the FSH molecule, which

contains a negative specificity site that only allows FSH to bind to FSH high affinity receptors on the target cells (Combarnous and Henge, 1981). Each FSH molecule may have various amounts of sialic acid bound to it, and equine FSH is thus polymorphic (Irvine, 1979; Combarnous and Henge, 1981; Bousefield and Nard, 1984). The more sialic acid present the more biologically active is the hormone (Vaitukaitis and Ross, 1971). In addition hormone molecules with sialic acid present are more slowly degraded by the liver, and thus have a long half life and stable plasma levels (Irvine, 1979). The amount of each form released varies with the degree of stimulation of the pituitary by GnRH (Hagen and McNeilly, 1975). Sialination is the last stage in FSH synthesis. Exogenous GnRH administration initially stimulates the release of sialinated FSH. Further stimulation results in the release of desialinated FSH.

When a stallion is castrated there is a subsequent rise in serum FSH levels, suggesting that hormones secreted by the scrotal contents inhibit FSH secretion in the normal stallion (Thompson et al 1979a). If castrated stallions receive testosterone or oestradiol therapy thirty days after castration, FSH levels gradually drop to levels just greater than precastration levels. These results suggest that, in the normal stallion, testosterone and oestradiol, secreted by the interstitial tissue of the testes, inhibit the release of FSH from the pituitary (Thompson et al 1979a).

The concentration of FSH in the pituitaries of

these steroid treated, castrated animals was higher than that in control castrates that received no treatment. This suggests that steroid treatment either, stimulated the synthesis of FSH in the pituitaries of the treated castrates, or that it inhibited the release of FSH from their pituitaries. Since the peripheral FSH concentrations were lower in the steroid treated animals, but the pituitary levels were higher, this tends to suggest that steroid treatment inhibits FSH release more than it inhibits FSH synthesis (Thompson et al 1979a). Such effects could be controlled by the hypothalamus, via GnRH release. When GnRH was administered to these steroid treated castrates there was a subsequent rise in peripheral FSH concentrations. However, the response of animals treated with oestradiol alone after castration was smaller than that of untreated animals, despite the higher concentrations of FSH in the pituitaries of the treated animals. This suggests that oestradiol reduces the responsiveness of the pituitary to GnRH and that this, in turn, affects pituitary function. On the other hand, animals treated with testosterone after castration responded to GnRH administration by releasing more FSH than untreated animals. This may reflect the large amount of FSH stored in the pituitaries of these testosterone-treated animals, or may indicate that less GnRH was secreted by the hypothalami of these treated animals, and that this was responsible for their reduced peripheral FSH levels (Thompson et al 1979a).

Thus it appears that FSH secretion is controlled by the hypothalamus and the pituitary, via feedback loops similar to those identified in other species, with testosterone acting at the hypothalamic level, and oestradiol acting at the pituitary level.

When castrated stallions were subsequently treated with greater than normal physiological levels of oestradiol, or testosterone, peripheral circulating levels of FSH did not drop to precastration levels (Thompson et al 1979a). This was in clear contrast to the larger corresponding drop in the LH levels in these animals. This suggested that a hormone, other than testosterone and oestradiol, was secreted by the scrotal contents of the normal stallion, and that this hormone inhibited FSH secretion by the pituitary. Such a hormone has been identified in the males of other species and has been named inhibin. Considerable evidence from other species suggest that inhibin is secreted by the Sertoli cells found within the seminiferous tubules (de Kretser, 1979). Sertoli cells have been shown to secrete a substance that inhibits FSH synthesis by cultured pituitary cells (Steinberger and Steinberger, 1976), and more recently Inhibin has been isolated in several species (Baker, 1983).

In adult stallions, seasonal variations in serum FSH levels parallel seasonal variations in serum testosterone. Both hormones reach peak levels in the spring, and then fall throughout the summer to trough

levels in the autumn and winter (Harris, Irvine and Evans, 1983; Johnson and Thompson, 1983; Burns et al 1984b). This seasonal pattern to peripheral FSH levels does not appear to be associated with changes in daylight, as manipulation of artificial lighting appears to have no significant effect upon FSH levels in the stallion (Burns et al 1982; Burns et al 1984b).

Equine FSH has very little LH like activity and plays little part in the control of Leydig cell function and gonadal steroid biosynthesis (Licht et al 1979). Equine FSH binds to rat seminiferous tubule preparations, and stimulates the production of cyclic AMP (Licht et al 1979). Therefore, as in other species, the FSH target tissue appears to be the germinal epithelium.

Recent studies report that vaccination of rats against FSH resulted in a total arrest of spermatogenesis (El Azab, El Hariri and El Azab, 1984). This suggests that FSH is essential for the maintenance of normal spermatogenesis. In addition, FSH may be needed for the normal secretion of Androgen Binding Protein (ABP) by the Sertoli cells. ABP concentrates testosterone within the epididymides and this stimulates normal sperm maturation (Hansson et al 1975). Therefore, FSH may be an essential stimulator of both spermatogenesis and sperm maturation. Some evidence for the role of FSH in maintaining stallion fertility is provided by Burns et al (1982), who reports lower FSH levels in stallions with poorer fertility.

Thus, in the normal stallion, protein hormones are responsible for maintaining normal libido and the normal activity of the tissues associated with reproduction.

LH secretion controls the synthesis and release of steroid hormones from the Leydig cells in the interstitial tissue of the testes. The testosterone secreted in this way maintains normal accessory gland metabolism, and the ability to ejaculate. With oestradiol, testosterone is also responsible for maintaining normal libido.

FSH secretion maintains normal spermatogenesis within the testes and sperm maturation within the epididymides.

1.3. THE CASTRATED MALE EQUINE

In certain circumstances the male equine is the preferred sex of the species, having speed, stamina, power and courage surpassing that of the female. For this reason the chosen charger for the cavalry, up until the advent of the "blitzkrieg", was the stallion (Meredith, 1891). Times change and the role of the horse has changed. The modern non-racing horse is kept for recreational purposes. Therefore, certain behavioural characteristics of the stallion, which previously were highly prized, are now undesirable. Even the keenest modern horseperson seems unable to find the time or expertise to train a stallion as a riding animal. In consequence it has become customary to castrate all colts not destined for stud work. Most colts, including those thoroughbreds that have not proven themselves on the flat, are castrated at 2 to 3 years of age. This allows the growth promotional effects, that result from high circulating levels of anabolic hormones in the entire animal, to have their effect before castration.

The effect of castration on the stallion is dramatic. Most of the aggressive, belligerent, proud and unpredictable behavioural characteristics are usually lost, and are replaced by a mare-like disposition. This effect is well recognised and, as a result, the vast majority of male equines in this country are now castrated stallions, or geldings as they are called.

However, all castrated animals do not adopt asexual behaviour. In the human, castration has little effect upon

behaviour or libido. Similarly, some geldings may retain facets of stallion-like behaviour. This is because stallion behaviour is not totally dependant on hormone levels, as is demonstrated by young colts, who can exhibit stallion like behaviour during play despite low circulating levels of androgens and oestrogens (Cox, 1979b).

During the surgical castration of the male equine the testes, epididymides and a section of the spermatic cord are removed, but the rest of the tubular tract and the accessory sex glands remain in situ. Seven percent of the total sperm reserves remain in the deferent ducts and ampullae following castration (Amann et al 1979). Therefore the total number of spermatozoa in the first two ejaculates, collected within the first four days after castration, is within the normal range for stallions (Shideler et al 1979). However, these spermatozoa are immotile and geldings are sterile within days of castration.

Following castration stallion behavioural patterns may persist for a variable period of time. In most cases ejaculation, the desire to mount and, finally, the ability to gain an erection are lost 2-3 months after castration (Thompson et al 1980). However, some animals maintain these activities for many months, or years, following castration (Cox, 1979b). The hormone levels in these animals are similar to levels in geldings that rapidly abandon such behaviour (Cox 1979b).

After castration the concentrations of testosterone and oestradiol in the peripheral circulation

fall rapidly, reaching stable levels by six hours postcastration (Thompson et al 1979a; Ganjam and Kenney, 1975). This low level of testosterone secretion is then maintained by adrenal tissue, located in the adrenal glands, and at ectopic sites, for the rest of the life of the castrate (Crowe et al 1977; Smith, 1974).

The low levels of steroid hormones results in the change from stallion to gelding behaviour and also removes the steroid hormone inhibition of gonadotrophin secretion. Since the brain is physically unaffected by castration, the removal of steroid inhibition results in increased LH and FSH release from the pituitary.

FSH levels rise slowly over the month following castration and then stabilise with a tenfold elevation above normal stallion peripheral plasma concentrations (Thompson et al 1979a).

LH levels rise more rapidly than FSH levels after castration, stabilising two weeks after castration (Thompson et al 1979a). The normal seasonal pattern of LH is abolished following castration, and over the subsequent years the LH concentration falls, and eventually stabilises at the precastration level (Irvine and Alexander, 1982).

In maintaining the high peripheral concentrations of gonadotrophins that are observed in the months following castration, the stores of gonadotrophins within the pituitary do not become depleted, and the administration of GnRH to castrated horses results in a further rise in LH and FSH levels (Thompson et al 1979a).

Since adrenal steroid secretion can be stimulated by the administration of substances with LH-like activity, such as Human Chorionic Gonadotrophin, the pituitary may be actively controlling steroid secretion in the castrate (Cox et al 1973).

1.4. THE CRYPTORCHID STALLION

The male embryonic gonads of the equine embryo differentiate at five and a half weeks gestation. Subsequently they undergo a massive hypertrophy to nearly adult size (Arthur, 1961). This hypertrophy is the response of the testicular interstitial cells to hormonal changes in the dam (Arthur, 1961). As the embryo grows, these relatively large testes migrate caudally so that by 5 months gestation they lie just proximal to the internal inguinal ring (Smith, 1975; Arthur, 1961). At this stage the ligaments attaching the testes to the vaginal processes of the scrotum (the gubernacular ligaments) are large and gelatinous, and completely fill the inguinal canals and vaginal processes, and prevent the testes from passing through the canals into the scrotum. By eight months gestation the gubernacular ligaments have shrunk considerably, but the testes are still too large to pass through the inguinal canals and into the scrotum. However as the foetus grows the tail of the epididymides are gradually drawn, by the gubernacular ligaments, into the inguinal canals. Then, finally, during the last month of gestation the testes follow the epididymides into the inguinal canals (Smith, 1975).

At birth the gubernacular ligaments are still large structures within the scrotum. In 50% of colt foals the testes may not have descended completely into the scrotum at birth, but lie within the inguinal canal

(Pickett, 1981a; Smith, 1975). Testes will fail to reach the scrotum if any of the changes described above fail to occur. Failure of one, or both, testes to descend into the scrotum results in the condition known as cryptorchidism. In the larger breeds of horse the right testis migrates within the embryo more quickly than the left testis (Pickett, 1981a). This may be the result of the right gonad lying more caudally in the embryo, so that the right testis has a shorter distance to travel to the scrotum. This may also explain why left sided testicular retention is more common in larger breeds (Cox, Edwards and Neal, 1979a; Smith, 1975). However, in pony breeds right sided retention occurs more frequently, suggesting that horse breed may affect the incidence of cryptorchidism. Another breed difference is that in some ponies right-sided retention resolves spontaneously at 2-4 years of age. This temporary retention within the inguinal canal is hypothesised as the aetiology of the unilateral testicular hypoplasia commonly observed in the Welsh pony breeds (Cox, Edwards and Neal, 1979a).

Retention of testes outside the scrotum results in a failure of normal postnatal and pubertal development of the testes. The most severely affected testes are those retained within the abdominal cavity. Here spermatogenesis is not initiated, and the seminiferous tubules contain a lining of a single layer of Sertoli cells and spermatogonia (Arthur, 1961; Bishop, David and Messervy, 1964; Arighi et al 1984). However the interstitial, or Leydig, cells

between these tubules are apparently unaffected, or are sometimes present in increased numbers (Arighi et al 1984).

The seminiferous tubules of testes retained within the inguinal canal appear to be less affected than those lying within the abdominal cavity. These testes may contain varying numbers of the cellular layers within their seminiferous tubules, and often have normal numbers of Leydig cells (Arighi et al 1984).

Because of these changes testes retained outwith the scrotum are smaller than normal, and those within the abdomen are smaller than those retained within the inguinal canal (Arthur, 1961). Although these gross and histological findings have traditionally been considered as degenerative changes, resulting from the exposure of testicular tissue to deep body temperature, they may represent a failure of the testes to develop in a normal way beyond the foetal stage. As embryonic structures develop under the chemotactic influence of their local microenvironment, failure to develop in these retained testes may be due to a lack of the right stimuli in the ectopic site. Failure to develop could also result from abnormal vascular supplies to these retained testes. In normal scrotal testes the steroid hormone concentration within the testes is higher than that in the peripheral circulation, and this results from a countercurrent exchange of steroids between the testicular veins and spermatic arteries within the pampaniform plexus (Setchell and Cox, 1982). Abnormal vasculature in the retained testes may reduce the

efficiency of such a mechanism, and result in steroid hormone levels within the testes that are insufficient for normal postnatal development.

Although spermatogenesis is arrested in abdominally sited cryptorchid testes, the Leydig cells of the interstitial tissue are less affected, and the concentration of testosterone in the peripheral plasma of animals with one or two testes retained outwith the scrotum is similar to that found in normal stallions (Ganjam and Kenney, 1975). However, lower than normal levels of hydroxyandrogens are present in the peripheral circulation of cryptorchid animals. Intratesticular levels of testosterone are higher in cryptorchid testes than in scrotal testes, and a higher percentage of testosterone in the hydroxyandrogens in the cryptorchid testicular tissue suggests that less metabolism of testosterone occurs in cryptorchid testicular tissue (Ganjam and Kenney, 1975). This is supported by the results of in-vitro work carried out with Leydig cells. Ryan and Raeside (1984) have shown that abdominal testicular tissue secretes more testosterone but less androstenedione, on a weight for weight basis, than scrotal testicular tissue.

The similarity between the levels of testosterone in the peripheral circulation of normal stallions and cryptorchids suggests that any changes in steroid metabolism are

compensated for by the "steroid hormone-pituitary hormone" feedback loop.

Retention of the testes outwith the scrotum, and the resultant inhibition of normal spermatogenesis, may cause less inhibin to be released into the peripheral circulation by the Sertoli cells. This would cause a reduction in the inhibin induced inhibition of pituitary secretion of FSH, and result in elevated peripheral FSH levels in cryptorchid stallions. Such an effect has been observed following the surgical induction of bilateral abdominal cryptorchidism in the rat (Caraty, Martinat and Blanc, 1981; Caraty, 1983), and the ram (Schanbacher and Ford, 1977). In man congenital cryptorchidism is accompanied by elevated FSH levels, in cases of both unilateral and bilateral cryptorchidism (Wu et al 1981; Purvis et al 1975).

Because FSH is probably secreted at a stable and constant rate, measurement of peripheral FSH levels in male equines may aid the diagnosis of cryptorchidism, with the presence of cryptorchid testicular tissue being accompanied by elevated plasma FSH concentrations.

1.5. OUTLINE OF STUDY

The initial investigations described in this thesis were concerned with the use of the measurement of testosterone levels in the blood, as a means of identifying whether testicular tissue was present in male equines. Detailed studies of the patterns of secretion of this hormone in entire and castrated horses were carried out, in an attempt to explain the variation in the circulating levels of testosterone reported in the literature.

Subsequently the use of FSH estimation as an alternative method of diagnosis was investigated and this involved the establishment of a suitable method for estimating FSH in equine blood.

Since many of the animals examined were subsequently castrated, their FSH levels before castration were compared with the spermatogenic activity present in histological sections of the testes removed.

This was followed by a study of the relationship between semen samples collected from two stallions, and the relevant FSH levels in their blood. This attempted to indicate whether changes in semen quality and quantity in individual stallions could be monitored by identifying changes in circulating FSH levels.

Finally the effect of vasectomy and castration on FSH levels in one stallion were investigated, to identify whether measurement of FSH concentration of blood samples could be used in the differential diagnosis of the aetiology of infertility in the stallion.

CHAPTER TWO

PLASMA TESTOSTERONE CONCENTRATION IN STALLIONS, GELDINGS AND CRYPTORCHID MALE EQUINES

CHAPTER TWO

PLASMA TESTOSTERONE CONCENTRATION IN STALLIONS, GELDINGS AND CRYPTORCHID MALE EQUINES

2.1. INTRODUCTION

The value of testosterone estimation as a diagnostic means of determining whether male equines had testicular tissue present or not was investigated.

Initially results of testosterone estimations of blood samples taken from normal stallions, geldings and cryptorchid stallions, were extracted from the hospital records. The patterns of secretion of testosterone in three stallions, and one gelding, were then investigated in detail.

Subsequently a series of animals admitted to the surgery department for castration were investigated. The value of Human Chorionic Gonadotrophin (HCG) administration in the diagnosis of whether testicular tissue was present in these cases, was assessed.

2.2. MATERIALS AND METHODS

2.2.1 Testosterone Radioimmunoassay

A liquid phase radioimmunoassay, method of Munro, Renton and Butcher (1979) was employed, with minor modifications.

2.2.1.1. Materials

1 Sodium Chloride; BDH.

2 Trimersal; Sigma.

3 Sodium dihydrogen orthophosphate; BDH.

4 Disodium hydrogen orthophosphate; Koch Light.

5 Gelatin; BDH.

6 Anti Testosterone 11 (Succinyl) BGG; Steranti Research Ltd, Batch 603/14.

7 1,2,6,7,H^3(n) Testosterone label; Amersham UK.

8 Norit A Charcoal; Sigma.

9 Dextran; Pharmacia.

10 Diethyl Ether; Pronalys, M&B.

11 Testosterone; Sigma.

12 Scintillator cocktail; Emulsifier-scintillator 299, Packard.

13 Polythene scintillation vials; Packard.

14 Flint Glass testtubes; Samco.

15 Scintillation counter; Packard A3000.

16 200µl and 1000µl variable pipettes; Eppendorf.

2.2.1.2. Reagents

a) Assay buffer;

This was a phosphate buffered saline solution, with gelatin added (known henceforth as PBS-gel).

Each 1 litre of solution contained;

Sodium Chloride	8g
Trimersal	0.1g
0.5M $NaH_2PO_4.2H_2O$	6.2ml
0.5M Na_2HPO_4	12.4ml

To each litre of this solution was added 1g of Gelatin.

This buffer was stored at $4^{o}C$ until use.

b) Working antiserum solution;

An Anti-Testosterone 11(Succinyl) BGG antiserum, raised in rabbits, was employed in this assay. A stock solution containing an antibody dilution of 1:100 was prepared, and this was stored at -20°C until use. The specificity of this antibody is shown in the following table;

ANTIGEN	RELATIVE SPECIFICITY
11beta-OH-testosterone	100%
11alpha hemisuccinate testosterone	38%
19 nortestosterone	24.7%
5alpha dihydrotestosterone	9.5%
5beta dihydrotestosterone	8.7%
11alpha hydroxytestosterone	6.2%
testosterone-3-0-CMO	4%
17beta hemisuccinyl testosterone	<0.2%
17beta oestradiol	0%

40ml of a 1:80,000 dilution of antibody in PBS-Gel was prepared immediately before use. This gave approximately 50% specific binding in the assay protocol described.

c) Working tracer solution;

1,2,6,7,H^3(n)testosterone with a specific activity of 90 Ci/mM was stored at -20°C in ethanol. This solution had an activity 250µCi per 50ml.

For each assay, 200µl of this 250µCi/50ml stock solution of labelled testosterone was evaporated to dryness, and the residue reconstituted to 10ml with PBS-Gel.

d) Dextran-coated-charcoal solution

Norit-A charcoal was washed with methanol and dried. 1g of this, and 0.1g of dextran, were added to 400ml of phosphate buffered saline, prepared as in (a), but with no gelatin added.

e) Standard solutions

Two solutions of testosterone in ethanol were stored at -20^{o}C. Solution 1 contained 2ng/ml of testosterone, and solution 2 contained 100pg/ml testosterone.

2.2.1.3. Assay protocol

a) Ether extraction step

The following tubes were prepared for each assay;

(i) 3 Total counts (TC) tubes; empty.

(ii) 2 Nonspecific binding (NS) tubes; empty.

(iii) 3 Total binding (B0) tubes; empty.

(iv) 7 Standard tubes in duplicate; these received appropriate volumes of standard solutions 1 and 2, to give tubes containing, 17.3, 34.7, 69.3, 173.4, 346.7, 693.4 and 1733.5, pmol of testosterone respectively. These were evaporated to dryness.

(v) Duplicate sample and control plasma tubes; 50µl of stallion plasma or control plasma, or 1000µl of gelding

plasma or control plasma.

All the tubes received 2.5ml of diethyl ether from an automatic dispenser, and were vortexed for 10 minutes. Following centrifugation, at 1750g for 10 minutes (MSE Mistral 6L), the tubes were frozen at -10^{o}C and the nonaqueous phases decanted into clean tubes. The ether was evaporated to leave tubes containing dried ether extracts.

b) Assay step

All tubes received 100µl of the working tracer solution. Standard, sample, control and BO tubes also received 500µl of the working antibody solution via an automatic dilutor. NS and TC tubes received 500µl of PBS-Gel in place of antibody. All tubes were vortexed and incubated overnight at 4^{o}C.

c) Separation step

All tubes, except the TC tubes, received 500µl of the dextran-coated-charcoal solution. The TC tubes received 500µl of PBS-Gel in place of charcoal. The tubes were vortexed for two minutes, incubated at 4^{o}C for a further 10 minutes, and centrifuged at 1750g for 30 minutes. 700µl of the resultant supernatent was pipetted into scintillation vials containing 10ml of scintillator. After thorough mixing, the scintillation within each vial was measured in the scintillation counter.

2.2.1.4. Estimation of extraction efficiency

To determine the efficiency of the ether

extraction step, three tubes containing either 50µl, or 1000µl Of PBS-gel (depending upon the volume of sample used), and 20µl of working tracer solution were extracted alongside the assay tubes. These tubes received PBS-Gel in place of tracer, antibody and charcoal in subsequent assay steps.

The percentage efficiency of the extraction step was then derived from the equation;

$$E = \frac{5 \times EXT}{TC}$$

where; E = Extraction efficiency

EXT = mean c.p.m. in EXT tubes

TC = mean c.p.m. in TC tubes

2.2.1.5. Interassay quality control

Three control samples were included in each assay. These were samples taken from large pools of stallion serum, gelding serum, and a 50:50 mixture of these pools.

2.2.1.6. Standard curve preparation and analysis

An Apple II Europlus microcomputer analysed the data from the scintillation counter (Rodbard and Lewald, 1970). This used a logit-log transformation of the standard results to determine the testosterone content of the samples. Results were corrected by multiplication by the extraction efficiency factor for that particular assay, and corrected for sample volume.

2.2.2. Results retrieved from hospital records

Case records of male equines admitted to the hospital over the last 12 years were retrieved using the Termatrex data retrieval system. The concentration of testosterone measured in plasma samples taken from these animals was recorded. Only the results from animals where the sexual status was either confirmed at surgery or established from a reliable previous history were included in the study. All animals were over two years of age.

Testosterone concentration was measured in all the samples using the radioimmunoassay already described, except that samples were assayed without duplicate, using a fixed sample volume of 200µl for all cases.
Histograms were constructed to illustrate the distribution of the testosterone concentrations measured in samples taken from the different types of animals. In addition, the mean testosterone concentration, and range of concentrations in each type of animal, were compared.

2.2.3. Animals studied in detail

Three pony stallions and one pony gelding were studied in detail.

2.2.3.1. Histories

Max: a 6 year old Welsh pony stallion standing 12.2h. He is a proven sire used regularly for teasing mares, semen collection, and natural service, and was maintained in good working condition.

Steptoe: a 22 year old, 12h Welsh cross stallion. He had not been used for stud work before he was purchased for this study. Although in a thin condition, he was keen to tease and serve mares, and was used for semen collection.
Blackie: a 14h, 3 year old Fell pony. He had not been used for stud work before purchase, but was keen to work, and was in good working condition.
Bonzo: a 6 year old 14h Welsh cross gelding pony. He had a history of teasing mares and being difficult to school in the presence of other horses.

2.2.3.2. Maintenance

The stallions were housed in looseboxes throughout the period of study. They received a ration of horse and pony cubes (Spillers), adlib hay and adlib water. All three were used for teasing mares resident in the equine unit, semen collection using an artificial vagina and, on occasion, natural service.

The gelding was maintained at grass, with hay supplementation during the winter months. For the duration of each bleed Bonzo was housed and received the same rations as the stallions. During blood sample collection the animals were exposed to continuous artificial lighting. During the periods between serial sample collection natural lighting was not supplemented.

2.2.3.3. Cannulation technique

A 20cm by 10cm rectangle of skin was clipped over the left jugular furrow. This area was scrubbed with an

aqueous solution of Pevidine, and rinsed with absolute alcohol. 5ml of 2% Lignocaine was infiltrated above the jugular vein using a 5ml syringe, and a 22g 3/4" needle, to provide a 3cm circular field of analgesia. After the application of a tourniquet, the jugular vein was penetrated, through this field, using a 60mm long 2.1mm diameter trochar and cannula (Vygon). This was advanced ventrally within the lumen of the vein, and the trochar withdrawn. The tourniquet was released, and the cannula flushed with 5ml of a 0.5% solution of heparin, in normal saline. Finally the cannula was sutured in place, using two 1.G monofilament nylon sutures.

Samples were withdrawn from the cannulae using heparinised syringes (Monovet). These samples were centrifuged at 1000g, for 10 minutes, in a Beckman TJ-6 centrifuge, and the plasma supernatent decanted and stored at $-20^{o}C$ until assay.

When samples were taken every hour the cannulae were flushed with 5ml of 0.5% heparin, after each sample was withdrawn. However the cannulae were not flushed when the samples were taken at 10 minute intervals.

Blocked cannulae were recannulated using sterile nylon stylets (Vygon).

2.2.3.4. Infrequent blood sample collection

Blood samples were collected by jugular venepuncture, using 19g 1.5" needles. Blood was withdrawn into heparinised syringes (Monovet), and was then

centrifuged at 1000g in a Beckman TJ-6 centrifuge for ten minutes. The plasma was then harvested and stored at -20^{o}C until testosterone assay.

2.2.3.5. Blood samples collected

Samples collected at 1 hour intervals:

Max; November and October

Steptoe; March

Blackie; March

Bonzo; October

Samples collected at ten minute intervals:

Max; Marchand December

Bonzo; December

2.2.4. Surgical cases

6 animals, admitted for castration during 1982-1983, were investigated.

In two cases blood samples were collected immediately before 6000IU of HCG (Chorulon; Intervet) was injected slowly into the left jugular vein. Jugular venepuncture and heparinised blood sample collection was then repeated 30, 60 and 240 minutes after HCG administration.

In the remaining four cases blood samples were taken at intervals before castration.

2.2.5. Statistical analysis

Statistical analysis was carried out using the

MINITAB statistics package (Penn. State. Univ., release 81.1). Analysis of variance was carried out using the analysis of variance one way command.

2.3. RESULTS

2.3.1 Testosterone concentration in blood samples taken from hospital cases

Figures 2.1., 2.2., and 2.3., show the frequency with which blood samples taken from normal stallions, cryptorchid stallions and geldings, contained testosterone concentrations within 0.25 nmol/l ranges. There was no significant difference between the mean concentration in samples taken from normal stallions, and samples from cryptorchid stallions ($P>0.05$). The frequency with which samples contained concentrations in these ranges had a similar, positively skewed, normal distribution.

The samples taken from the geldings had a significantly lower mean concentration ($P<0.05$). Some gelding samples contained testosterone concentrations within the ranges of concentrations found in normal stallion and cryptorchid stallion samples.

Table 2.1. demonstrates the overlap in the ranges of concentrations of testosterone, measured in samples taken from normal stallions, cryptorchid stallions, geldings.

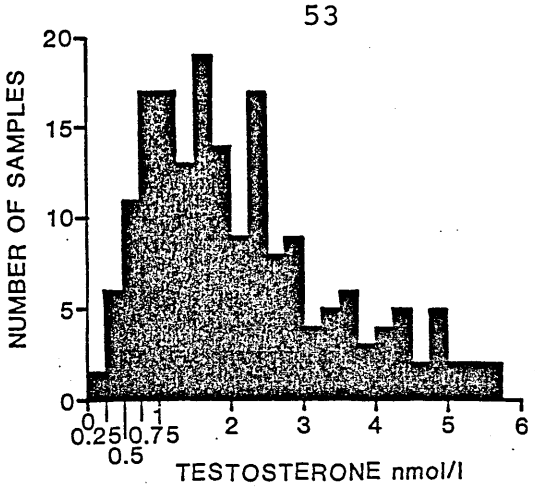


FIGURE 2.1.
TESTOSTERONE CONCENTRATION IN 194 BLOOD SAMPLES TAKEN FROM 40 NORMAL STALLIONS

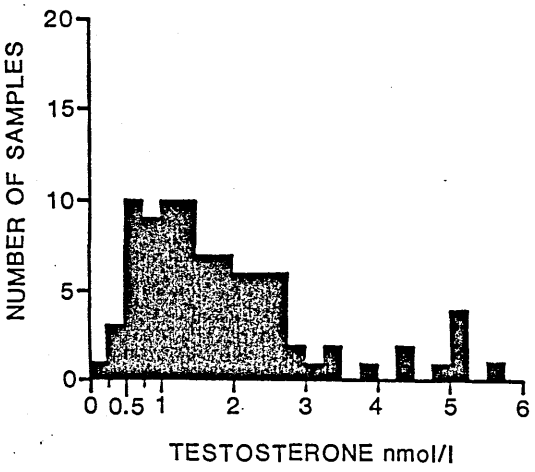


FIGURE 2.2.
TESTOSTERONE CONCENTRATION IN 94 BLOOD SAMPLES TAKEN FROM 32 CRYPTORCHID STALLIONS

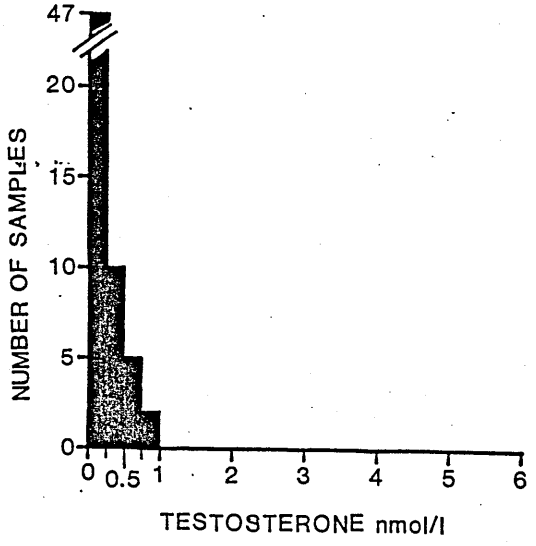


FIGURE 2.3.
TESTOSTERONE CONCENTRATION IN 64 BLOOD SAMPLES TAKEN FROM 19 GELDINGS

TABLE 2.1. MEAN AND RANGE OF TESTOSTERONE CONCENTRATIONS IN SAMPLES FROM NORMAL STALLIONS, CRYPTORCHID STALLIONS, AND GELDINGS

Samples assayed singly, using a sample volume of 200µl for all samples.

ANIMAL TYPE	TESTOSTERONE CONCENTRATION, nmol/l	
	MEAN	RANGE
NORMAL STALLION	2.61	0.31-16.1
CRYPTORCHID STALLION	2.16	0.32-16.1
GELDING	0.22	0.01-0.82

2.3.2. Testosterone concentration in blood samples taken at one hour intervals

Figure 2.4. shows the concentration of testosterone in samples taken at one hour intervals from the stallions, Max (November), and Steptoe (March), and the gelding Bonzo (October). The two stallions show similar patterns of secretion during different seasons of the year. Both demonstrate three peaks of secretion at approximately 9pm, 4am, and 1pm, and three troughs at approximately 4pm, 1am, and 10am. There was no significant difference between the concentration of testosterone in samples taken during the night, and samples taken during the day, in either stallion ($P>0.05$).

A larger plasma sample volume was employed in the assay of samples taken from the gelding Bonzo. All these samples contained significantly less testosterone than was present in the stallion samples ($P<0.05$).

FIGURE 2.4.

MAX (NOVEMBER), STEPTOE (MARCH) AND BONZO (OCTOBER)

TESTOSTERONE CONCENTRATION IN BLOOD SAMPLES COLLECTED AT ONE HOUR INTERVALS

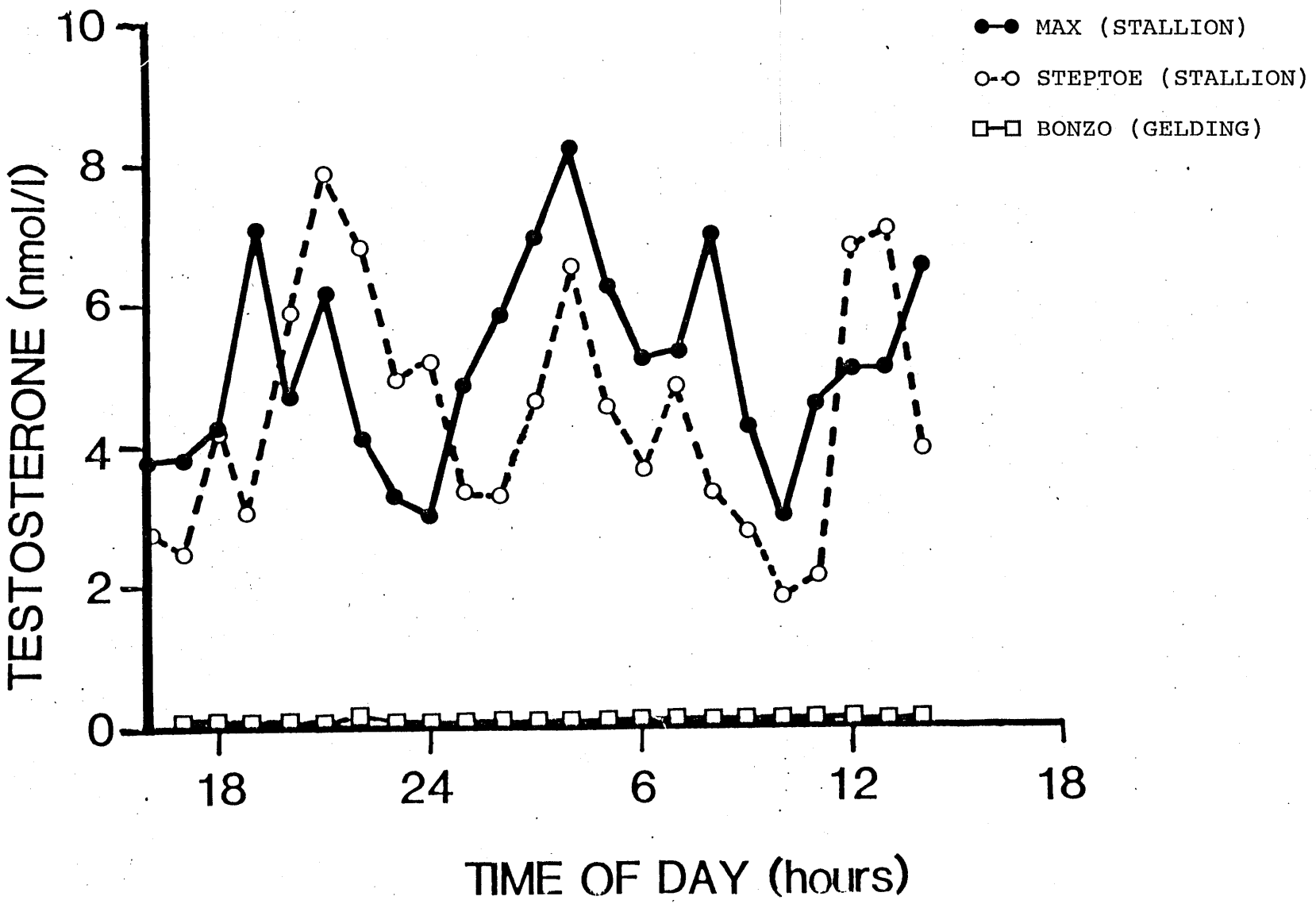


Figure 2.5. illustrates the results of testosterone assay of blood samples taken at one hour intervals from the stallions Max (October) and Blackie (March).

The stallions showed similar patterns of secretion during different seasons of the year, with trough levels at mid-day and a single peak during the night. In these cases there was a significantly higher concentration of testosterone in samples taken during the night ($P<0.05$). There was no significant difference in the mean concentration of testosterone in samples taken from the three stallions in figures 2.4. and 2.5., during different seasons of the year ($P>0.05$).

Different patterns of secretion were observed in the stallions during the same season of the year.

Figure 2.6. illustrates the concentration of testosterone measured in samples taken from the gelding Bonzo (October), using a plasma volume of 1000µl, to increase assay sensitivity. The pattern of secretion is different to that observed in the stallion samples, with very little variation around a much lower mean concentration. There was no significant difference in the concentration of testosterone in samples taken during the night and samples taken during the day ($P>0.05$).

FIGURE 2.5.

MAX (OCTOBER), BLACKIE (MARCH)

TESTOSTERONE CONCENTRATION IN BLOOD SAMPLES COLLECTED AT ONE HOUR INTERVALS

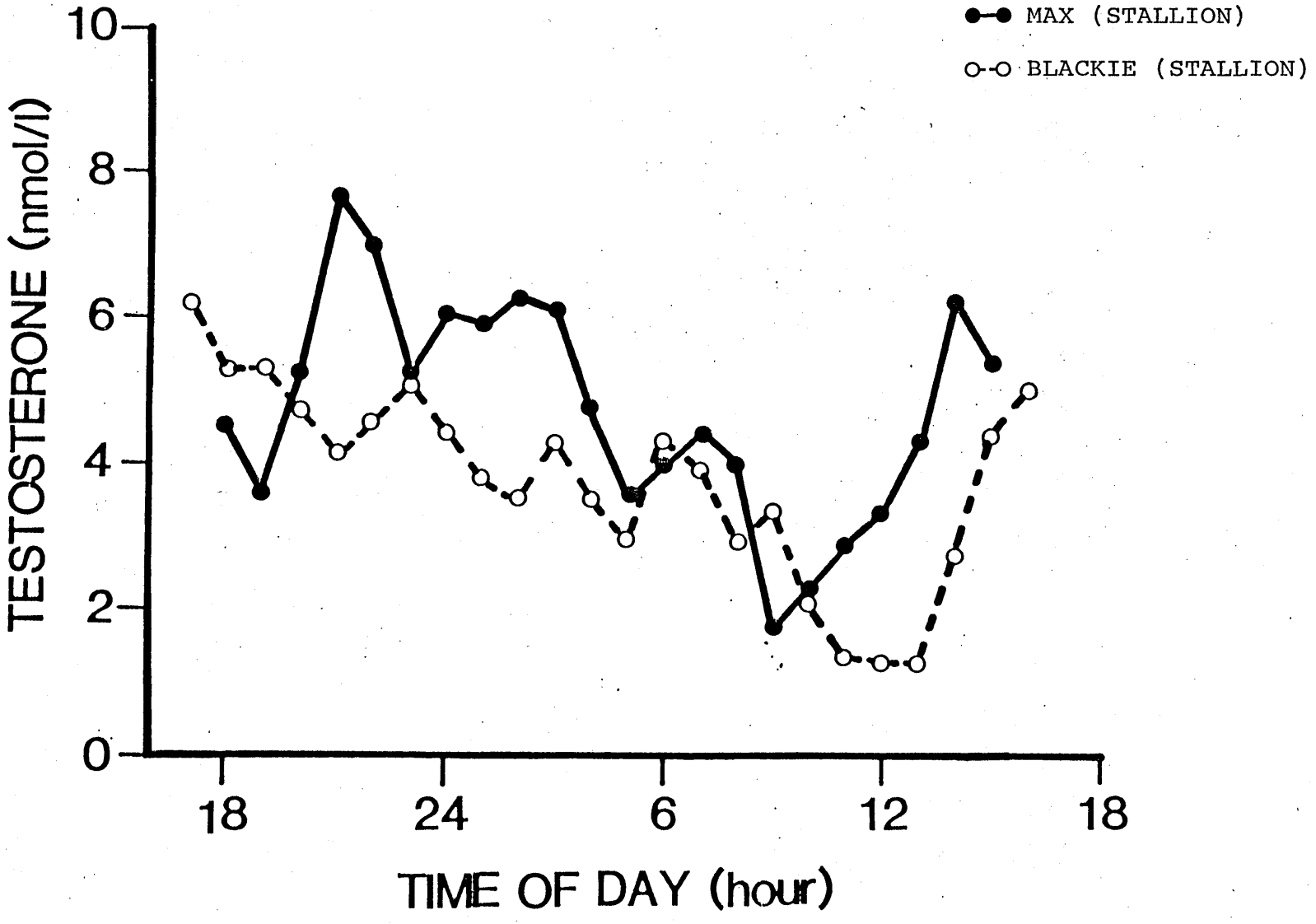
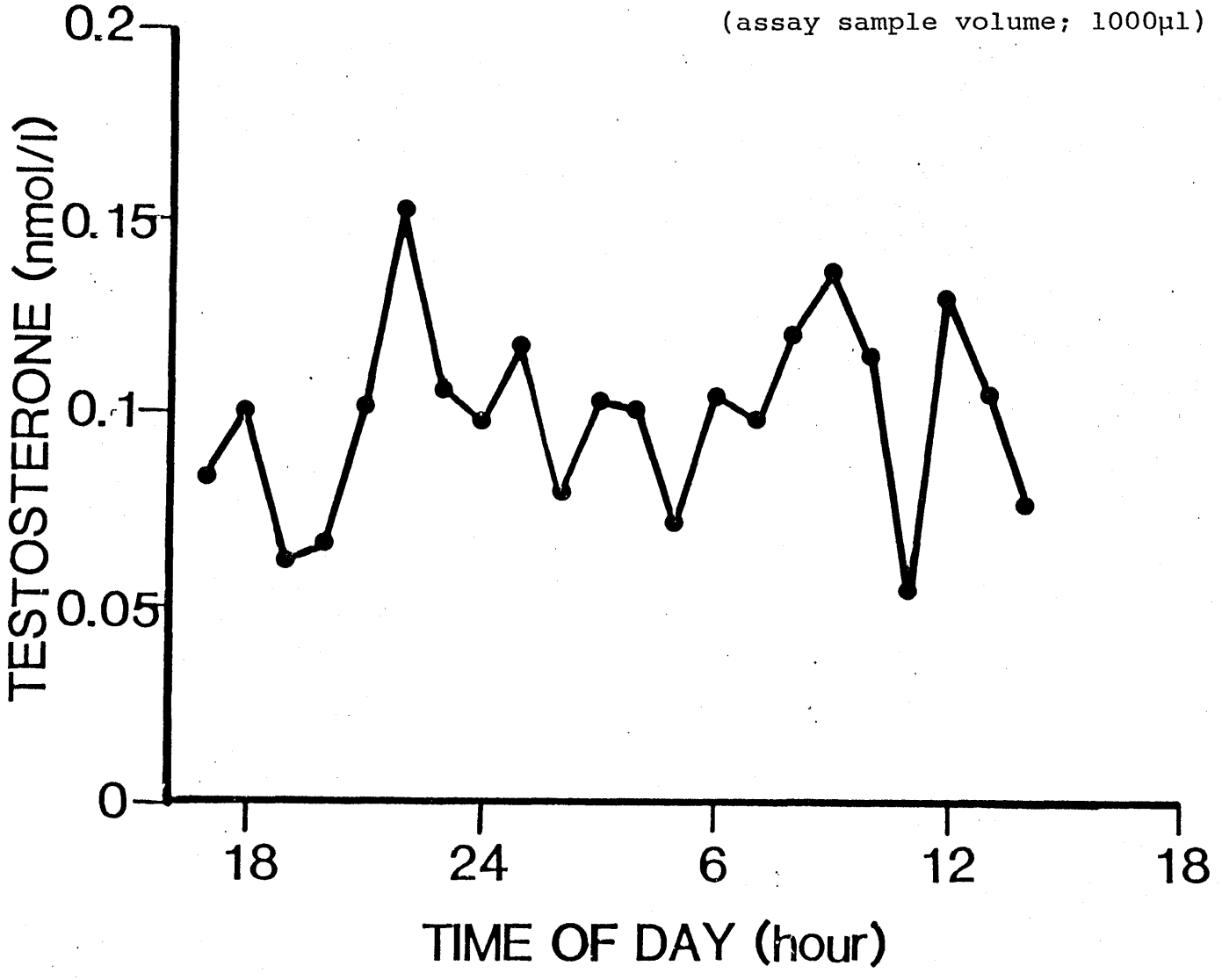


FIGURE 2.6.

BONZO (OCTOBER)

TESTOSTERONE CONCENTRATION IN BLOOD SAMPLES COLLECTED AT ONE HOUR INTERVALS

(assay sample volume; 1000µl)



2.3.3. Testosterone concentration in blood samples taken at ten minute intervals

Figure 2.7. illustrates the concentration of tesosterone estimated in samples taken at ten minute intervals from the stallion Max (December and March), and the gelding Bonzo.

There were two different trends to the concentrations of testosterone measured in the same stallion at different times of the year. In March testosterone levels fell gradually over the entire period of study, but in December levels fell gradually to trough levels at 4-30am, and then rose again. There was a significant difference between the concentrations of testosterone estimated in this stallion during these two studies ($P<0.05$).

The samples taken from the gelding, and assayed using a sample volume of 1000µl, contained significantly lower testosterone concentrations than were found in the stallion samples ($P<0.05$).

FIGURE 2.7.

MAX (DECEMBER and MARCHAND BONZO (DECEMBER)

TESTOSTERONE CONCENTRATION IN BLOOD SAMPLES TAKEN AT TEN MINUTE INTERVALS

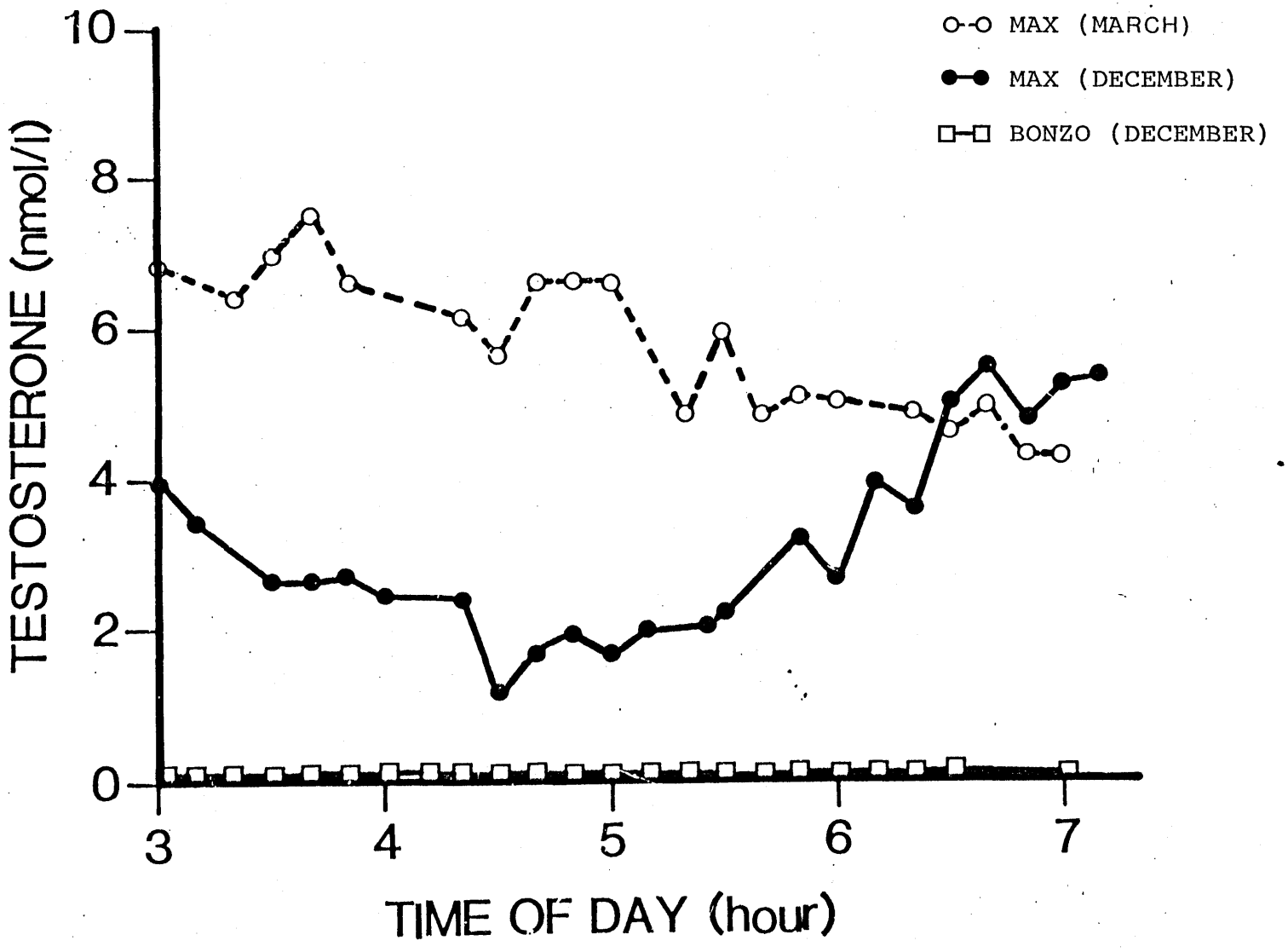


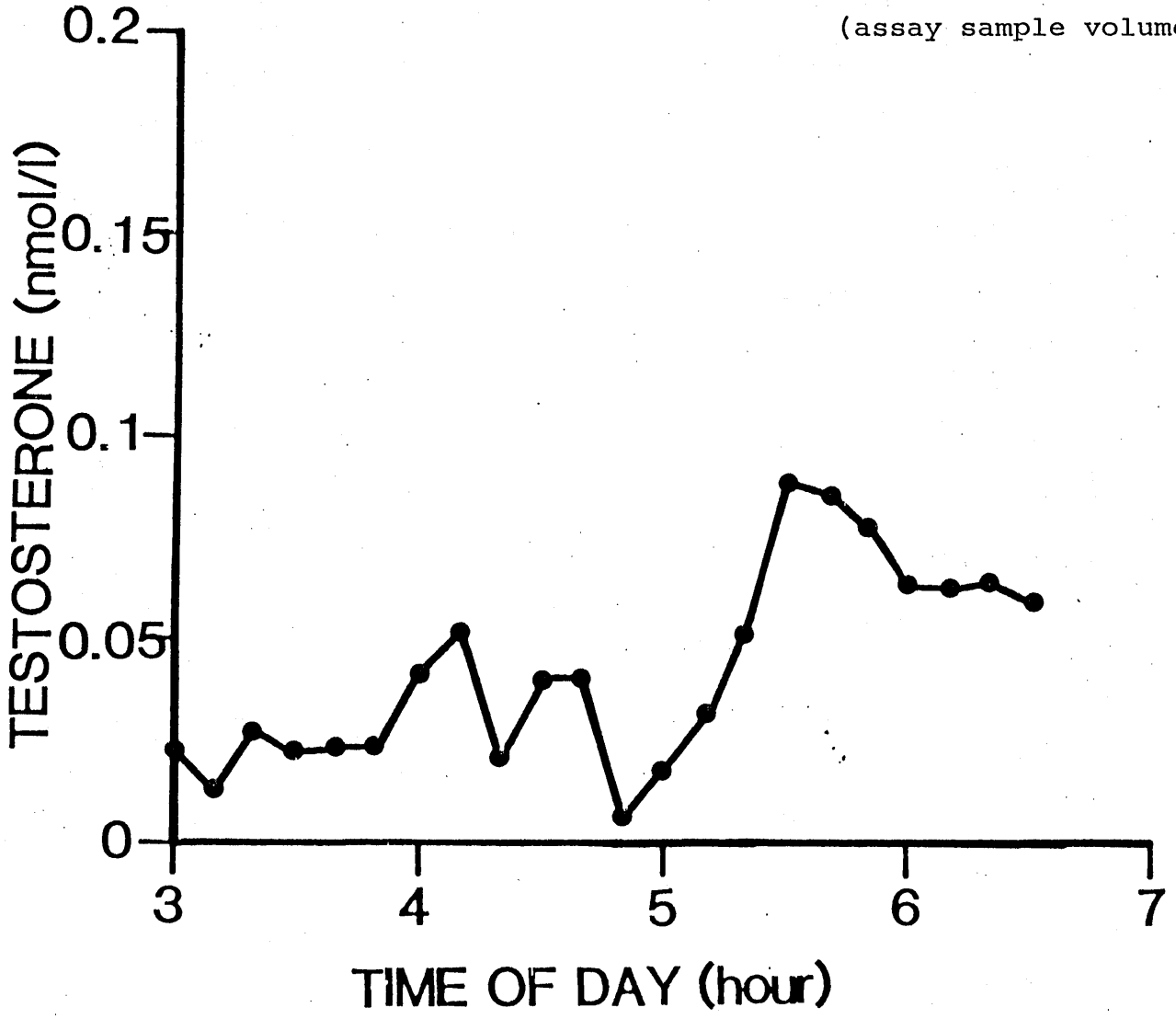
Figure 2.8. illustrates the results of the assay of samples taken at ten minute intervals from the gelding Bonzo (December), using a larger plasma volume to increase assay sensitivity. There was a much lower mean concentration of testosterone, compared with the concentrations found in the stallion samples, with little deviation from the mean concentration. The concentrations in these winter samples were significantly lower than those estimated in the hourly autumn samples in the same animal ($p<0.05$).

FIGURE 2.8.

BONZO (DECEMBER)

CONCENTRATION OF TESTOSTERONE IN BLOOD SAMPLES COLLECTED AT TEN MINUTE INTERVALS

(assay sample volume; 1000µl)



2.2.4. The effect of HCG administration upon peripheral plasma testosterone concentrations in one cryptorchid and one normal stallion

Table 2.2 shows that the normal stallion and the cryptorchid stallion responded to HCG administration by secreting more testosterone. The response of the normal stallion was greater than that of the cryptorchid stallion.

The pre HCG sample taken from the cryptorchid stallion contained very low levels of testosterone.

TABLE 2.2

CIRCULATING PLASMA TESTOSTERONE CONCENTRATIONS IN ONE NORMAL STALLION AND ONE CRYPTORCHID STALLION, DURING OCTOBER, FOLLOWING THE ADMINISTRATION OF HCG.

ANIMAL IDENTITY	TYPE OF STALLION	TIME	TESTOSTERONE CONCENTRATION nMol/l.
89169	NORMAL	11-40*	1.88
		12-10	9.26
		12-40	16.48
		15-40	8.49
82646	CRYPTORCHID	11-45*	0.31
		12-15	1.37
		12-45	2.89
		15-45	2.87

* DENOTES TIME OF HCG ADMINISTRATION.

2.3.5. Peripheral plasma testosterone concentrations in a series of cases admitted to the surgery department for castration

Table 2.3. presents the concentration of testosterone, estimated in plasma samples taken at intervals from three cryptorchid stallions and one gelding. The four samples taken from the gelding contained significantly less testosterone than was present in samples taken from the cryptorchid stallions ($P<0.05$). These gelding samples contained more testosterone than was present in the pre HCG sample taken from case 82646 in Table 2.2..

The concentration of testosterone in the samples taken from the hemicastrate cryptorchid, case 92837, was similar to, or greater than, the concentrations measured in stallions with two testes present.

Testosterone concentrations in stallion Rig6, measured during May, showed considerable variation during the hour of study. Concentrations in the other stallions were more stable. Testosterone concentrations were highest in the stallion whose samples were collected in May.

TABLE 2.3.
TESTOSTERONE CONCENTRATIONS IN PLASMA SAMPLES TAKEN FROM THREE CRYPTORCHID STALLIONS AND ONE GELDING AT VARIOUS INTERVALS DURING THE DAY WITHOUT HCG ADMINISTRATION

ANIMAL IDENTITY.	TYPE OF STALLION.	MONTH	TIME.	TESTOSTERONE CONCENTRATION nMol/l.
92874	CRYPTORCHID	OCTOBER	13-50	1.48
			14-10	1.32
			10-00	1.24
RIG6	CRYPTORCHID	MAY	10-10	3.30
			10-35	2.67
			10-45	3.03
			11-05	5.37
92837	HEMICASTRATE CRYPTORCHID	OCTOBER	15-15	1.94
			15-35	2.04
			16-00	2.16
			16-15	2.10
RIG8	GELDING	SEPTEMBER	10-55	0.32
			11-15	0.41
			11-30	0.46
			11-55	0.40

2.4. DISCUSSION

The circulating plasma testosterone levels estimated in samples taken routinely from male equines over a number of years, agreed with those of other workers (Harris, Irvine and Evans, 1983; Turner and Kirkpatrick, 1982; Burns et al 1982; Irvine, Alexander and Hughes, 1984; Crowe et al 1977; Cox et al 1973; Ganjam, 1979; Squires et al 1978). However, the lowest level recorded for stallions in this group of blood samples (figure 2.1.), was higher than the lowest concentration of testosterone recorded in stallions by Cox et al (1973). It is recognised that the results of radioimmunoassay techniques carried out in different laboratories can vary, due to variation in type of antibody, and the sensitivity of each assay method. This could explain this difference in concentration range.

The large standard deviation about the mean concentration of these groups of samples is also in agreement with others (Ganjam, 1979; Cox et al 1973; Turner and Kirkpatrick, 1982; Harris, Irvine and Evans, 1983). There are several explanations for this wide range of testosterone concentrations. Individual animals may persistently produce samples that have consistently high or low levels of this hormone, resulting in between-stallion variation. On the other hand, a variation may occur within stallions, where each stallion may produce different samples containing one of range of testosterone concentrations. To ascertain the importance of between

stallion variation, samples would have to be taken from a large number of stallions simultaneously, and their testosterone concentrations compared. This was not undertaken in these studies. However evidence of within stallion variation was provided by the data from the samples taken at regular intervals from the three stallions, Max, Steptoe and Blackie.

Sharma (1976), Cox et al (1973), and Carson and Thompson (1979) have reported a smooth biphasic diurnal rhythm to the circulating plasma testosterone levels in stallions. Although this was not observed in the hourly samples taken from these three stallions, there was a significant difference in the circulating plasma testosterone concentrations between those samples taken during the night and those taken during the day in figure 2.5.. In all four bleeds carried out the circulating plasma testosterone concentration was higher in the late afternoon (figures 2.4. and 2.5.). In Max, in October, and Blackie, in March, a single peak in circulating plasma testosterone concentrations occurred during the night (figure 2.5.). Basal levels of this hormone were found in both stallions during the day. However on other occasions two peaks were observed during the night (Max and Steptoe, figure 2.4.). Therefore, although a diurnal variation was apparent in two cases, no definite pattern was identified in the three stallions investigated. These findings are in agreement with Ganjam (1979).

If such changes in the circulating plasma testosterone concentration do occur in most stallions, then samples taken at different times of the day could contain significantly different levels, and this would explain the wide range found in the accrued results.

The nighttime rises in circulating plasma testosterone concentration in the stallions investigated could be associated with changes in ambient lighting. However, as all three stallions were under constant lighting throughout the period of sampling, this suggests that changes in the level of lighting were not the reason for the diurnal changes in circulating plasma testosterone concentration in these stallions.

However all three stallions were considerably more stressed during the night than during the day. This was manifested by restlessness and patchy sweating. Cox and Williams (1975a) have reported that the administration of adrenocorticotrophic hormone (ACTH) to stallions, results in increased circulating plasma testosterone concentrations. They have suggested that this was the result of changes in testicular blood flow. Stress may thus have accounted for some of the variation in the circulating plasma testosterone concentrations in the three stallions investigated. It is unlikely however, that the taking of a single blood sample from a stallion would be sufficiently stressful to account for the vast range of concentrations in the samples submitted to the routine biochemistry

department over the last 12 years.

In more recent publications, Ganjam (1979), and Cox and Williams (1975a), have suggested that the circulating plasma testosterone concentration in the stallion fluctuates randomly throughout the day and night and, on average, shows no consistent night time increase. The results from the stallion Max, in November, and Steptoe, in March, tend to agree with this statement, as in both, multiple peaks were seen during both the night and, in Max's case, during the day as well. If random episodes of increased circulating plasma testosterone concentration can occur, for reasons as yet unknown, then these could mask or confuse any underlying smooth diurnal patterns that may exist. Such random incidents may even be responsible for any of the night time peaks observed. Therefore it is unlikely to be possible to describe a definite pattern of testosterone secretion in this domestic species.

It is widely believed that stallions show a seasonal variation in semen quality (Van der Holst, 1975; Pickett, Faulkner and Voss, 1975c) and in circulating gonadal steroid hormone levels (Johnson and Thompson, 1983; Burns et al 1984b; Ganjam and Kenney, 1975; Harris, Irvine and Evans, 1983). The winter is referred to in the literature, as a period of sexual rest for the male equine, accompanied by low levels of gonadal steroid hormones in the peripheral circulation. During the spring semen production is said to increase, and the circulating plasma

testosterone concentration is said to rise, reaching a peak in May. Season may demonstrate its effect by changes in; a) the level of testosterone secretion, or b) the pattern of secretion, or by changes in both a) and b). The mean concentration of testosterone in the results recorded from samples taken at hourly and ten minute intervals at different times of the year, indicate a statistically significant difference in the concentration of testosterone during different months. These results agree with the hypothesis that there is a seasonal variation in testosterone levels, since the lowest levels were found in the winter months, and the highest levels in May. Therefore seasonal changes could explain some of the variation seen in the routine laboratory results.

It has been reported that stallions exposed to an artificial daylength during the winter have temporarily elevated levels of circulating plasma testosterone, suggesting that photoperiod may play a part in the seasonal regulation of testosterone metabolism in the stallion (Burns et al 1982; Burns et al 1984b). The pituitary gland may be involved in the seasonal variation of circulating plasma testosterone concentration, as LH levels reach a nadir during the spring (Irvine and Alexander, 1982; Burns et al 1984b; Harris, Irvine and Evans, 1983; Johnson and Thompson, 1983). The effect of photoperiod was not investigated in this study. Factors such as available nutrition and ambient temperature, may also play a part in

producing such changes.

Although there was a difference in the amount of testosterone secreted during the different seasons, the pattern of testosterone secretion did not vary with the seasons, in the results recorded. Indeed similar patterns of secretion were observed in different months, and different patterns of secretion were observed in the same month (figures 2.4. and 2.5.). However, the number of stallions studied was too few to draw any firm conclusions with regard to seasonal differences in the wider equine population.

Therefore, it would appear from the studies undertaken that the circulating plasma testosterone concentration may vary in samples of a stallion's blood with the time of day, the time of year, and because of a variety of other extraneous factors.

The mean level and range of testosterone concentrations, in samples taken from geldings over the last 12 years (table 2.1.), showed a range similar to that already reported in the literature (Cox, 1975b; Cox and Williams, 1975a; Ganjam and Kenney, 1975; Arighi et al 1984). The highest levels found in these results were higher than those reported by other workers, probably for the same reasons already referred to, in the discussion of the normal and cryptorchid stallion results.

In these samples the highest levels found in samples from geldings, were higher than the lowest levels

found in samples from stallions. This seems strange in the gelding, which possesses no testicular interstitial tissue. In the gelding some testosterone synthesis is thought to take place in adrenal tissue, located both within the adrenal glands and at ectopic sites (Crowe et al 1977; Cox et al 1973; Bishop, David and Messervy, 1964).

In the gelding Bonzo, the detailed study of circulating plasma testosterone concentrations showed no smooth biphasic diurnal pattern (figure 2.6.), and there was no significant difference in the circulating plasma testosterone concentration during the day and night. Again, as with the stallions, the gelding Bonzo was under artificial lighting during the sampling period, and this may have affected the circulating plasma testosterone concentration. The results of the samples taken at ten minute intervals from this same animal (figure 2.8.) showed random episodic increases in the circulating plasma testosterone concentration, similar to those seen in the hourly samples. These episodes appeared to occur at random throughout the periods of study, and were of a somewhat similar pattern to those found in the three stallions studied here, and also in stallions studied by Ganjam (1979).

The mean concentration of testosterone in samples taken from Bonzo in December was significantly lower than that found in samples taken in October (figures 2.6. and 2.8.). This may suggest that, as in the entire animal,

season may play a part in the control of testosterone secretion in the gelding.

There was no significant difference in the range or mean concentration of testosterone in samples taken from stallions, and samples taken from cryptorchid animals, in the samples submitted to the laboratory for routine analysis. This agrees with the findings of Ganjam and Kenney (1975), and Cox and Williams (1975a), and suggests that testis location does not affect the level of Leydig cell testosterone secretion. This finding has also been demonstrated in vitro by Ryan and Raeside (1984).

The group of cryptorchid animals, providing the laboratory data, included animals with either one or two testes present. Cox et al (1973), has demonstrated that hemicastrated animals have testosterone levels similar to those in animals with two testes. The similarity between the concentration of testosterone in samples taken from cryptorchids and samples taken from stallions in this study supports these findings.

The two cases that received HCG both responded by releasing more testosterone into the peripheral circulation (table 2.2.). This response has been widely reported (Arighi et al 1984; Cox et al 1973; Cox and Williams, 1975a; Cox, 1975b; Crowe et al 1977), and is caused by HCG binding to LH receptors on the cell membrane of Leydig cells (Evans, Rosen and Miukis, 1982). The response of the normal stallion in this study was greater than that of the

cryptorchid equine (table 2.2.). This agrees with Cox and Williams (1975a) who suggest that testes location is the major factor determining testes response to HCG.

In those cases where HCG was not administered, the testosterone level in the hemicastrate animal was similar to that seen in the two animals with two testes present (table 2.3). This suggests that this animal had compensated for the loss of one testis, by increasing testosterone secretion from the remaining testis.

The concentrations of testosterone in samples taken from the cryptorchids in October, were slightly lower than those in samples taken in May. This suggests that, as in the entire animals, there is a seasonal variation in plasma testosterone levels in the cryptorchid horse. A similar pattern has been reported by Cox (1975b), who notes that testosterone levels are especially low in hemicastrated animals during the winter months.

One of the reasons for this study was to investigate whether in male equines the estimation of testosterone concentration in plasma samples could be used to demonstrate whether or not testicular tissue is present. This would enable the identification of geldings whose behaviour did not respond to castration, because of some psychopathology, and who continued to exhibit sexual and aggressive behaviour in the presence of other equines (and who will be referred to henceforth as psychopathic geldings). Such a test would prevent misdiagnosis by

veterinary surgeons, who suspect that any animal that has no testes present in the scrotum, but that exhibits sexual and agressive behaviour, has one or two testes retained within the abdomen or inguinal canal. As misdiagnosis may lead to fruitless and potentially harmful surgery, the development of such a blood test would be of great benefit. The development of such an approach has already received much attention. Ganjam and Kenney (1975) suggested that measurement of the concentration of testosterone in a single blood sample can serve to distinguish the badly behaved equine that is a psychopathic gelding, from the animal whose behaviour is due to retained testicular tissue.

Cox et al (1973) has reported that geldings who continued to show stallion like behaviour after castration, have circulating plasma testosterone concentrations identical to those of normal geldings. Using the assay employed in these studies, the range of circulating plasma testosterone concentrations in normal geldings was determined, and this therefore should also represent the range of circulating plasma testosterone concentrations in psychopathic geldings.

From the results described in this thesis it is clear that if the concentration of testosterone in a single blood sample taken from a badly behaved male equine is greater than the highest concentration recorded in the laboratory data for normal geldings, then that badly

behaved animal must possess a testis. On the other hand if the concentration of testosterone in a single sample from such a case is lower than the lowest concentration recorded in the laboratory for an animal that possesses testicular tissue, then that case must be a psychopathic gelding. Therefore in some cases this single sample test, originally described by Ganjam and Kenney (1975), would successfully differentiate the two types of animal. However, there was an overlap between the higher testosterone concentrations in gelding blood samples and the lower testosterone concentrations in blood samples from normal and cryptorchid stallions. Therefore a definite diagnosis could not be reached if a problem animal produced a single sample with a testosterone concentration within this overlap range.

Therefore this single sample test may fail to identify whether a badly behaved male equine is a gelding or not. In four of the cases that were investigated surgically, the first samples taken from each animal had plasma testosterone concentrations higher than the highest concentration of testosterone measured in samples taken from geldings, indicating that these cases were not psychopathic geldings, but that they possessed testicular tissue. This proved correct at surgery.

However in two cases (82646 and rig 8) no diagnosis would have been reached using this single sample test, as the concentration of testosterone in the initial blood samples taken fell within the overlap range of

testosterone concentrations. It was subsequently proved that one of these animals did possess testicular tissue, and the other did not. These results illustrate the major flaw in this test procedure.

Most authors agree that, while circulating plasma testosterone concentrations increase in stallions following HCG administration, they do not do so in geldings (Cox et al 1973; Cox and Williams, 1975a; Cox et al 1975b; Crowe et al 1977). In 1973, Cox et al described a test based on this principle for the identification of psychopathic geldings that continue to show agressive male behaviour in the absence of testicular tissue. Using this approach, animals that failed to respond to HCG administration were diagnosed as psychopathic geldings.

In the studies described here, two cases did respond to HCG administration and demonstrated a subsequent rise in circulating plasma testosterone concentration. Both cases were therefore correctly diagnosed as possessing testicular tissue.

However, misdiagnoses may occur using this test, as various other factors can affect the response of male animals to HCG administration. Crowe et al (1977) and Cox and Williams (1975a) have both suggested that cryptorchid testes show a reduced response to HCG stimulation, compared with normal scrotal testes. The degree of stimulation achieved following the administration of HCG may also

depend upon an animal's initial resting circulating plasma testosterone concentration. Cox et al (1973) have suggested that entire animals respond with a larger rise in concentration of testosterone if the pre-HCG sample concentration of testosterone is in the lower end of the normal range. This suggests that if a high circulating plasma testosterone concentration is present prior to HCG administration, then there will be little response to HCG. Both these situations could result in a poor response to HCG, and a misdiagnosis.

In 1975 Cox and Williams described a test which ignored the changes in concentration, and depended upon the absolute levels of testosterone, in samples taken before and after HCG administration, to reach a diagnosis. In this later "Cox" test blood samples are taken before and after HCG administration, and the concentration of testosterone measured in both samples are evaluated in the same way as in the single sample results obtained for the "Ganjam" type test. The HCG stimulates those badly behaved problem animals that possess testicular tissue to produce post-HCG blood samples with concentrations of testosterone higher than that recorded for any gelding. Surgical intervention, and removal of the testicular tissue may then be carried out on these animals. On the other hand, if stimulation does not occur, and either sample contains a concentration of testosterone lower than the lowest concentration recorded in the laboratory for animals that did possess

testicular tissue, then such a problem animal would be diagnosed as a psychopathic gelding, and no surgery would be carried out. However, a minority of animals may still fail to be diagnosed, if both samples contain a concentration of testosterone that falls into the overlap range of concentrations. By administering HCG the number of cases falling into this group are fewer than when the single sample test without HCG stimulation was adopted. Two of the cases investigated surgically in this study were diagnosed correctly as possessing testicular tissue, using this test. However, if HCG stimulation had not been carried out, and the pre HCG samples were used for a single sample test, then a diagnosis would only have been possible in one case. This offers proof of the value of HCG administration.

Therefore the studies undertaken for this thesis indicate that the most efficient approach available at present for differentiating the various forms of problem equines, would appear to require the administration of HCG. However, this test requires the collection of two blood samples for radioimmunoassay during a total on stable visit time of 1 hour. Even with this expense, a diagnosis cannot be guaranteed in every case. In addition, HCG is a highly antigenic drug, and therefore, repeat testing of animals which produce inconclusive results at the first attempt should be avoided, to prevent the occurrence of anaphylactic shock

and, possibly death. Therefore a better method is required to be established.

The other gonadal steroids and their metabolites show secretion patterns similar to that of testosterone, with many extraneous factors influencing their secretion. Despite this Cox (1982) has recently advocated the measurement of Oestrone sulphate concentration in single samples to identify whether badly behaved male animals are psychopathic geldings or not. This approach was not studied in this thesis.

The use of other gonadal steroids and their metabolites, as a means of identifying the different conditions, have similar drawbacks to those found in the use of testosterone estimation. They show secretion patterns similar to that of testosterone, and many extraneous factors influence their secretion (Ganjam 1979).

An alternative method would be to measure the concentration of gonadotrophins in these problem geldings, as these hormones have long half lives and stable levels within the peripheral circulation. Of the two gonadotrophic hormones, LH shows more variation than FSH. LH concentration have been shown to vary with the season of the year (Irvine and Alexander, 1982; Burns et al 1984b; Harris, Irvine and Evans, 1983; Johnson and Thompson, 1983), ambient lighting (Burns et al 1982; Burns et al 1984b) and sexual stimulation (Irvine, Alexander and

Hughes, 1984). There is also evidence that, although LH levels rise immediately after castration, this change is not maintained indefinitely. During the years following castration, peripheral LH concentrations appear to return to precastration levels (Irvine and Alexander, 1982). This would make it impossible to differentiate long term psychopathic geldings from animals that possess testicular tissue, by measuring the concentration of this hormone in plasma samples.

Although seasonal variation has been reported in FSH concentrations, (Harris, Irvine and Evans, 1983; Burns et al 1984b) fewer other factors influence FSH secretion. There is also no evidence available to suggest that FSH levels return to precastration levels in the long term castrate. In other mammals, the concentration of FSH in the peripheral circulation of cryptorchid males, is higher than that in normal males, and so measurement of this hormone may differentiate normal from cryptorchid stallions, and psychopathic geldings from animals that possess testicular tissue (Caraty, 1983; Caraty, Martinat and Blanc; 1981; Schanbacher and Ford, 1977; Wu et al 1981; Purvis et al 1975). Work in man also suggests that the measurement of this hormone may aid diagnosis in cases of male infertility (de Kretser, 1979; Wu et al 1981; Purvis et al 1975; Rosen and Weintraub, 1970; Hunter et al 1974).

Therefore, it was decided to investigate whether FSH measurement could be used for diagnosis in problem gelding cases, and or, in infertile stallions.

CHAPTER THREE

ESTABLISHING AN FSH ASSAY

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3.1. INTRODUCTION

In order to undertake the investigation into changes in circulating blood concentrations of FSH, it was necessary to establish a suitable method of assay. Two methods in particular were thought suitable. These were; (a) Radioreceptor assay, and (b) Radioimmunoassay. Both approaches were carried out, and their results compared.

3.2 RADIORECEPTOR ASSAY

3.2.1. Introduction

A heterologous radioreceptor assay was established, using the method of Cheng (1975), with minor modifications. Bovine testis preparation, porcine FSH standards, and radioactively labelled human FSH, were employed in the assay.

3.2.2. Materials and methods

3.2.2.1. Materials

1 Tris; Trisma Base, reagent grade, Sigma.

2 Hydrochloric acid; May and Baker.

3 Sucrose; Formachem.

4 Magnesium Chloride; BDH.

5 Bovine Serum Albumen; Sigma.

6 Polyethylene Glycol; Sigma.

7 Glass distilled water.

8 Human FSH labelled with Iodine^{125}, supplied by; Dr R. Chapman, Department of Pathological Biochemistry, Glasgow Royal Infirmary.

9 Porcine FSH, 50IU vial; Sigma.

10 Flint Glass testtubes; Samco.

11 Fresh bovine testes, supplied by; the Glasgow University, Lanark Veterinary Practice. These were taken from 6-12 month old stirks and stored on ice. Receptor preparation took place within 12 hours.

3.2.2.2. Buffers and reagents

(a) Stock 0.5M Tris buffer

50.55g of Trizma base was disolved in 500ml of distilled water and 1M hydrochloric acid was added to give a pH of 7.2. This solution was made up to 1 litre and stored at $4^{o}C$.

The follwing buffers were prepared from this stock solution. The pH of each buffer was checked just prior to use.

Receptor buffer

Each litre contained;

Stock Tris buffer	50ml
Sucrose	171.2g
Distilled water to	1000ml

This was a 0.025M Tris-HCl buffer at pH 7.2, containing 0.5M sucrose.

Assay buffer

Each litre contained;

Stock Tris buffer	50ml
Magnesium Chloride	2.03g
BSA	1g
Distilled water to	1000ml

This was a 0.025M Tris-HCl buffer at pH 7.2, containing 0.01M magnesium chloride, and 0.1% bovine serum albumin (BSA) w/v.

Dilution buffer

Each litre contained;

Stock Tris buffer	500ml

BSA 1g

Distilled water to 1000ml

This was a 0.25M Tris-HCl buffer at pH 7.2, containing 0.1% BSA w/v.

Receptor suspension buffer

Each litre contained;

Stock Tris buffer 50ml

Magnesium chloride 2.03g

Distilled water to 1000ml

This was a 0.025M Tris-HCl pH buffer, containing 0.01M magnesium chloride.

(b) Polyethylene glycol

20g of polyethylene glycol was dissolved in distilled water to 100ml, to produce a 20% w/v, polyethylene glycol solution.

(c) Porcine FSH standard solution

50IU of porcine FSH was dissolved in 5ml of assay buffer. 100µl aliquots of this solution were dispensed, and stored at -20^{o}C until use. Prior to use an aliquot was thawed, and 2.4ml of assay buffer was added to each aliquot. Serial, 1 in 2 dilutions, of this solution were prepared using the same buffer, giving standard solutions equivalent to porcine FSH concentrations of; 40, 20, 10, 5, 2.5 and 1.25 mIU per ml.

(d) Serum pools

1 litre of blood was collected; (a) from a 6 year old, Welsh pony stallion, and (b) from a pony gelding. This

blood was allowed to clot overnight at 4°C, and was centrifuged at 1000g in a Mistral 6L centrifuge. The serum was harvested, and 1ml aliquots of the stallion or gelding serum were frozen at -20°C, for use as control sera.

(e) Non-specific binding solution

5ml of assay buffer was added to a 50IU vial of porcine FSH. 300µl aliquots of this solution were dispensed, and stored at -20°C until use.

(f) Working tracer solution

Aliquots of the stock tracer solution were diluted with reaction stopping buffer, to give a solution containing 10,000 counts per 100µl.

3.2.2.3. Receptor preparation

All procedures were performed at 4°C, or on ice.

50g of testicular parenchyma was isolated from its covering tunics, sliced into small pieces, and rinsed with receptor buffer. This was homogenised for 30 seconds in a Silverson homogeniser, set at maximum speed.

The resultant homogenate was further homogenised for 10 seconds, with 250ml of receptor buffer, to give 300ml of blended homogenate. This emulsion was filtered through 4 layers of cheesecloth, to remove larger pieces of tissue, and then filtered through a further 8 layers of cheesecloth.

The filtrate was poured into Beckman type 335449 polyallomer bottles, and centrifuged at 12000g for 30

minutes, in a Beckman L2-65B ultracentrifuge. The resultant supernatent was decanted into Beckman type 336090, thick walled polycarbonate tubes, and centrifuged at 100,000g, for a further 1 hour.

The pellets were resuspended using the Silverson homogeniser, in suspension buffer, at a rate of 1ml of buffer per 6ml of supernatent centrifuged in the second centrifugation step. This suspension was divided into 0.5ml aliquots and stored at -60^{o}C until use.

For use the stock suspension was diluted to a working 1 in 2 dilution, and homogenised using a hand, ground glass, homogeniser. This optimum working dilution was determined from receptor dilution curves.

3.2.2.4. Receptor titration

100µl of serial dilutions of the receptor preparation were incubated with 100µl of working tracer solution, and 300µl of assay buffer, overnight at room temperature. These tubes then received 1ml of 20% Polyethylene glycol, prior to centrifugation at 1750g in a Beckman TJ-6 centrifuge. The supernatent was aspirated, and the pellet counted in a Packard 5230 scintillation counter. Non specific binding was determined by adding 100mIU (a gross excess) of porcine FSH to similar tubes prior to incubation.

The specific binding of tracer with each dilution of receptor was then determined and dilution curves prepared. Dilution curves were also obtained for the

receptor preparation in the presence of 100µl of gelding or stallion pool serum, and 200µl of assay buffer.

3.2.2.5. Preparation of low FSH serum

10ml of stallion pool serum was incubated with 10ml of a 1 in 2 dilution of receptors, for 2.5 hours at room temperature. After centrifugation at 100,000g for 1 hour, the supernatent was dispensed into 1ml aliquots, and stored at -20°C until use, as an FSH free serum.

When this solution was assayed, 200µl of the treated solution was added to 100µl of working receptor solution, 100µl of working tracer solution, and 100µl of assay buffer.

3.2.2.6. Assay protocol

Tubes were prepared as follows;

DAY 1.

3 Total counts tubes (TC).

100µl working tracer.

400µl assay buffer.

3 Total binding (B0) tubes.

100µl working tracer.

100µl stallion pool serum.

100µl working receptor solution.

200µl assay buffer.

2 Nonspecific binding (NS) tubes.

100µl working tracer solution.

100µl stallion pool serum.

100µl assay buffer with 1IU porcine FSH per ml.

100µl working receptor solution.

100µl assay buffer.

6 duplicate Standard (S) tubes.

100µl working tracer solution.

100µl working receptor solution.

100µl stallion pool serum.

100µl of porcine standard FSH in assay buffer.

100µl assay buffer.

Sample tubes in duplicate.

100µl working tracer solution.

100µl serum sample.

100µl working receptor solution.

200µl assay buffer.

After vortexing, all tubes were incubated at room temperature (20°C) for 20 hours.

DAY 2 (Separation of bound and free tracer)

Following incubation the reaction was stopped in all the tubes except the Total Counts tubes, by adding 3ml of dilution buffer, at 4°C. Each of these tubes then received 1ml of 20% polyethylene glycol, and was vortexed.

After spinning at 1000g in a Mistral 6L centrifuge for 30 minutes, the supernatent was aspirated and the pellet counted in a Packard 5230 scintillation counter.

3.2.2.7. Standard curve preparation and analysis

Assay data was analysed using an Apple IIe microcomputer, and a programme after Rodbard and Lewald (1970).

3.2.3. Results

3.2.3.1. Receptor dilution curves in the presence of stallion and gelding sera

Figure 3.1. shows that both curves had a sigmoid shape. A maximum specific binding of 14.5% was observed in the presence of stallion serum, and the steepest slope of each curve coincided with a receptor dilution of 1 in 4.

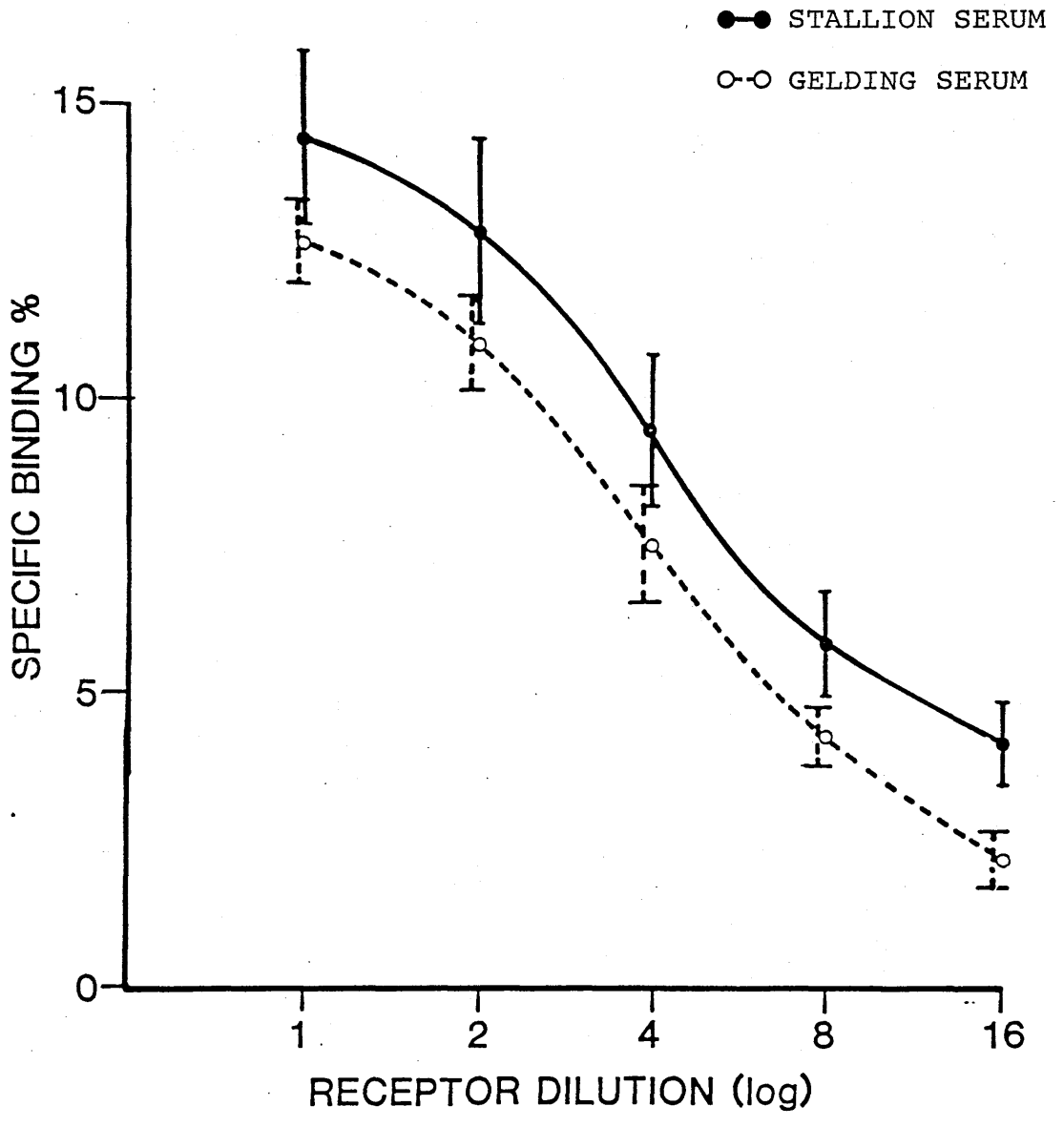
There was a parallel displacement of the dilution curves in the presence of pooled sera, that contained different concentrations of FSH.

There was an overlap in the large standard errors of these results, at the lower dilutions of receptors.

With a 1 in 2 dilution of the receptors, the dilution curve was moderately steep, there was more than 10% specific binding of tracer, and wide displacement of the curves in different sera. This was the dilution of receptor selected for the subsequent preparation of standard curves.

FIGURE 3.1.

SPECIFIC BINDING (± S.E.M.) OF HUMAN FSH TRACER, IN THE PRESENCE OF SERIAL DILUTIONS OF RECEPTOR PREPARATION, AND STALLION AND GELDING SERUM



3.2.3.2. Cumulative standard curve

Figure 3.2. illustrates the cumulative standard curve for five assays. There was a relatively large standard error, around the mean percentage of binding, in the presence of each standard solution. The mean total binding (± s.e.m) in these assays was 17.1% ± 1.5%.

There was a rather flat curve, with a range of binding from 75% to 35%, over the range of standards. 50% binding was obtained in the presence of the standard containing 10mIU/ml of porcine FSH.

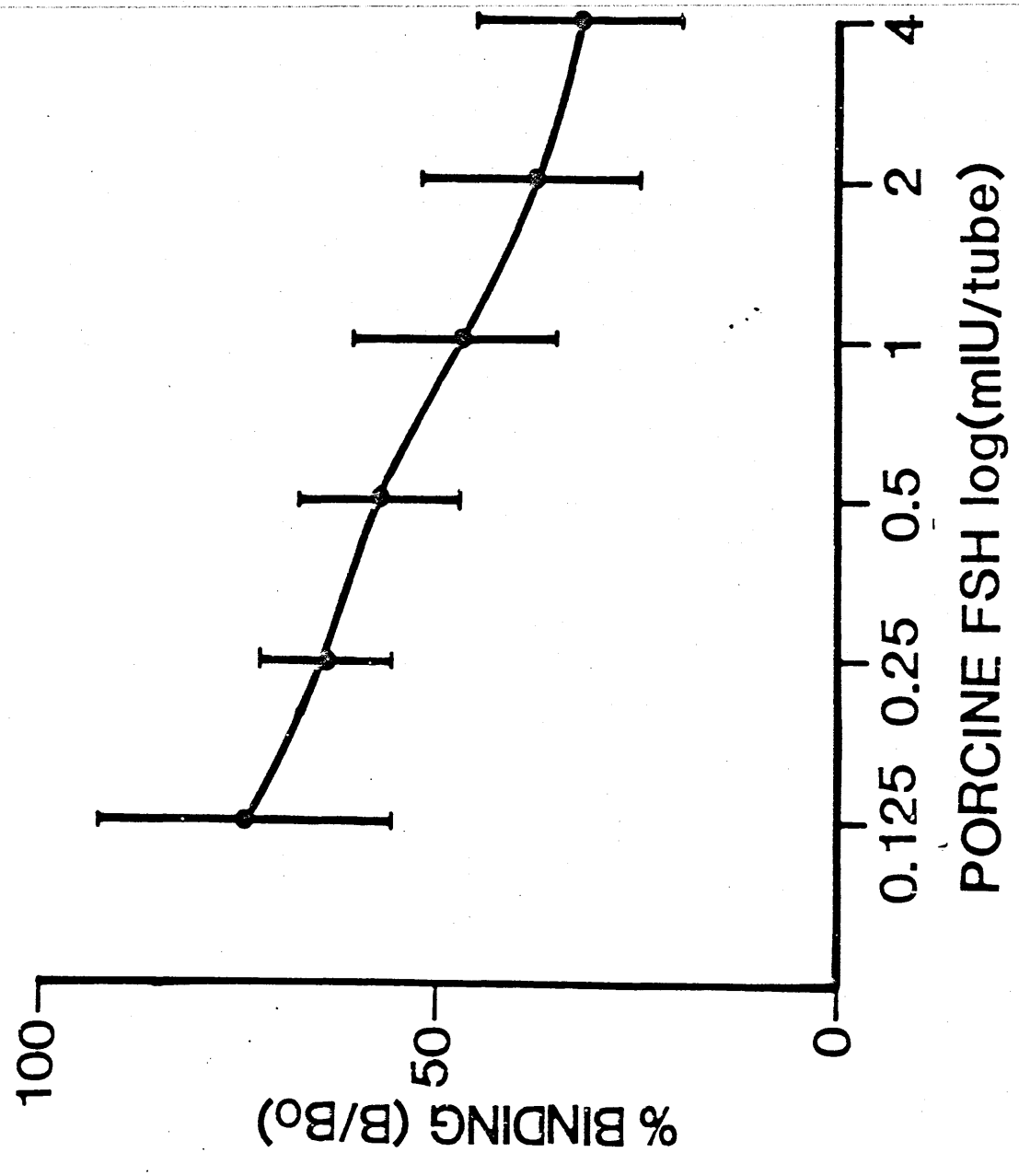
In the five assays represented in this cumulative curve the mean non specific binding (± s.e.m) was 2.9% ± 0.43%.

3.2.3.3. Assay sensitivity

The limit of sensitivity of the assay, defined as the value at twice the standard deviation from the binding obtained in the presence of the pooled stallion serum only (in the BO tubes), was calculated by extrapolation from the cumulative standard curve (figure 3.2.), as 2.0mIU / tube.

FIGURE 3.2.

THE COMPOSITE STANDARD CURVE FOR 5 RADIORECEPTOR ASSAYS USED TO CALCULATE THE LIMIT OF SENSITIVITY OF THE ASSAY. EACH POINT IS THE MEAN ± S.E.M..



3.2.3.4. Specific binding in the presence of various sera and solutions

Table 3.1. demonstrates that the specific binding, in the presence of serum from stallions A and B, was higher than the binding in the presence of stallion pooled serum. The human "blank" serum inhibited FSH binding to a greater extent than the stallion serum.

Incubation of stallion pool serum with receptors, followed by the removal of the receptors (3.2.2.5.), for use as a blank serum reduced the specific binding, compared with that obtained with untreated stallion pool serum.

The "blank" sera did not increase the binding in the assay tubes to exceed that recorded for stallions A and B.

Table 3.1.

Specific binding (B/BO) in the presence of various sera and solutions.

SERUM/SOLUTION	SPECIFIC BINDING (%)
Stallion pool	100
Stallion 'A'	116
Stallion 'B'	121
Human, low FSH serum	98
Stallion pool serum after-incubation with receptors	88

3.3. RADIOIMMUNOASSAY

3.3.1. Introduction

A homologous radioimmunoassay was established using a method described by Dr R. Chapman (personal communication, 1983). The assay employed an antiequine FSH antibody, radioactively labelled equine FSH, and equine FSH standard solutions.

3.3.2. Materials and methods

3.3.2.1. Materials

1 Disodium hydrogen orthophosphate; Analar

2 Sodium dihydrogen orthophosphate; Analar.

3 Bovine Serum Albumin; Sigma.

4 Sodium Azide; BDH.

5 Triton-X-100; BDH.

6 Glass distilled water.

7 Carrier free Iodine^{125}; Amersham International, 1mCi per 10µl.

8 Solid Phase Lactoperoxidase, supplied by; Dr R. Chapman, Dept. Pathological Biochemistry, Glasgow Royal Infirmary.

9 Highly purified Equine FSH, Batch E99B, 100µg supplied by; Professor Papkoff, University of California, San Francisco, U.S.A..

10 Anti Equine FSH antibody; RIA (UK) Ltd, Washington, Tyne and Wear; Crude/Glycerolated antibody, Batch 813, product 1544. The immunogen was eFSH (I044), prepared by UCB Bioproducts (Ltd). eFSH (I044) contains <1% eLH and eTSH, and <0.1% eGH and ePRL. It has a ratio of alpha to beta subunits similar to that of other pure eFSH preparations, and has a molecular weight of 32,000.

11 Sac-Cel, Antirabbit, Solid Phase Second antibody, coated on a cellulose suspension; Wellcome RD70.

12 Hamilton 100µl fixed needle syringe.

13 0.02cm internal diameter polythene tubing; Sterilin.

14 LP3 plastic testtubes; Luckhams.

15 100 vol Hydrogen peroxide; Analar.

16 200µl and 1000µl variable volume pippettes; Eppendorf.

17 Automatic diluter/dispenser; Dilutrend, Bohringer.

18 Multivortex shaker; Baird and Tatlock.

19 Scintillation spectrometer; Packard 5230.

3.3.2.2. Reagents

a) Assay Buffer

Each 2 litres of buffer contained;

Na_2HPO_4	11.5g
$NaH_2PO_4.2H_2O$	2.97g
BSA	5g
Sodium Azide	0.2g
Triton-X-100	1g

This 0.05M phosphate buffer had a pH of 7.4, and was stable at 4°C, for at least 4 weeks.

b) Phospate buffer

Each 200ml contained;

Na_2HPO_4	11.5g
$NaH_2PO_4.2H_2O$	2.97g

This 0.5M phospate buffer had a pH of 7.4, and was stored in aliquots at -20°C until use.

c) FSH preparations

The 100µg of FSH supplied by Professor Papkoff was dissolved in 0.5ml of double glass-distilled water. The actual volume of water added was determined by weight, and 10.5µl aliquots of the solution, containing 2µg of equine FSH were prepared, and frozen at -20°C until use. These aliquots were used either for iodination, or for stock standard solution preparation.

Stock standard solution was prepared by adding 7990µl of assay buffer to a 2µg vial of FSH. This gave a standard stock solution containing 250ng E99B/ml. 0.5ml aliquots of this solution were then prepared, and stored at -20°C until use.

For each assay standard curve, serial 1 in 2 dilutions of these stock standard vials were made, with assay buffer, using a 'Dilutrend' diluter. This gave 7 standard solutions containing; 250, 125, 62.5, 31.2, 15.6, 7.81, and 3.9 ng of equine FSH, per ml, respectively.

d) Working antibody solution preparation

The anti equine FSH antibody was raised in rabbits, and supplied in a crude glycerolated form. 65µl of a 1 in 10 dilution of the serum was sufficient for 1000 assay tubes, at a recommended final dilution of 1 in 75,000.

Dilution curves were prepared for each batch of antibody purchased.

1.56ml of assay buffer was added to each 65µl vial

of antibody. 162µl aliquots of this, 1 in 250 dilution, were prepared and stored at -20°C until use. Just prior to use, a vial of stock antibody was rinsed out into 25ml of assay buffer. When 250µl of this solution was dispensed to each assay tube this gave a working dilution of 1 in 75,000.

3.3.2.3. Iodination of FSH

Iodination materials;

a) 1 vial containing 2µgm of Equine FSH.

b) 5ml 0.5M phospate buffer.

c) 200µl of solid phase lactoperoxidase suspension, agitated before use.

d) 200 ml of distilled water, to which was added 10µl of 100vols Hydrogen peroxide just prior to use.

e) A vial of Carrier free Iodine^{125}, 100µCi per µl, containing at least 20µl of solution.

Iodination method;

Using a 100µl, glass Hamilton syringe, the following were drawn up into a 30cm length of plastic tubing of, I.D. 0.02cm;

10µl 0.5M phospate buffer

10µl solid phase Lactoperoxidase

10µl Hydrogen peroxide

10µl Iodine^{125} (checked to contain 2000cps at 60cm from a minimonitor)

These were expelled into the vial containing 2 µg of equine FSH. A clock was started and the vial vortexed.

After 30 minutes incubation, 200µl of assay buffer were added and the vial vortexed. Using a Pasteur pipette, this solution was transferred to a 40cm column of Ultragel AC54. Buffer was pumped through the column, at a flow rate of 40ml/hr, with each fraction collected over 1.5 minutes.

The counts per second in each tube were subsequently measured with a lead-shielded minimonitor, and the counts per second per tube plotted against fraction number (figure 3.3). The fractions constituting the first peak were then divided into three groups; (a) the 3 tubes constituting the rising part of the peak, (b) the 3 tubes constituting the peak proper, and the beginning of the falling part of the peak, and (c) the 3 tubes on the falling edge of the peak. 10 µl aliquots of a, b, and c, were counted in a Packard 5200 scintillation spectrometer. Aliquots calculated to contain 1000,000 cpm were then prepared, for each part of the peak, and were stored at $-20^{o}C$.

The % specific binding of each part of the peak was then determined (section 3.3.2.5.), and aliquots of the highest binding section of the peak were then used for subsequent assays.

The percentage incorporation of the $Iodine^{125}$, was estimated from the areas under the first and second peaks. The specific activity was calculated using the equation;

$$S = \left[\frac{B + D}{B + D + E}\right] \times \frac{A}{1550} \times \frac{500}{C}$$

where; S = specific activity

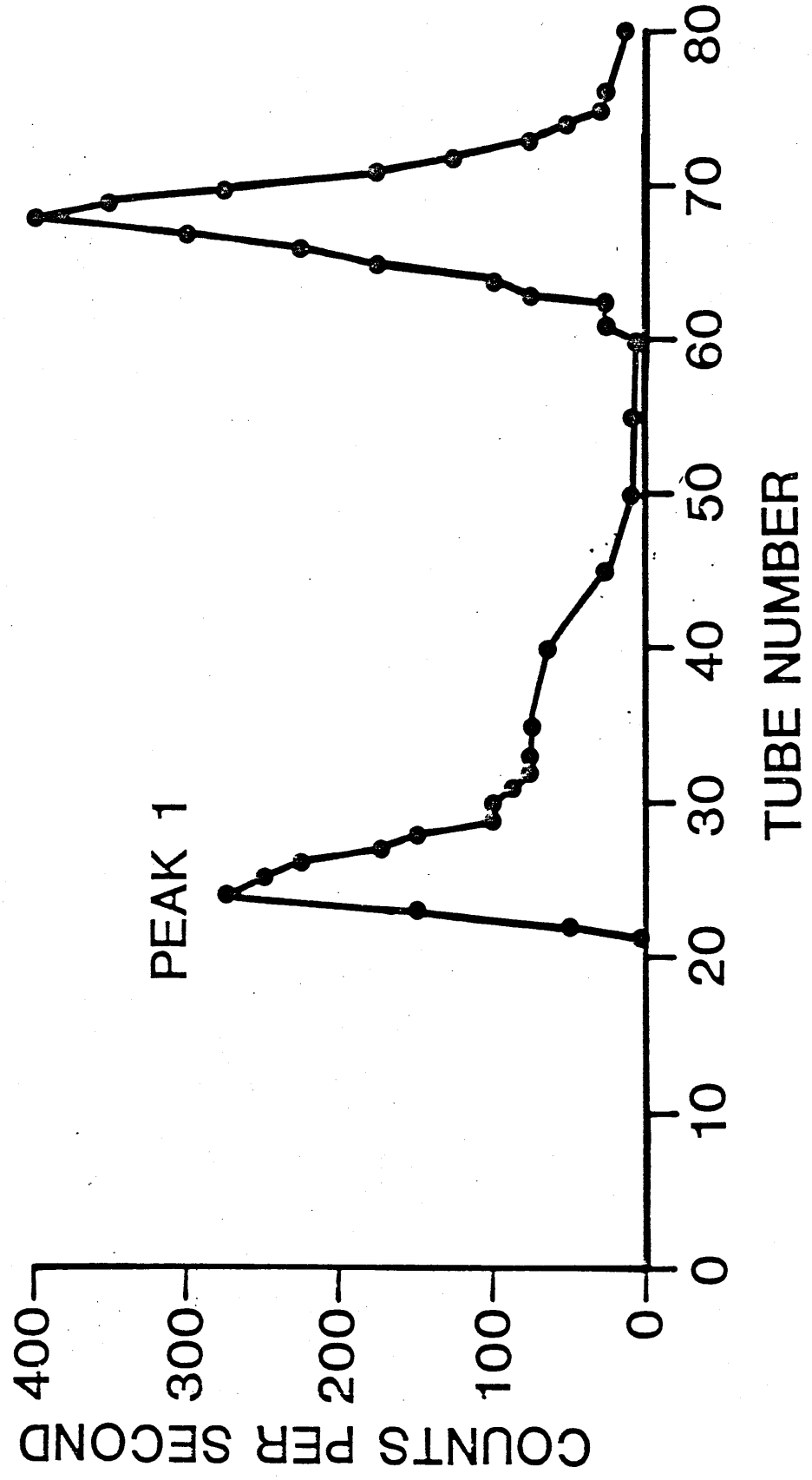


FIGURE 3.3.

ELUTION PROFILE FOR FSH IODINATED USING SOLID PHASE LACTOPEROXIDASE

A = cpm in reaction vessel

B = cpm in empty reaction vessel

C = Amount of FSH µg

D = cpm in protein peak

E = cpm in Iodine peak

3.3.2.4. Dilution curves

Dilution curves were prepared by determining the specific binding of serial dilutions of stock antibody (see; section 3.3.2.5.).

Although the antibody dilution curve, figure 3.5, suggested that a dilution less than 1 in 75,000 may be optimal, as the curve steepens at lower dilutions, cost dictated that a 1 in 75,000 dilution was employed in subsequent assays. This dilution gave a mean specific binding $\pm$ sem of 21% ($\pm$ 0.85%).

3.3.2.5. Assay protocol

The following tubeswere prepared;

DAY 1.

3 Total count tubes;

empty on day 1.

2 Non specific binding (NS) tubes;

300µl of assay buffer.

3 Total binding (B0) tubes;

50µl assay buffer

250µl working antibody solution.

Duplicate Standard (S1-S7) tubes;

50µl of working standards containing 250, 125, 62.5, 31.2, 15.1, 7.8 and 3.9ng/ml equine FSH respectively.

250µl working antibody solution.

Duplicate Control (CH and CL) tubes;

50µl of control gelding pool or stallion pool serum respectively

250µl working antibody solution.

Duplicate sample tubes;

50µl Sample serum.

250µl working antibody solution.

DAY 2.

100µl of working tracer solution added to all tubes.

DAY 3.

Separation of free and bound tracer

A suspension of antirabbit antiserum fixed to cellulose particles was purchased for the second antibody separation step. Each tube, except the TC tubes, received 100µl of solid phase second antibody via a Dilutrend automatic dispenser. The tubes were vortexed in a multivortex rack, and incubated at room temperature for 30 minutes. 1ml of deionised water was added to each tube and, without a further vortex, the tubes were centrifuged at 1000g for 30 minutes in a Mistral 6L centrifuge. After aspiration of the supernatent, the pellets were counted in a Packard 5230 scintillation spectrometer.

This separation step gave a mean nonspecific binding percentage $\pm$ sem of 4.05% ($\pm$ 0.98%).

In assays containing more than 100 tubes, a standard curve was included at both ends of the assay, to identify any assay drift.

3.3.2.6. Standard curve preparation and analysis

The concentration of FSH in the unknown samples (expressed as ng of equine FSH E99, per ml) was determined by interpolation from the standard curve, after logit (Y), and log (dose) transformations. This was performed using an Apple IIe microcomputer and a programme after Rodbard and Lewald (1970).

3.3.3. Results

3.3.3.1. Iodination performance

20-50% of the iodine was incorporated into the protein peak. This fraction had a specific activity of 150μCi/μg.

3.3.3.2. Specificity

The specificity of the antibody was ensured by the suppliers. Specificity was expressed as the percentage inhibition of tracer binding, at the ED50 point of the standard curve. Cross reactions with UCB Bioproducts (Ltd) hormone preparations were; 9.5% for eLH (I043) and 2.4% for eCG (I040). eLH (I043) has <2% eFSH contamination.

3.3.3.3. Accuracy

For an assessment of accuracy known amounts of equine FSH (between 7.8 and 31.2 ng/ml) were added to the stallion pool serum, and included in several assays. After adjustment for the stallion pool FSH concentration, the mean recovery figure, ± sem, was 113.6% ± 8.3%.

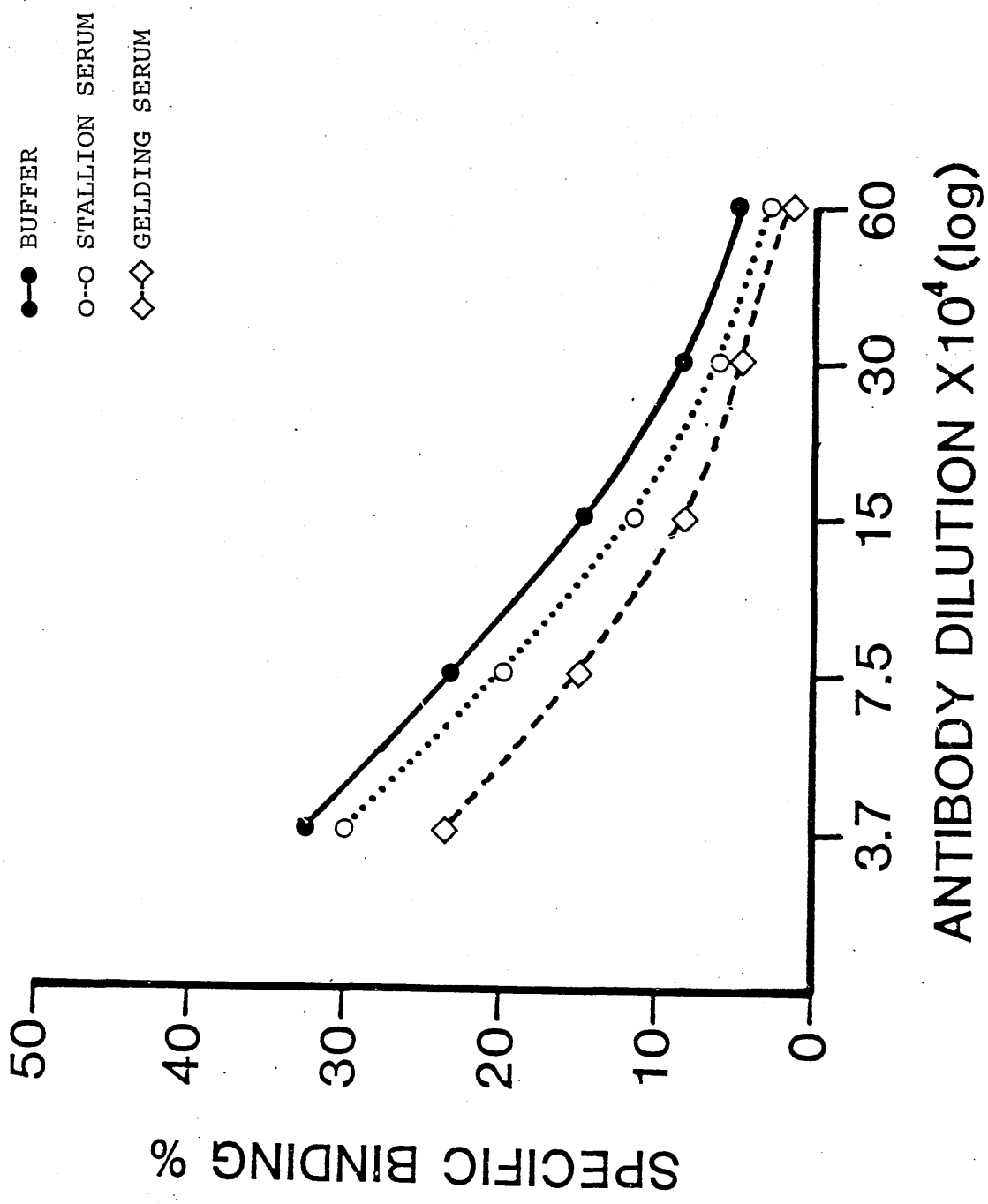
3.3.3.4. Parallelism of dilution and standard curves

Dilution curves for the antiequine FSH antibody, in the presence of stallion serum, gelding serum, buffer and known standards, were all parallel (figure 3.4.).

The presence of different volumes of gelding pool serum produced binding inhibition parallel to that of the standard curve (figure 3.5.).

FIGURE 3.4.

ANTIBODY DILUTION CURVES PREPARED IN THE PRESENCE OF BUFFER, STALLION SERUM AND GELDING SERUM (S.E.M. OF ALL POINTS <1%)



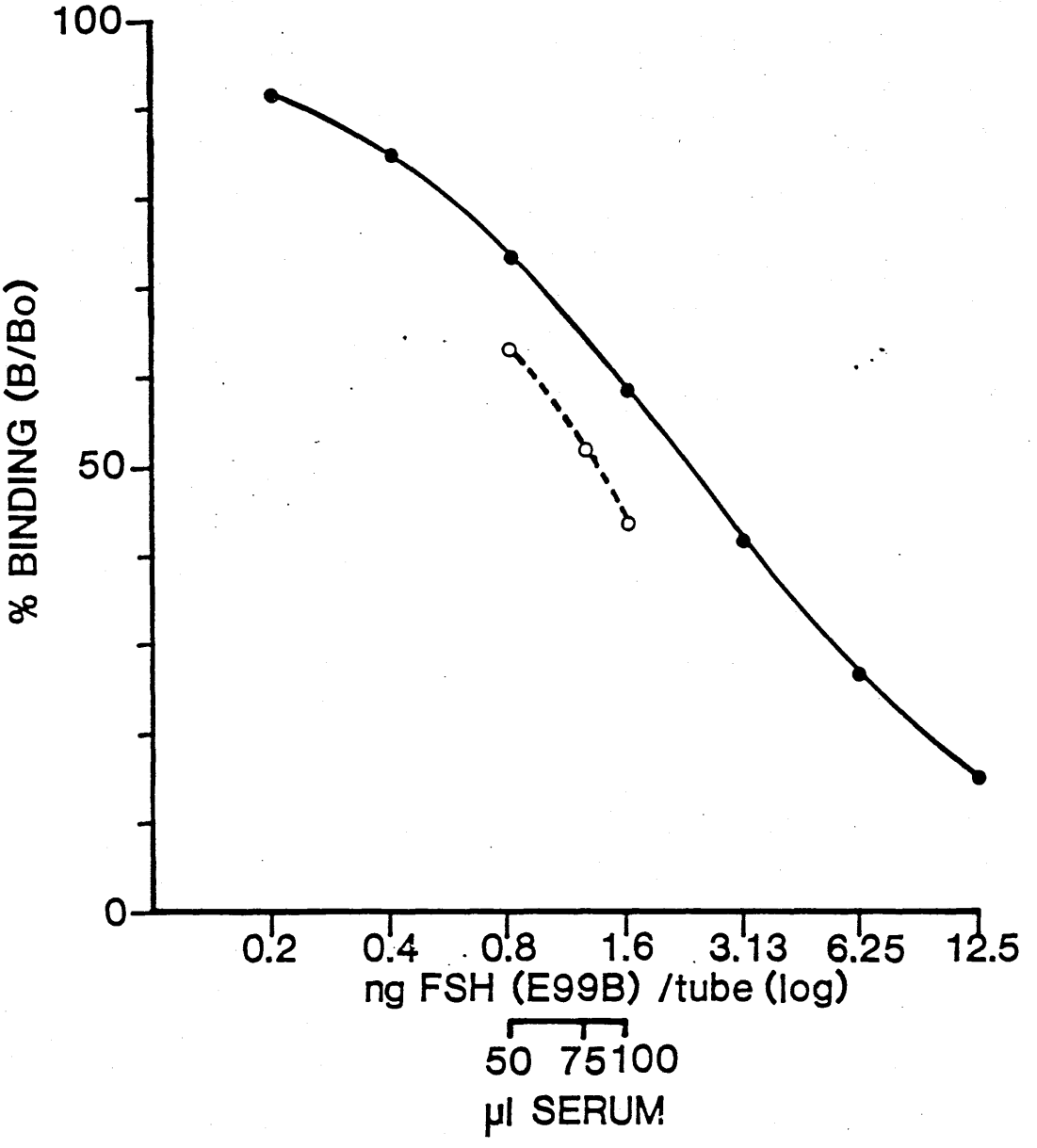


FIGURE 3.5.

THE CUMULATIVE STANDARD CURVE FOR 20 ASSAYS (●—●) USED TO CALCULATE THE LIMIT OF SENSITIVITY OF THE ASSAY (S.E.M. OF ALL POINTS <1%).

DOSE-RESPONSE CURVE (o- -o) FOR THREE VOLUMES OF GELDING POOL SERUM IS ALSO ILLUSTRATED

3.3.3.4. Sensitivity

The limit of sensitivity for the assay, defined as the value at twice the standard deviation from the binding obtained with zero concentration of FSH, was 250 pg/tube. This was calculated from the cumulative standard curve, and the B0 tube data from all the assays included in this study.

3.3.3.5. Repeatability

As shown in figure 3.5. the FSH standard curve was highly repeatable, with standard errors for all points less than 0.85%.

The inter-assay coefficient of variation (CV) was calculated from the concentration of FSH measured in the two control sera in each assay. The inter-assay CV for the high and low controls were 18.4% and 21.4% respectively, with a mean CV of 19.0%.

The intra-assay coefficient of variation, calculated from the standard deviations of 100 randomly selected, sample duplicate pairs, was calculated from the equation;

$$\text{Standard deviation} = \sqrt{\frac{\leqslant d^2}{2n-1}}$$

where; d is the difference between the two values for each duplicate pair.

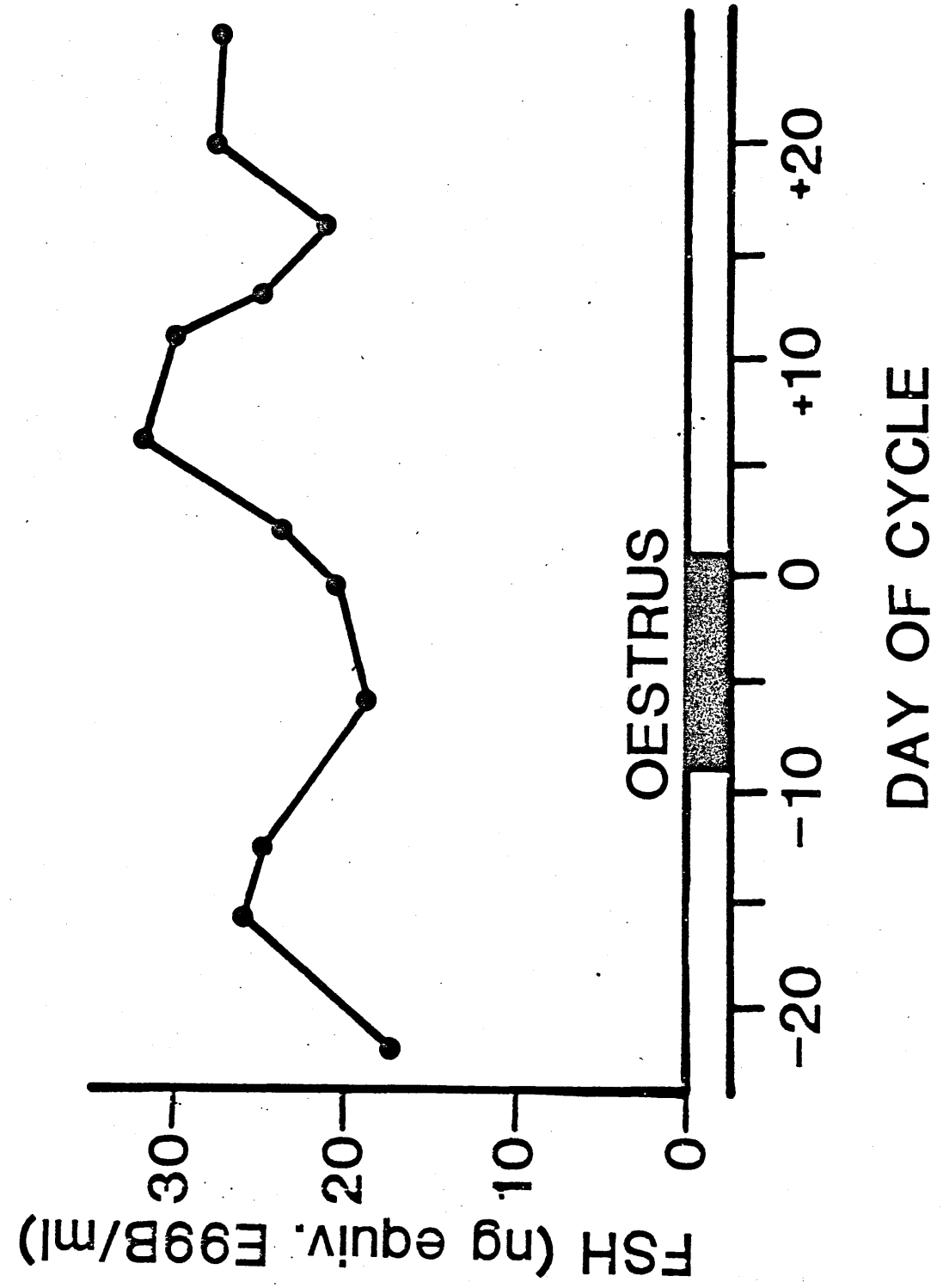
The intra-ssay CV was calculated thus to be 5.94%.

3.3.3.6. FSH levels measured in plasma samples taken from a mare at different times during the oestrus cycle

The changes in concentration represented by the results in figure 3.6. concur with those of other workers using heterologous assay systems.
A similar diphasic pattern of FSH secretion, with peaks during late oestrus and mid-dioestrus has been reported by Evans and Irvine (1975).

FIGURE 3.6.

CONCENTRATION OF FSH IN BLOOD SAMPLES TAKEN FROM A MARE DURING THE COURSE OF AN OESTROUS CYCLE



3.4. DISCUSSION

Equine FSH molecules are composed of two glycopeptide subunits, bound together by covalent bonding (Irvine, 1979; Combarnous and Henge, 1981; Bousefield and Nard, 1984). The amount of carbohydrate, especially sialic acid, bound to each unit varies, and purified equine FSH is thus a polymorphic mixture of molecules. The amount of sialic acid present on each molecule determines the biological activity of that molecule (Irvine, 1979; Vaitukaitis and Ross, 1971). Therefore, the assay method for equine FSH that provides the most clinically significant data, is one that specifically measures the amount of the most biologically active forms of FSH. For this reason the first assay method investigated in this study was a bioassay system, namely a heterologous radioreceptor assay (RRA).

The pilot radioreceptor assay technique described in this thesis made use of readily available materials; bovine testes, labelled human FSH tracer, and porcine FSH standard, to establish whether such an assay could be used to estimate equine FSH in serum samples. This was to determine whether further work in this area should be undertaken, if purified equine FSH became available. The results presented in this thesis indicate that this radioreceptor assay system could have been further developed for use in estimating serum FSH concentration. Similar conclusions were drawn by Combarnous and Henge

(1981), and Sairam (1979). Parallel displacement of receptor dilution curves was seen in the presence of sera containing different FSH concentrations (figure 3.1.), and standard curves were prepared using porcine FSH standard solutions (figure 3.2.). However, the potential advantages of the use of this biological system, were outweighed by technical difficulties in assay procedure.

Although a receptor preparation technique described by Sairam (1979) yielded larger batches of receptors than the original method described by Cheng (1975), these batches were still only sufficient for a few hundred assay tubes. When each of these fresh batches of receptors was "characterised" by producing dilution curves, the slope of these curves varied from batch to batch. This would have lead to between-batch variations in control sample results. Subsequently, aliquots of each batch of receptor preparation had to be resuspended by hand homogenisation before use, and variations in homogenisation and suspension uniformity may have contributed to the large standard errors observed for the points on the cumulative standard curves.

Since hormone binding at receptor sites is particularly sensitive to pH and the concentration of specific ions, this poor repeatability of the standard curves may also have been partly due to a tendency for the pH of the Tris buffers to fall during storage. These effects would have produced a large within-assay

variability in sample results.

Another technical difficulty was that the method of Cheng (1975) produced standard curves in the presence of low FSH plasma, to allow for nonspecific binding to serum proteins. The selection of a large pool of low FSH plasma proved difficult, and this resulted in many assays where samples contained less FSH than the zero standard, BO, tubes.

Because of these technical problems it was decided to investigate a homologous Radioimmunoassay (RIA), when equine FSH became available, from Professor Papkoff (University of California, San Francisco), and an antiequine FSH antibody became available from RIA (UK) Ltd (Washington, Tyne and Wear). This RIA system had the advantages of simplicity, and stability. The antibody was supplied with guaranteed specificity, and in a consistent form, that merely required dilution before use. This antibody and the equine FSH were then used in an established human FSH assay system (Dr R. Chapman, personal communication), using a commercially available and prevalidated, solid phase second antibody system. The iodination procedure employed techniques established by Dr R. Chapman (personal communication) over many years, and which produced a consistent supply of iodinated equine FSH label.

This homologous assay overcame problems associated with heterologous systems, such as those using antiovine

FSH antibody (Urwin and Allen, 1982), or antihuman FSH antibody (Evans and Irvine, 1976; Freedman, Garcia and Ginther, 1979). These antibodies were not raised against equine FSH molecules. Therefore they may selectively bind the minority forms of equine FSH in plasma samples, that most closely resemble either ovine, or human FSH, and may not bind the most important equine FSH molecules (Hagen and McNeilly, 1975). This may be especially true for assays using antiovine FSH antibody. Ovine FSH contains few sialic acid residues, compared to equine FSH. Antiovine FSH antibody will therefore most probably selectively bind the least sialized, and least biologically active forms of equine FSH.

The sensitivity of the RIA described here was higher than that of Driancourt and Palmer (1982) or Freedman, Garcia and Ginther (1979) when allowance is made for the different standard preparations employed. The percentage recovery of standard FSH in the presence of serum was similar to that reported by Urwin and Allen (1982), and the intra assay coefficient of variance was similar to, or lower than, those reported by Urwin and Allen (1982), Driancourt and Palmer (1982), and Freedman, Garcia and Ginther (1979). However, although the inter assay coefficient of variance was lower than that reported by Driancourt and Palmer (1982), it was higher than that reported by other authors. This may reflect either the recent establishment of the assay and technical

inexperience, or the relatively short life of each labelled FSH preparation. This effect was probably due to the high specific activity of the label preparation causing denaturation of the FSH, and adding to interassay differences between assays using fresh label, and assays using older label (Dr R. Chapman, personal communication).

The disadvantage of the RIA system, compared with the earlier RRA system, is that the most immunopotent forms of FSH are not necessarily the most biologically active, or important forms of FSH (Irvine, 1979; Vaitukaitis and Ross, 1971; Athineos, Thornton and Winzler, 1962). Although the homologous assay described here is an improvement on earlier heterologous systems, only an assay based upon biological activity, or binding at receptor sites, can accurately measure the amount of biologically active hormone present in a serum sample. However, due to difficulties inherent in the bioassay of large numbers of samples, the homologous RIA was selected as the method of choice for the estimation of circulating FSH concentrations in male equines. This system was employed in all subsequent studies.

CHAPTER FOUR

PERIPHERAL CIRCULATING FSH CONCENTRATIONS IN NORMAL STALLIONS, CRYPTORCHID STALLIONS, AND CASTRATED MALE EQUINES

CHAPTER FOUR

PERIPHERAL CIRCULATING FSH CONCENTRATIONS IN NORMAL STALLIONS, CRYPTORCHID STALLIONS, AND CASTRATED MALE EQUINES

4.1. INTRODUCTION

Initially, the concentrations of FSH in blood samples collected previously, from three stallions and one gelding (chapter 2), were estimated to determine the pattern of FSH secretion during the course of the day.

The pattern of FSH secretion over a period of months was then investigated in three stallions and one gelding.

The concentration of FSH in samples submitted from a variety of stallions, and geldings were also compared.

Subsequently, blood samples were taken from a series of stallions admitted for castration, and the histological appearance of their testes were compared with their peripheral circulating levels of FSH prior to castration.

4.2. MATERIALS AND METHODS

4.2.1. Blood samples collected at ten minute and one hour intervals

The samples collected from the stallions Max, Steptoe, and Blackie, and from the gelding Bonzo, in section 2.2.3.5., were analysed using the FSH RIA.

4.2.2. Blood samples collected at longer intervals

4.2.2.1. Animals used

The stallions Max, and Steptoe, and the gelding Bonzo, have already been described (2.2.3.1.). A 3 year old thoroughbred stallion, Dick was also studied. This stallion had not worked at stud prior to purchase. He was in good working condition throughout the period of study.

4.2.2.2. Maintenance

The stallions Max, Steptoe, and Dick, were housed in loose boxes throughout the period of study, and received a ration of horse and pony cubes (Spillers), adlib hay, and adlib water. These stallions were occasionally exposed to artificial lighting during the winter, as photoperiod experiments were carried out on mares in adjacent barns. All three stallions were used daily to tease mares, and Max and Steptoe were both used for natural service during December 1983. These two stallions were also used for weekly semen collection during subsequent months. The stallion Dick was used intermittently for natural service

during the spring months.

The gelding Bonzo was maintained at grass with hay supplementation during the winter months.

4.2.2.3. Blood samples collected

Blood samples were collected as previously described (2.2.3.4.). Samples were collected at least once weekly. Max and Steptoe were sampled more frequently than Dick, and during several months these two stallions tolerated daily sample collection.

Less frequent sample collection was possible from the gelding Bonzo, running free at grass.

4.2.3. Blood samples submitted from normal stallions and geldings

Blood samples were taken from 19 different stallions as previously described (2.2.3.4.). These were either normal stallions admitted to the Veterinary Hospital for castration, or normal stallions resident at a collaborating stud.

Samples were also made available from 21 geldings resident either in the Department of Veterinary Pharmacology, or at a nearby Equine Blood Bank.

4.2.4. FSH RIA

All samples were stored at -20^{o}C until assay. Assay method was as described in the previous section (3.3.2.). A volume of 50µl was used for all serum samples.

4.2.5. Testicular histology

4.2.5.1. Case histories

9 stallions were castrated at the veterinary hospital, and two were castrated by a local general practitioner. The details of these cases are presented in table 4.1.. Prior to castration blood samples were collected as described previously (2.2.3.5.).

TABLE 4.1.

DESCRIPTION OF ANIMALS CASTRATED DURING THE PERIOD OF STUDY

IDENTITY	BREED	AGE, yrs	HISTORY
JLA	PO	0.75	Normal colt
JLB	TB	1.5	Normal colt
W1	PO	1.5	Normal colt
W2	PO	1.5	Normal colt
89619	PO	5	Normal stallion
Lad	TB	5	Proven Normal stallion
95514	PO	16	Normal stallion
92874	PO	1	Unilat cryptorchid
82646	TB	2	Unilat cryptorchid
95367	PO	3	Unilat cryptorchid
92837	PO	3	Hemicastrate cryptorchid

PO = Pony

TB = Thoroughbred

Unilat = Unilateral cryptorchid stallion

4.2.5.2. Castration technique

Normal stallions under two years of age were castrated in the standing position, using an open technique (Heinze, 1966). Older normal stallions were castrated under general anaesthesia, using a closed technique (Heinze, 1966). Emasculators were employed in both techniques, to sever the spermatic cord (Vickers-Blake, 1893). In cases castrated using a closed technique double transfixation ligation of the spermatic cord with No. 1. chromic catgut, was also employed.

All cryptorchid stallions were castrated under general anaesthesia. The cryptorchid testis was always removed first in unilateral cryptorchids, using an open castration technique employing both emasculators and ligatures. Where necessary the cryptorchid testes were retrieved from the inguinal canal, or abdomen, by careful dissection around, and traction on, the inguinal extension of the gubernaculum testis (Valdez et al 1979; Adams, 1964). Six inch sponge forceps were used to assist in the exteriorisation of these testes. In unilateral cases, the scrotal testis was then removed using a closed castration technique.

After removal, the testes were isolated from their vaginal tunics, and 1cm cubes of testicular parenchyma were prepared for the preparation of histological sections.

4.2.5.3. Preparation of sections of testicular parenchyma for histological examination

Materials used;

a) Buffered Neutral Formalin (BNF);

Formaldehyde 40% w/v	10ml
Acid Sodium phosphate monohydrate	0.4g
Anhydrous disodium phosphate	0.65g
Water	100ml

b) Haematoxylin solution;

Haematoxylin	1g
Distilled water	1000ml
Ammonium alum	50g
Sodium Iodate	0.2g
Citric acid	1g
Chloral hydrate	50g

c) Eosin solution;

A saturated Eosin solution was prepared by adding 3g of Eosin to 100ml of absolute alcohol.

Methods used;

a) Sample collection;

1cm cubes of testicular parenchyma were taken from representative parts of testicular parenchyma. These were fixed in 50ml of BNF for 12 to 24 hours.

b) Processing;

This was carried out using an automatic Histokine 24 hour tissue processor (Shandon Elliot LTD). The complete process took 29 hours. The different stages of the process were;

70% Methylated spirit	2hr

70% Methylated spirit + 5% Phenol	2hr
90% Methylated spirit + 5% Phenol	2hr
Absolute alcohol + 5% Phenol (1)	2hr
Absolute alcohol + 5% Phenol (2)	1hr
Absolute alcohol + 5% Phenol (3)	1hr
1% Celloidin in Methyl Benzoate	4hr
Histoclear (1)	1hr
Histoclear (2)	1hr
Histoclear (3)	1hr
Paraffin wax (1)	6hr
Paraffin wax (2)	6hr

c) Cutting of sections;

5µm thick sections were cut from the wax blocks containing the specimens, using a microtome (Optical Spencer 821). These sections were mounted on glass slides, dried in an oven at 56^{o}C for 1 hour and then stained.

d) Staining procedure;

Sections mounted on glass slides were agitated in Xylene for 1-2 minutes, and then transferred to absolute alcohol. After 1 minute, they were soaked in Methylated spirit for a further minute, and then rinsed with water. A few drops of Iodine were then placed on the slides for 1-2 minutes, before rinsing in water. The slides were then placed in 5% Sodium thiosulphate for 1 minute, washed in water, and then placed in the Haematoxylin solution, for 5 minutes. After rinsing in water, and 3 quick immersions in acid alcohol, a further water wash was followed by

"blueing" in Scotts tap water, for 90 seconds, and then another 2 minute water wash. They were then rinsed in methylated spirit, prior to staining in saturated alcoholic Eosin, for 20 seconds. Finally, they were rinsed in methylated spirit, dehydrated, cleared, and mounted.

4.2.5.4. Examination of stained sections

The slides were examined using a microscope (Wild), at X40 magnification, fitted with a ground-glass obscurer. On the obscurer screen was positioned a transparency, marked with 1mm squares. Using the moving stage of the microscope, true transverse sections of seminiferous tubules, with a circular outline, were centred on the grid. The vertical and horizontal diameter of ten such transverse sections were estimated, in mm, from the transparent grid. In addition the number of spermatogonia and primary spermatocyte and secondary spermatocyte nuclei, lying along, or adjacent to, the vertical and horizontal axes of the tubules, was estimated. Finally the presence or absence of spermatozoa within the lumen of the seminiferous tubules was noted.

Having calibrated the transparent grid, the actual diameter of the tubules was calculated.

The number of tubules studied on each slide, was determined by the number of circular sections of tubules available on the poorest slide. Therefore, ten true transverse sections were examined on each slide.

4.3. RESULTS

4.3.1. Concentration of FSH in blood samples taken at ten minute intervals

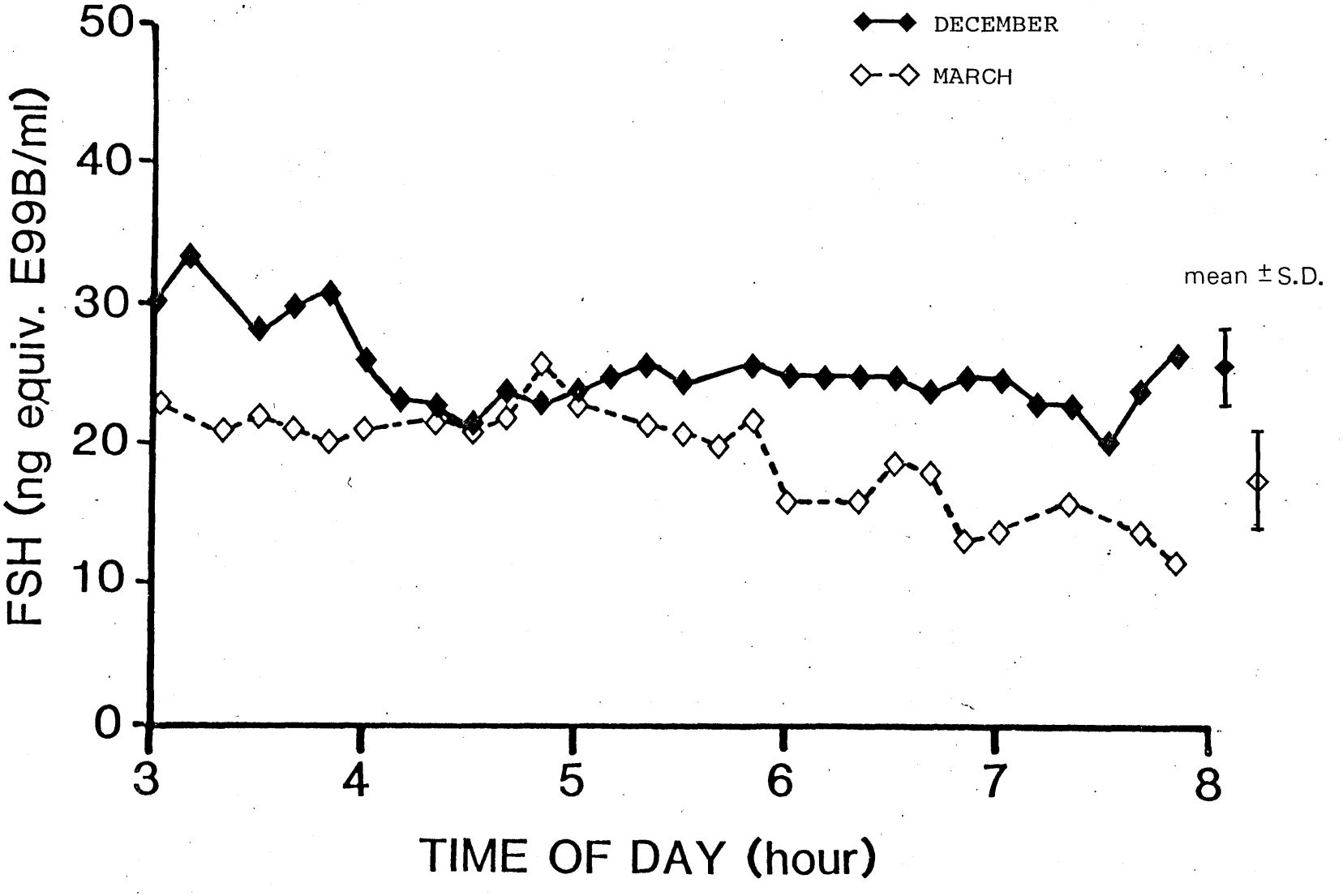
Figure 4.1. illustrates the concentration of FSH in blood samples taken at ten minute intervals from the stallion Max in December and in March. There was little change in peripheral FSH levels during either of the studies and the patterns of secretion were similar during both months. There was no significant difference between the mean FSH concentration in the two groups of samples ($P>0.05$).

The samples taken at ten minute intervals from the gelding Bonzo (figure 4.2.) contained significantly higher FSH concentrations ($P<0.05$) than were observed in the stallion samples (figure 4.1.). Figure 4.2. demonstrates that there was little variation in FSH levels during the period of study, although there was slightly more variation than was seen in samples taken at similar time intervals from the stallion Max.

FIGURE 4.1.

MAX (DECEMBER AND MARCH)

CONCENTRATION OF FSH IN BLOOD SAMPLES TAKEN FROM A STALLION AT TEN MINUTE INTERVALS



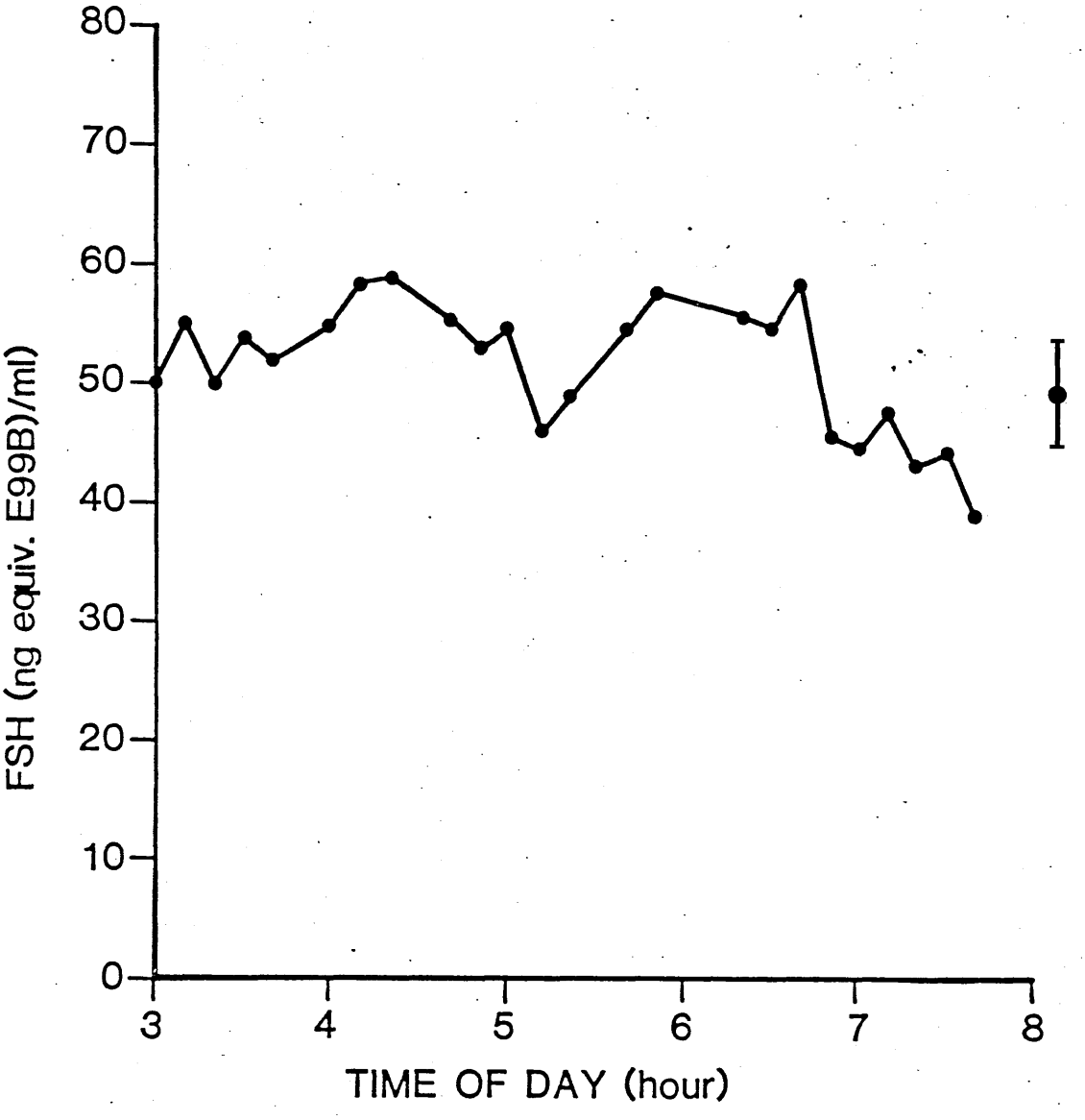


FIGURE 4.2.

BONZO (DECEMBER)

CONCENTRATION OF FSH IN BLOOD SAMPLES TAKEN FROM A GELDING AT TEN MINUTE INTERVALS

4.3.2. Concentration of FSH in blood samples taken at one hour intervals

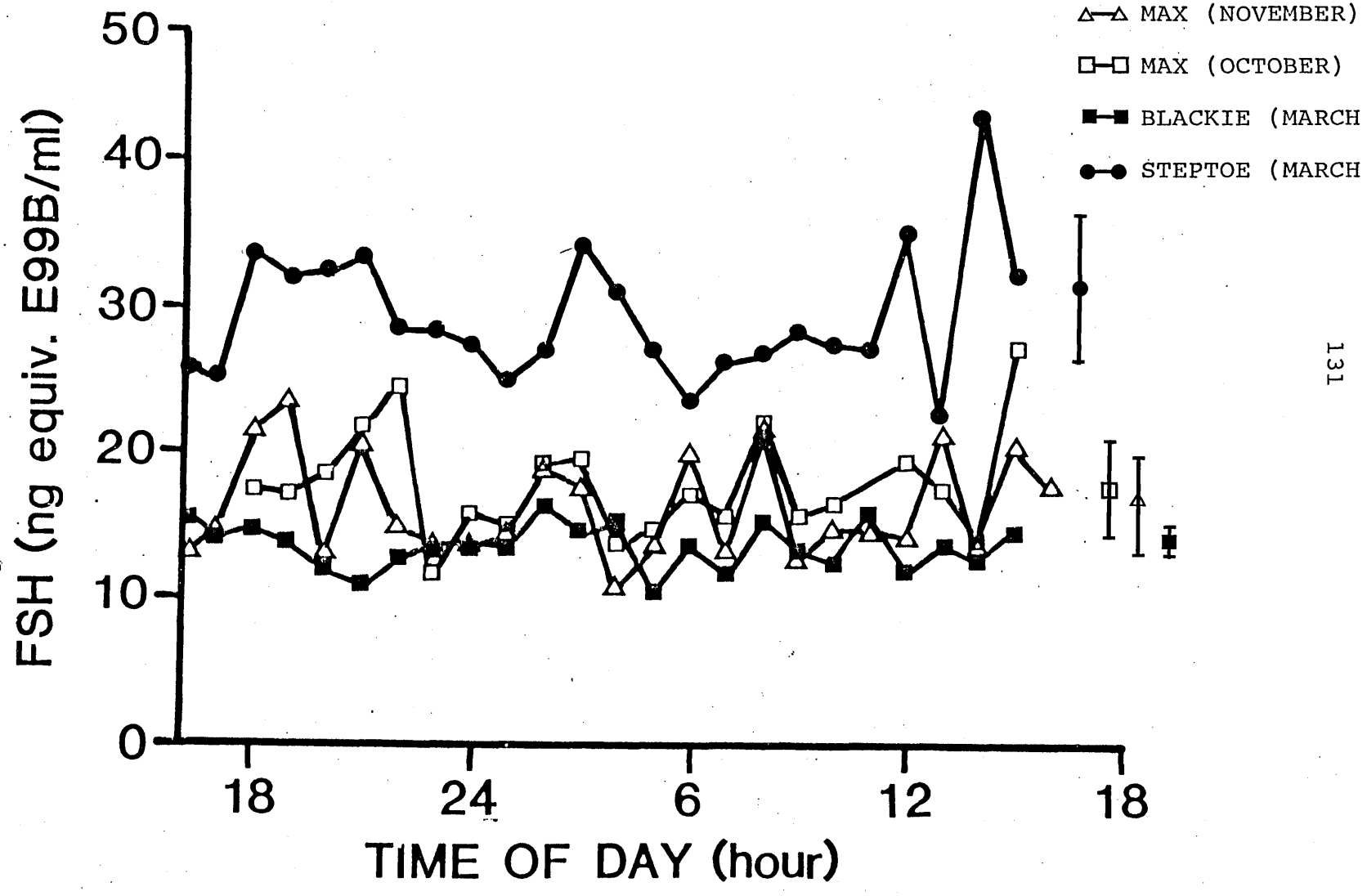
Figure 4.3. shows that samples taken at one hour intervals from the stallion Max on two occasions, and the stallion Blackie on one occasion, contained similar concentrations of FSH. The patterns of secretion on these occasions were similar. Although there was little variation around the mean daily concentration for each animal, some episodes of increased secretion of FSH did occur (Max, between 6pm and midnight on both occasions). The samples taken from the stallion Steptoe contained significantly more FSH than the samples taken from the other two stallions ($P<0.05$). There was also variation in the concentration of FSH estimated in the samples from Steptoe, with episodes of increased secretion occuring between 5pm and midnight, and between 1am and 6am.

Figure 4.4. shows that the samples collected at hourly intervals from the gelding Bonzo contained significantly more FSH ($P<0.05$) than the samples from the three stallions (figure 4.3.). Slightly more variation in FSH level was observed in the samples taken from the gelding. Levels fell from 6pm to midnight, then rose to a peak at 6am, and then fell again (figure 4.4).

FIGURE 4.3.

MAX (NOVEMBER AND OCTOBER), BLACKIE (MARCH) AND STEPTOE (MARCH)

CONCENTRATION OF FSH IN BLOOD SAMPLES TAKEN FROM THREE STALLIONS AT ONE HOUR INTERVALS



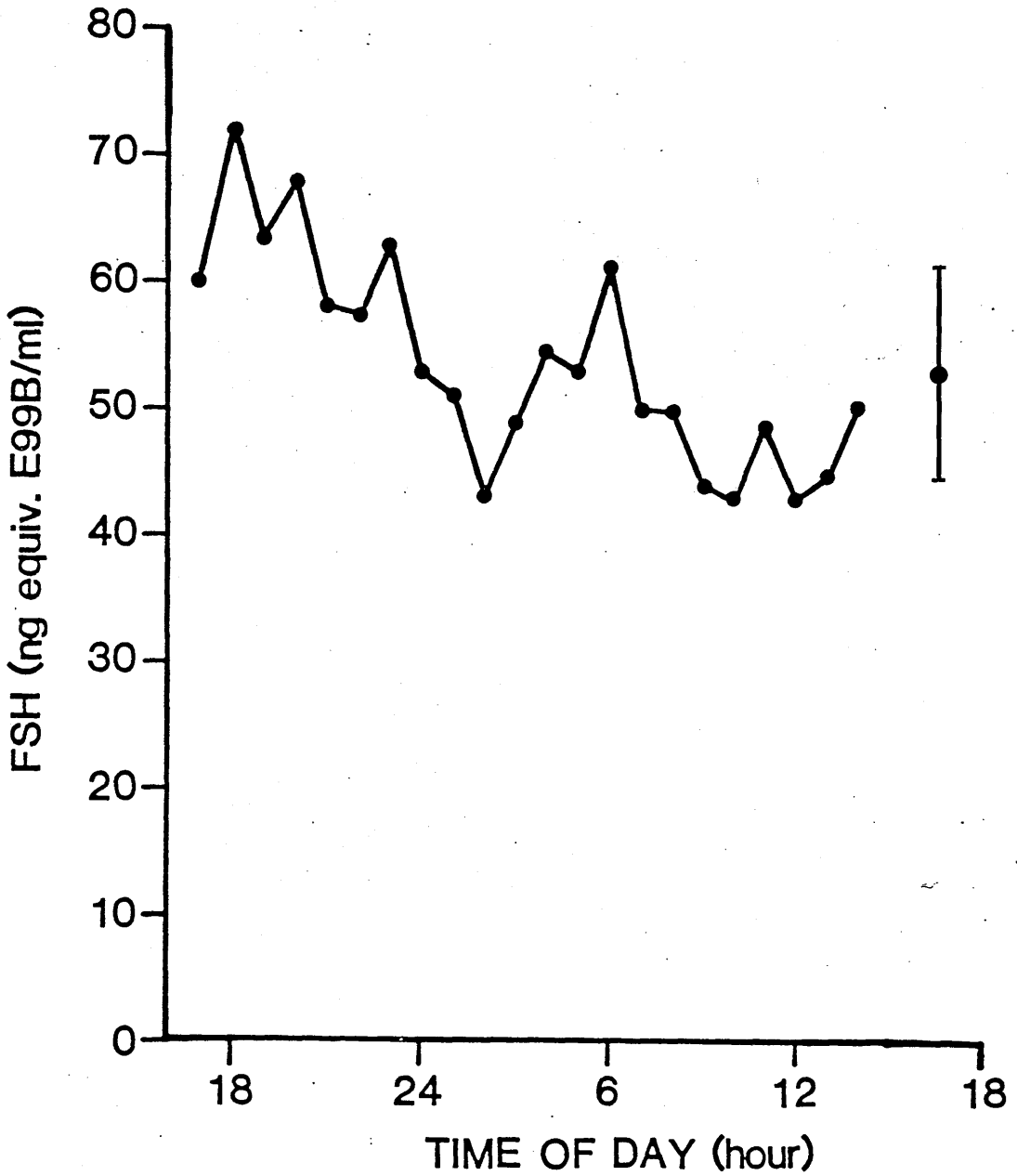


FIGURE 4.4.

BONZO (OCTOBER)

CONCENTRATION OF FSH IN BLOOD SAMPLES TAKEN FROM A GELDING AT ONE HOUR INTERVALS

4.3.3. Concentration of FSH in blood samples taken during different months

Figure 4.5. illustrates the concentration of FSH in samples collected during different months in 1984/85, from the stallions Max and Dick. There was less variation between the consecutive samples taken from Dick, and FSH concentrations were especially variable in Max between November and December. In Dick FSH levels fell slightly from October to January, and then rose slightly from March to May. There was less of a monthly trend in the 1984/85 results for Max, but levels did rise slightly between March and May.

The results of the measurement of FSH concentration in samples taken from the stallion Max in 1983/84 are illustrated in figure 4.6.. These showed a pattern similar to that seen in the stallion Max in 1984/85 (figure 4.5.), with little month by month change except for a slight increase in FSH concentration between March and May. The samples taken from the stallion Steptoe contained significantly more FSH than was present in the samples taken from the other two stallions, Max and Dick ($P<0.05$). The concentration in the samples taken from the stallion Steptoe was more variable than in the other stallions, and a more pronounced between-month trend was obvious. Levels of FSH fell between November and January and then rose between March and June.

FIGURE 4.5.

MAX (1984/1985) DICK(1984/1985)

CONCENTRATION OF FSH IN BLOOD SAMPLES TAKEN FROM TWO STALLIONS DURING TWO TEN MONTH PERIODS

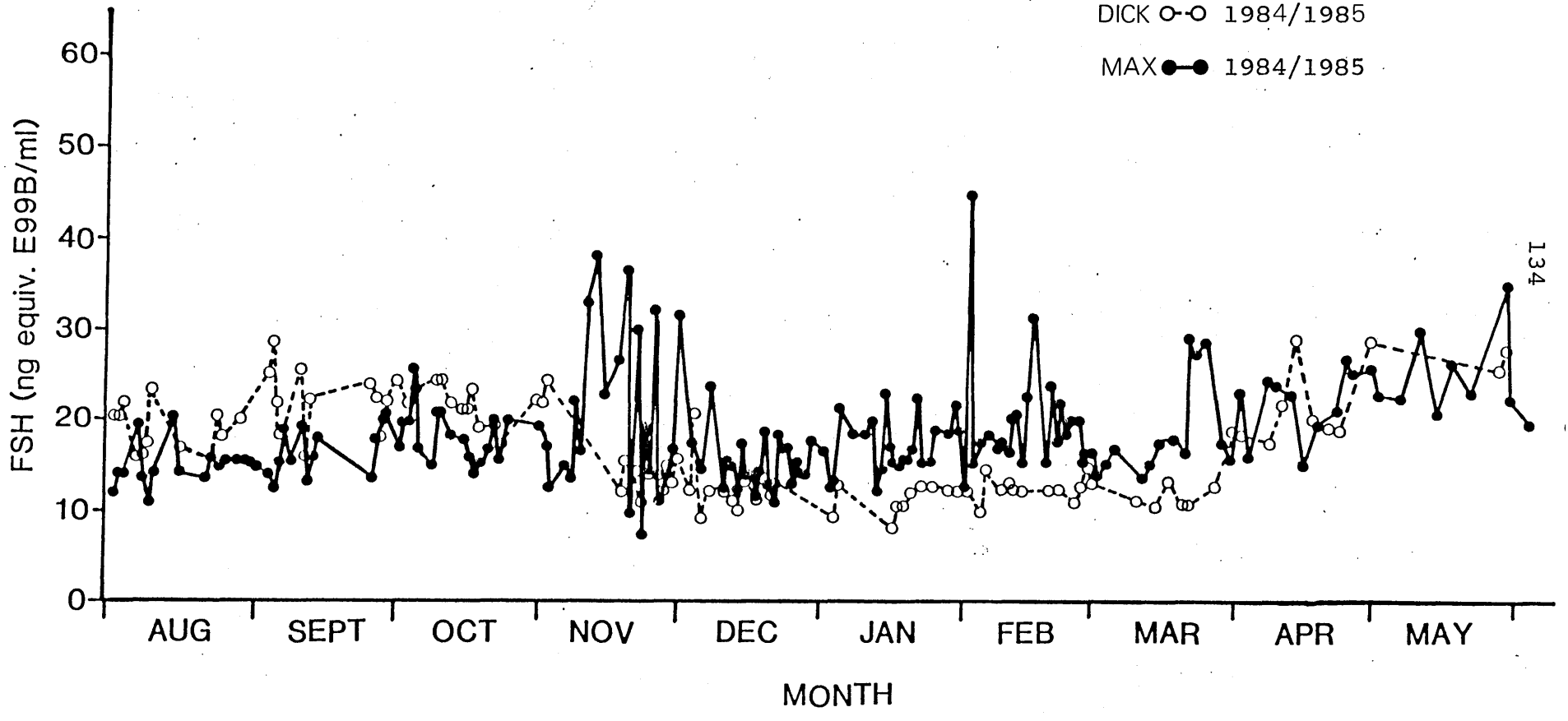


FIGURE 4.6.

MAX (1983/1984)

STEPTOE (1983/1984)

CONCENTRATION OF FSH IN BLOOD SAMPLES TAKEN FROM TWO STALLIONS

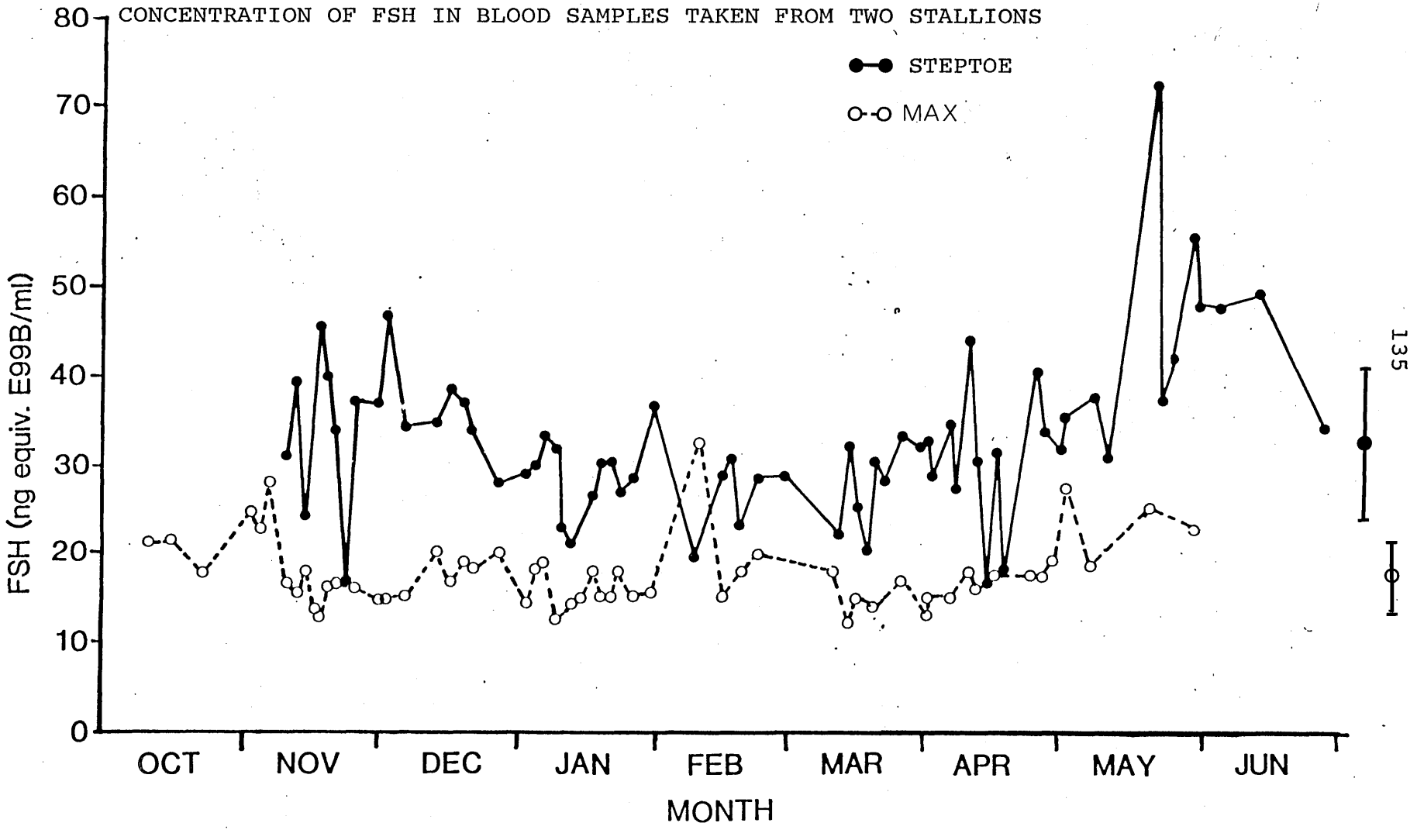
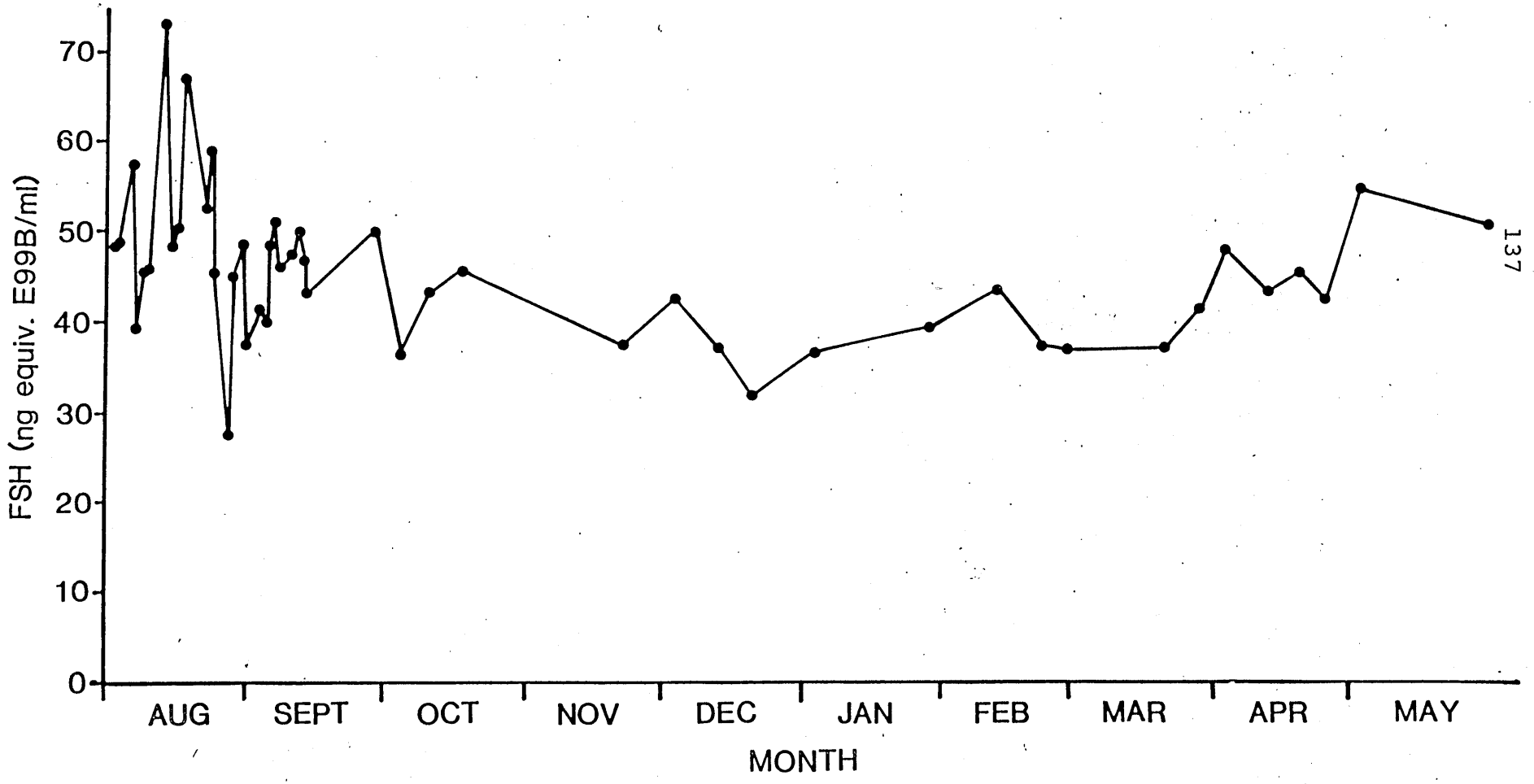


Figure 4.7. shows the results of FSH estimation of blood samples taken during different months from the gelding Bonzo. Fewer samples were collected from this animal. The mean concentration of FSH in these samples was significantly greater than the concentration measured in the samples taken from the three stallions ($P<0.05$). Variations between concentrations in consecutive samples, similar to those seen in the stallion Steptoe (figure 4.6.), occurred during more frequent sampling in August/September. A monthly trend similar to that seen in Max (1984/85, figure 4.5.) and Dick (figure 4.5.) was evident, with FSH levels falling slightly in December/January, and rising slightly between February and May.

FIGURE 4.7.

BONZO (1984/1985)

CONCENTRATION OF FSH IN BLOOD SAMPLES TAKEN FROM A GELDING BETWEEN AUGUST 1984 AND MAY 1985



4.3.4. FSH concentration in samples submitted from a mixed group of stallions and geldings

Table 4.2. shows the concentration of FSH in blood samples taken from a variety of stallions. FSH concentrations ranged between 8 ng/ml and 31 ng/ml. The concentrations appeared not to be related to month of year in these different stallions. Most samples contained less than 25 ng/ml of FSH, and the standard error around the mean concentration was small.

The samples taken from geldings and listed in table 4.3. contained a larger range of FSH concentrations than the stallion samples in table 4.2.(between 13 and 40 ng/ml). 50% of samples from geldings contained less than 25 ng/ml of FSH. The age of castration was not available for these geldings.

TABLE 4.2.

eFSH CONCENTRATION IN SERUM SAMPLES COLLECTED FROM 19 NORMAL STALLIONS AT VARIOUS TIMES OF THE YEAR

STALLION	AGE	MONTH	FSH ng/ml
EA		DECEMBER	16
EK		DECEMBER	14
ES		DECEMBER	14
EW		MARCH	20
ESU		MARCH	19
EM		MARCH	8
EPOO		MARCH	12
EWT		MARCH	21
EB		MARCH	18
EL		MARCH	27
W1	1.5	APRIL	23
W2	1.5	APRIL	28
P1		MAY	10
P1		JULY	18
FA		OCTOBER	15
C		OCTOBER	15
L10	5	OCTOBER	17
89425	16	OCTOBER	18
89619	5	NOVEMBER	20

MEAN $\pm$ S.D. 19 $\pm$ 4 ng/ml

S.E.M. = 1 ng/ml

TABLE 4.3.

eFSH CONCENTRATION IN SERUM SAMPLES SUBMITTED DURING OCTOBER FROM 21 GELDINGS OF KNOWN OR UNSPECIFIED AGE

ALL SAMPLES TAKEN IN OCTOBER.

GELDING	AGE IN YEARS	eFSH ng/ml
SQUIRE	6	40
JETHRO		39
WILLIAM		33
TOMMY		32
SARN		32
BEN	17	30
IAN		31
BARGAIN	5	29
OSCAR		27
DIRK	14	26
BRIAN		26
GOODY		24
ALEX		24
TRASH		22
SMOKEY	10	20
TIPPERARY		19
PRINCE		18
ROBBIE		15
TOBY		14
DRYSDALE		14
BOFFIN		13
MEAN $\pm$ S.D.		25 $\pm$ 9 ng/ml

S.E.M. = 2 ng/ml

4.3.5. Circulating peripheral FSH concentration and testicular histology

Table 4.4. allowed a comparison between testicular histology and peripheral FSH concentration. The number of nuclei lining the tubules, the tubule diameter, and the occurrence of spermatozoa within the tubules, was similar for all the normal stallions (plate 4.1.), despite a considerable age range. Peripheral FSH concentrations ranged between 10ng/ml and 28ng/ml. Tubule characteristics and FSH concentration appeared not to be correlated in this group of stallions.

The three unilateral inguinal cryptorchid stallions possessed scrotal testes with a histological appearance similar to that of the normal stallions (plate 4.1.). This group of unilateral cryptorchid stallions included one stallion where the tubule morphology of the "cryptorchid" testis was similar to that of the normal stallion testes (plate 4.2.). The concentrations of FSH estimated in a blood sample from this stallion was similar to the concentrations measured in the normal stallions.

In the other two cases in this group, the inguinal testes contained tubules which were narrower than the tubules in normal scrotal testes. These were lined by three or four layers of nuclei, and which were devoid of spermatozoa (plate 4.3.). The concentrations of FSH in these two cryptorchid stallions were also similar to those

estimated in blood samples from normal stallions.

The hemicastrate abdominal cryptorchid possessed one testis which contained narrow tubules. These were lined by three rows of nuclei, and which contained no spermatozoa (plate 4.4.). The concentration of FSH in the peripheral circulation of this animal was higher than the concentration estimated in blood samples from normal stallions and cryptorchid stallions that possessed scrotal testes.

TABLE 4.4.

TESTIS HISTOLOGY OF SEVEN NORMAL STALLIONS, THREE UNILATERAL INGUINAL CRYPTORCHID STALLIONS AND ONE HEMICASTRATED UNILATERAL ABDOMINAL CRYPTORCHID STALLION, AND PERIPHERAL CIRCULATING FSH CONCENTRATION

TABLE 4.4.

STALLION INDENTITY	TESTIS LOCATION LEFT	RIGHT	NUMBER OF NUCLEI LINING TUBULES LEFT	RIGHT	SEMINIFEROUS TUBULE DIAMETER; µm. LEFT	RIGHT	% TUBULES WITH SPERMATOZOA LEFT	RIGHT	PERIPHERAL FSH CONCENTRATION ng/ml.
JLA	SCR	SCR	12	12	41.5	38.5	100	80	14
JLB	SCR	SCR	13	13	44	44	80	80	10
W1	SCR	SCR	11	14	36.9	42.3	100	90	23
W2	SCR	SCR	12	13	39.4	40.9	90	100	28
89619	SCR	SCR	10	9	38.4	38.6	90	70	20
LAD	SCR	SCR	13	13	38.6	40.6	100	80	17
95514	SCR	SCR	12	13	42.7	44.4	100	90	18
92874	SCR	ING	13	15	38.8	37	100	80	26
82646	ING	SCR	4	13	19.7	32.4	0	100	19
95367	SCR	ING	11	3	40.7	22.7	100	0	22
92837	ABD	-	3	-	24.6	-	0	-	52

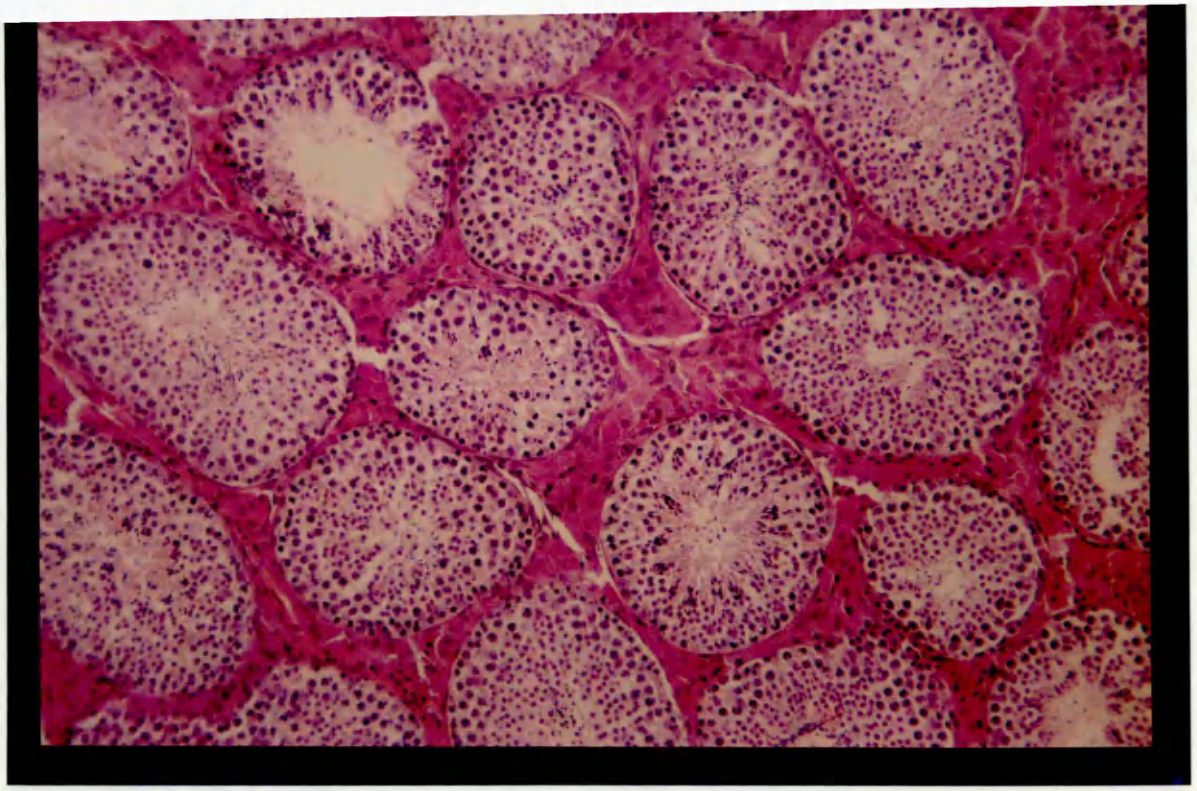


PLATE 4.1.
HISTOLOGICAL SECTION PREPARED FROM LEFT SCROTAL TESTIS OF CASE 95514

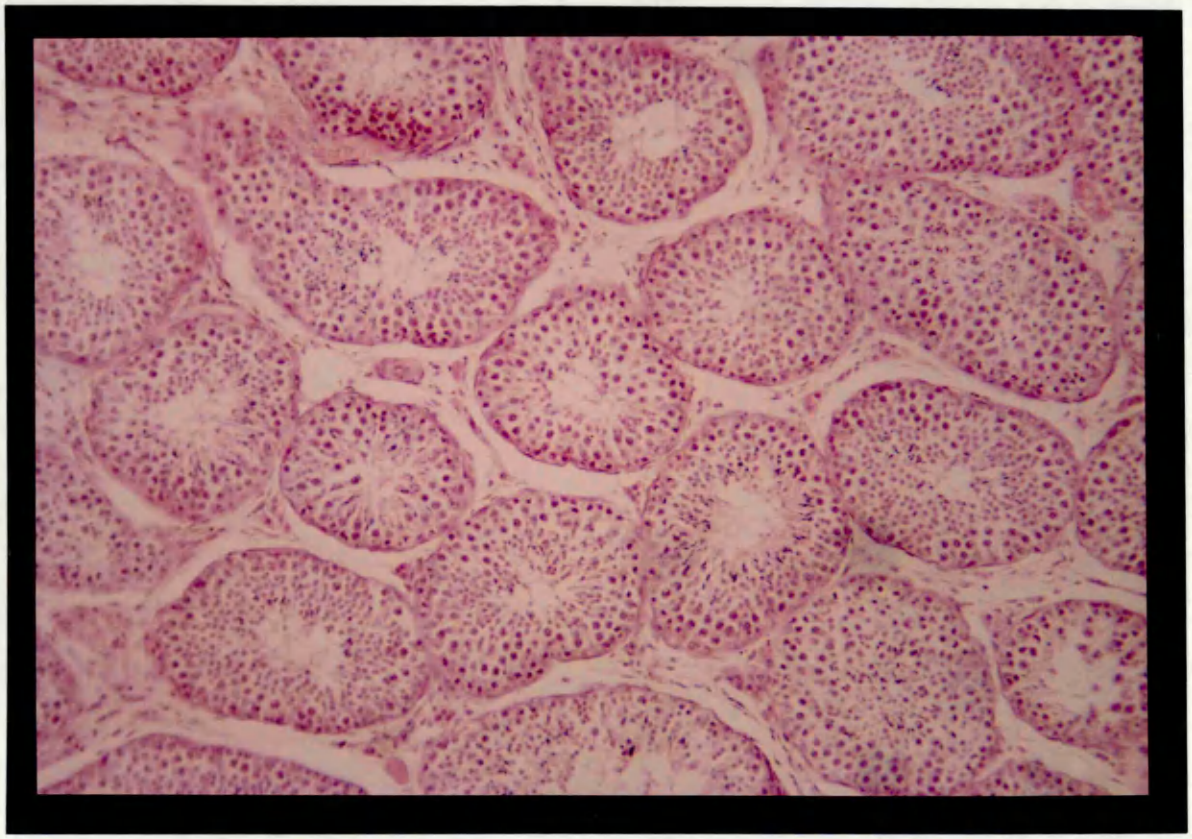


PLATE 4.2.
HISTOLOGICAL SECTION PREPARED FROM RIGHT 'INGUINAL' TESTIS OF CASE 92874

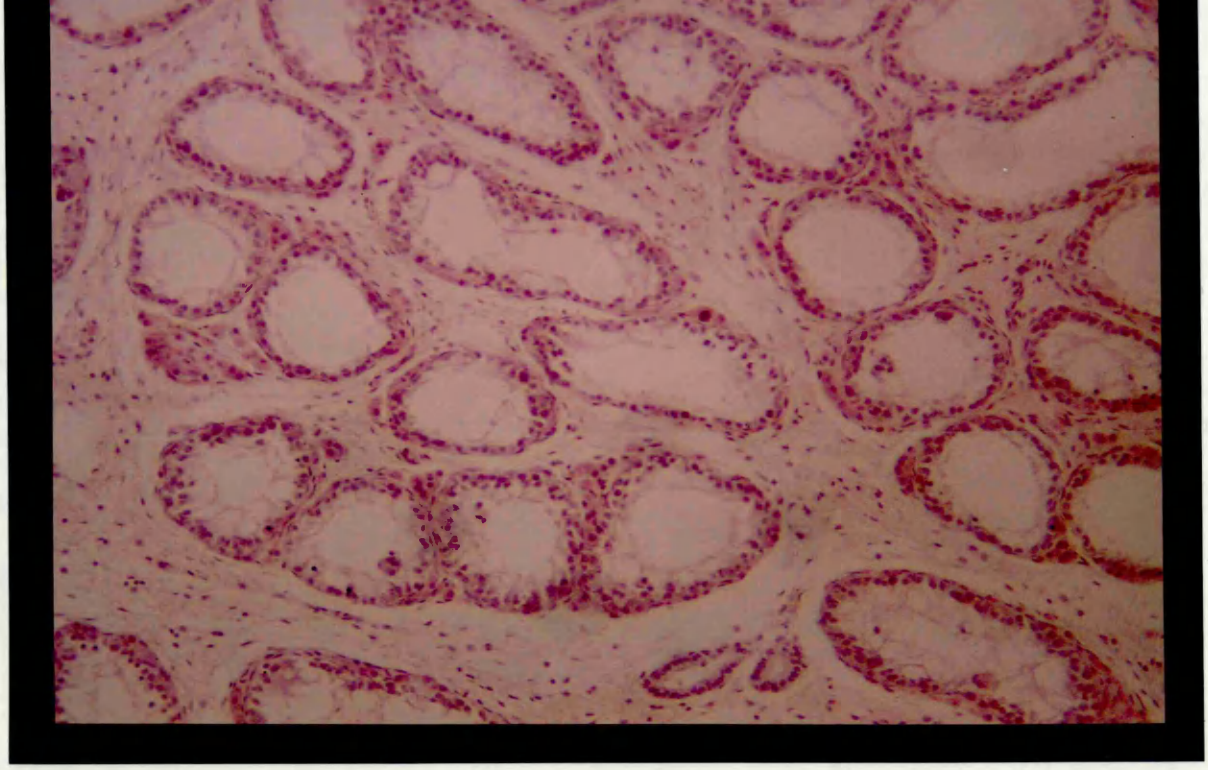


PLATE 4.3.
HISTOLOGICAL SECTION PREPARED FROM THE LEFT INGUINAL TESTIS OF CASE 82646

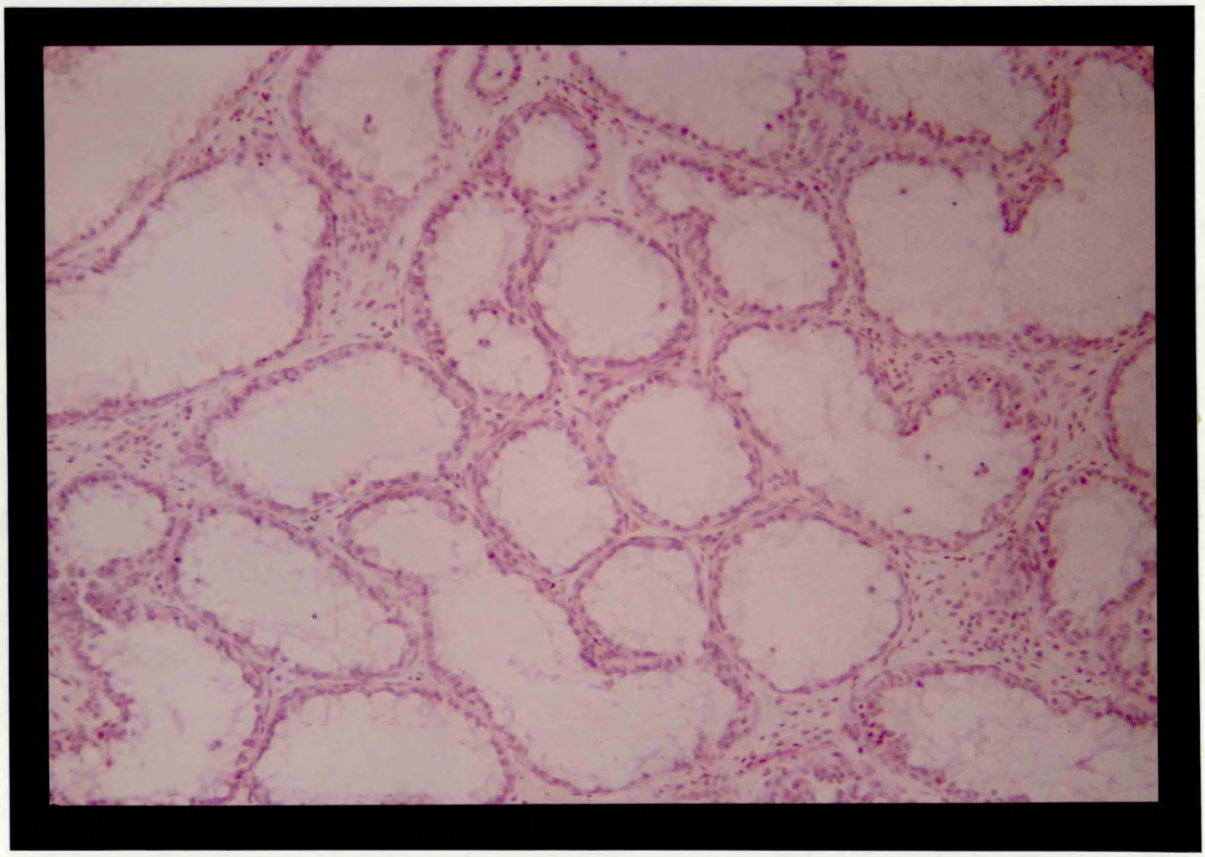


PLATE 4.4.
HISTOLOGICAL SECTION PREPARED FROM THE LEFT ABDOMINAL TESTIS OF CASE 92837

4.4. DISCUSSION

The reproductive hormones in male animals are secreted in various recognised patterns. One such pattern is a pulsatile pattern, in which rapid 2 to 8 fold rises in concentration occur, over periods of 5-10 minutes (Ellis and Desjardins, 1982). LH in the ram (D'Occhio, Schanbacher and Kinder, 1982) and bull (Schanbacher, 1982) is secreted in this fashion. Another recognised pattern of secretion is an episodic pattern, where changes in concentration of similar magnitude occur over 3-6 hour periods. Testosterone secretion in the ram and bull occurs in this way.

In this study the samples taken at ten minute intervals from three stallions revealed no evidence of pulsatile changes in FSH concentration (figure 4.1.). No evidence of a pulsatile pattern of secretion has been reported, as yet, for FSH secretion in the normal male of any species. However, samples taken at hourly intervals from these three stallions did show an episodic pattern of secretion (figure 4.3.), similar to that described previously for testosterone (section 2.3.2.), but with a lower amplitude. The frequency with which these episodes occurred, and the times of day at which they occurred, appeared to vary at random in the different stallions at different times of the year. Similar episodic patterns have recently been reported by Thompson et al (1985). Such episodic elevations in the peripheral FSH concentrations of

these stallions could be the result of episodic changes in either FSH catabolism by the liver, or of changes in FSH secretion by the pituitary.

FSH catabolism may be hampered by competition for binding sites on liver cells from other glycoproteins, or may be hampered by liver dystrophies, hepatitis, or cirrhosis (Irvine, 1979). However, such effects are unlikely to occur episodically.

When considering changes in pituitary release of FSH as an explanation of the episodic nature of FSH secretion, we must accept that various factors may affect pituitary FSH secretion. One such factor is that pituitary FSH release is controlled by GnRH release from the hypothalamus. A pulsatile pattern of GnRH release has been recorded in the ewe, (Levine et al 1981) and monkey (Carmel, Araki and Ferin, 1976). If similar pulsatile patterns of GnRH secretion occur in the stallion, they could be responsible for an episodic pattern of FSH secretion by the pituitary.

Pituitary secretion can also be affected by feedback mechanisms, and the peripheral concentration of other circulating plasma hormones (Thompson et al 1979a). Testosterone secretion in the stallion occurs in an episodic fashion (2.3.2.), and thus testosterone could be responsible for episodic pituitary inhibition, and so the patterns of FSH secretion observed in this study.

However, results obtained by researchers in other

species tend not to support either of these explanations. In the ewe, where pulsatile GnRH secretion has been observed, stable FSH levels have been recorded. In addition, exogenous testosterone administration to geldings induces pituitary inhibition over a period of days, rather than minutes (Thompson et al 1979a).

One other explanation for the pattern of FSH secretion found, could be that FSH synthesis and release are controlled by the pituitary cells themselves, and that the processes involved occur in an episodic fashion.

If this episodic pattern of FSH release is of importance, it may be associated with the mechanism by which the pituitary controls the processes of gonadal function, associated with the action of FSH. Such messages, coded into changes in episode frequency, could not be distorted by changes in liver FSH catabolism as easily as messages coded by prolonged changes in FSH levels could be.

The amplitude of the FSH episodes were small when compared to the amplitude of the episodic changes in testosterone concentration in the stallion (2.3.2.). Therefore the FSH concentration in single blood samples taken at random from stallions during the day, appear to be reasonably representative of the daily average FSH concentration in that animal.

In two of the three stallions studied in detail, the results of blood samples taken during different months of the year showed no definite seasonal pattern in

peripheral FSH concentrations (Max, figure 4.5., and Dick, figure 4.5.). A shallow seasonal pattern was only observed in one aged stallion (Steptoe, figure 4.6.), with FSH levels falling slightly during the winter, and rising during the spring and summer months. Although too few animals were studied to allow definite conclusions to be drawn, these results suggest that seasonal changes in FSH levels may not be as universal in the equine population as is implied in the literature (Harris, Irvine and Evans, 1983; Burns et al 1984b; Johnson and Thompson, 1983). Previous reports have described the winter months as a period of sexual rest for the stallion, with low peripheral FSH levels being associated with reduced rates of spermatogenesis in the testes (Harris, Irvine and Evans, 1983; Johnson and Thompson, 1983; Burns et al 1984b). These reduced spermatogenic rates are considered to cause the production of little semen of poor quality at this time (van der Holst, 1975; Pickett, Faulkner and Voss, 1975c; Johnson and Thompson, 1983; Harris, Irvine and Evans, 1983). Several environmental factors have been thought to be responsible for controlling these seasonal changes. These include changes in daylight length, in nutrition, in climate, and in sexual stimulation. The three stallions investigated in this study were housed during the winter, received an adequate diet, and were subjected to urban lighting during the night. They were also used regularly for natural service and semen collection. Therefore, their

failure to demonstrate a seasonal pattern of FSH secretion could be because they were not exposed to changes experienced in a more "natural" environment. However, other studies in which low winter levels of FSH were recorded were conducted with stallions maintained at commercial studs (Harris, Irvine and Evans, 1983; Burns et al 1984b).

Contradictory evidence suggests that changes in photoperiod may (Burns et al 1984b), or may not (Burns et al 1982), influence FSH secretion. Weight of evidence suggests that photoperiod has little effect, which suggests that the light exposure of the stallions in this study was not responsible for the aseasonality in FSH secretion observed here. Thus, the reasons why some, but not all, stallions demonstrate a seasonal FSH pattern has yet to be established. Such individuality has not been reported for other species where seasonal changes in male reproductive potential are accompanied by seasonal changes in FSH levels (Sanford, Palmer and Howland, 1977; Mirarchi et al 1978).

The aged stallion, Steptoe, consistently yielded blood samples containing higher FSH concentrations than were present in any of the other stallions (figures 4.3. and 4.6.). Other researchers have reported a correlation between stallion age and peripheral FSH levels, and the higher levels seen in this aged stallion may reflect this trend (Burns et al 1984b; Johnson and Thompson, 1983).

Elevated baseline levels of FSH in the peripheral circulation are associated with either reduced FSH catabolism, due to liver dysfunction, or are associated with increased FSH secretion by the pituitary. As there was no evidence of liver dysfunction in the aged stallion in this study, and as there is no evidence that liver failure is endemic in the older stallion population, increased pituitary FSH secretion would appear to be responsible for this age-related effect. Possible causes of increased pituitary FSH secretion in ageing stallions include; spontaneous hypersecretion by the gonadotrophe cells, increased GnRH secretion by the hypothalamus, increased sensitivity of the gonadotrophe cells to normal levels of GnRH, reduced steroid hormone and inhibin secretion by the testes, or reduced sensitivity to these inhibiting hormones. There have been no reports of studies into changes of gonadotrophe cell sensitivity with age, or reports of increased GnRH secretion in the older stallion. Secretion of testosterone, and other steroids, is not correlated with age, but there is evidence to suggest that the rate of spermatogenesis does slow slightly, in the aged stallion (Johnson and Thompson, 1983). If this slowing was associated with reduced Sertoli cell activity, and reduced inhibin secretion, it could offer one possible explanation for this ageing effect. A similar ageing pattern has not been observed for the other gonadotrophin, LH, and elevated FSH levels are not associated with ageing

in other species. Further studies are required to clarify the mechanism and significance of this correlation in the stallion.

When blood samples from a larger number of different stallions were analysed (table 4.2.), it appeared that most of the stallions yielded samples with concentrations within the range for the younger stallions studied in detail. However, further studies are required to investigate whether higher levels in some stallions reflected an age effect, or some specific pathology of the testes or liver.

The samples taken at ten minute intervals from the gelding Bonzo demonstrated a pulsatile pattern of secretion (figure 4.2.). The samples taken at hourly intervals also demonstrated an episodic pattern of secretion (figure 4.4.). The pulses had a higher amplitude than the episodes, and appeared to occur both during, and between, the episodes of increased peripheral FSH concentration. Such changes were larger than those observed in the three stallions. As oestradiol has been shown to reduce pituitary responsivness to GnRH (Thompson et al 1979a), these larger variations in peripheral FSH concentration in the gelding may reflect the low levels of oestradiol present in the circulation of the castrate. A similar increase in FSH concentration variability is seen in the ram (Schanbacher and Ford, 1977) and rat (Caraty, 1983) following castration.

In addition to these differences in pattern of secretion, there was also a significantly higher mean FSH concentration in the samples collected from the gelding compared to those collected from the stallions (figures 4.1., 4.2., 4.3., and 4.4.). Such changes in pattern, and baseline level of secretion, have been reported previously in the stallion (Thompson et al 1979a) and other species, following castration (Caraty, 1983; Schanbacher and Ford, 1977). The elevated baseline levels are thought to result from the release of the pituitary from the inhibitory effects of inhibin, and the steroid hormones secreted by the testes of the entire animal (Schanbacher and Ford, 1977).

Samples taken at different times of the year from the same gelding showed more variation in FSH concentration than those taken from the stallions (figure 4.7.). This could have been due to either the less frequent sampling of the gelding, or the greater hourly variation already described. The FSH concentration in samples from the gelding Bonzo demonstrated a shallow seasonal trend, with slightly lower FSH levels in the winter. This trend was similar to the pattern observed in the stallion Steptoe, in the earlier study, and demonstrates that seasonal changes in FSH seen in some stallions may not be dependent upon the presence of steroid hormones. Further work is needed to clarify the mechanism of seasonal changes in FSH secretion in the stallion, but these findings in one gelding suggest

that the changes in the testes associated with season may be controlled by seasonal changes in FSH, and that changes in the testes are not inducing the changes in pituitary FSH secretion.

The results from this one gelding suggested that FSH estimation could be used to differentiate samples taken from stallions and geldings, as significantly lower levels were present in the stallion samples. However, when samples taken from a number of different geldings were estimated, many contained FSH concentrations similar to those found in samples from stallions (table 4.3.). Since the age of these geldings differed markedly, this might indicate that the postcastration rise in FSH is not maintained indefinitely in the horse, and that over the years following castration the pituitary adapts to the absence of inhibitory hormones, and FSH secretion returns to precastration levels. Such adaptation could result from a decline in GnRH secretion, a decline in sensitivity of the pituitary to GnRH, or an intrinsic slowing of pituitary metabolism in the long-term gelding. A similar decline in LH levels has also been observed in the male equine, during the 2 to 3 years following castration (Irvine and Alexander, 1982), and the mechanism of adaptation may be common to both gonadotrophins. Further work is required to investigate how this adaptation, which may be unique to the horse, occurs.

This effect has important implications, as it prevents the use of FSH estimation for the discrimination

between stallions and geldings. Long term castrates may contain levels of FSH similar to those in young stallions, and less than those in older stallions. It may also suggest that cases of infertility which were initially associated with elevated FSH levels, may present years later with normal-stallion levels of FSH, as a result of similar pituitary adaptation.

Although samples taken at intervals over one hour from cryptorchid stallions contained similar FSH concentrations, relatively few samples were available for these studies. Therefore, further studies are required to establish whether single samples accurately reflect the daily mean FSH levels in these animals. Assuming that single samples did reflect the average daily FSH concentration allowed a comparison of testis morphology and FSH levels to be made in these animals.

The morphology of testicular parenchyma from 7 normal and 4 cryptorchid stallions, were compared by examination of histological sections, using a technique described by Arighi et al (1984). The three parameters measured were; the number of nuclei lining the seminiferous tubules, the diameter of the tubules, and the percentage of tubules containing spermatozoa (table 4.4.). There was little difference in cellularity, tubule diameter, or occurrence of spermatozoa, in sections taken from normal entire male equines whose ages ranged from between 9 months and 16 years. Reports in the literature indicate

that testis weight and width increases with age (Burns et al 1984b; Thompson, Pickett and Squires, 1979b; Cox, 1982b). The results obtained in these investigations suggest that such increases do not result from an increase in the diameter, or the cellularity, of individual seminiferous tubules. Instead they must result either from increases in tubule length, increases in the number of tubules per testis or from an increase in the mass of interstitial tissue within the testicular parenchyma. Johnson and Neaves (1981), and Johnson and Thompson (1983) have also reported that tubule diameter does not vary with age, but that tubule length and volume does increase with age.

FSH concentrations in all these normal entire animals were similar to those reported in the younger stallions, discussed previously. The small differences in circulating FSH concentration between these cases, and the small differences in the histological appearance of the testicular parenchyma sections from these cases, appeared not to be correlated. These differences could be accounted for by errors in the histological assessment of the slides, due to the small number of true cross sections available for study, and the difficulty in assessing the number of rows of spermatogonia lying along the X and Y axes of these tubules.

The results of the examination of the sections from testes taken from the inguinal region, demonstrated

that the degree of developmental arrest was related to the position of the abnormally sited testes.

In one unilateral case, one testis was located under the skin, at the exterior inguinal ring (the "high-flanker" position) and the other testis was located in the scrotum. Examination of histological sections from both these testes produced similar results, and moreover, these results were similar to those observed for normal stallions. Therefore, histologically the sections from this one high flanker testis and the scrotal testes were indistinguishable using these techniques. Such findings have been reported previously (Bishop, David and Messervy, 1964). This particular animal yielded blood samples which contained concentrations of FSH similar to those found in blood samples from the normal stallions.

Therefore here the histological appearance of the testes were consistent with normal circulating plasma FSH levels.

The results of the examination of the sections taken from testes retained within the inguinal canal, or at the interior inguinal ring, were different from those recorded for either the contralateral scrotal testes in the same cases, or the normal stallions. The retained testes contained tubules with narrower diameters, containing no spermatozoa. These were lined by fewer cells, compared with the normal scrotal testes. This histological appearance of cryptorchid testes has been reported

previously (Arighi et al 1984; Arthur, 1961; Bishop, David and Messervy, 1964). In addition, the histological appearance of these testes removed from the inguinal canal, or internal inguinal ring, was similar to that seen in the histological sections from an abdominal testis, recovered from the only hemicastrate cryptorchid stallion studied. This suggests that in this series of cases, inguinal and abdominal retention resulted in the same degree of arrest of testicular developement.

The histological appearance of sections prepared from the contralateral scrotal testes of the unilateral inguinal cryptorchids, were similar to those of the normal stallions. When the circulating peripheral concentration of FSH in these particular unilateral cryptorchid stallions was estimated, it was apparent that despite the presence of a cryptorchid testis, these animals produced samples with FSH concentrations similar to those found in normal stallions.

Adaptation of the pituitary following prolonged hypersecretion of FSH apparently occurs in the gelding some time after castration, as has already been discussed. However, these animals were probably too young for these low FSH levels to be the result of a similar adaptation of the pituitary. Other explanations for the low levels of FSH in these particular animals could be that either inhibin secretion by the Sertoli cells in the inguinal testes is continuing normally, despite an arrest of spermatogenesis,

or that a contralateral increased secretion of inhibin by the scrotal testis, has compensated for reduced secretion by the inguinal testis. Surgically induced bilateral abdominal cryptorchidism in the ram and rat, is always associated with elevated FSH levels (Schanbacher and Ford, 1977; Caraty, 1983), and this is thought to result from reduced inhibin secretion by cryptorchid testes. Therefore in the two cryptorchids studied here, the latter explanation, that normal stallion levels of circulating plasma FSH result from contralateral hyperplasia of the scrotal testis, appears to be the most likely explanation. A contralateral increase in scrotal testis size, has been observed in unilateral cryptorchids by other workers (Arthur, 1961; Cox, 1982b). Although no evidence of a contralateral increase in tubule diameter, or cellularity was seen in these cases, hyperplasia associated with an increase in the number or length of the tubules, similar to the age and seasonal changes reported by Johnson and Thompson (1983), may still have occurred. This could increase the mass of sertoli cells in the contralateral scrotal testis, and result in an increased amount of inhibin production by the scrotal testes, returning FSH secretion to normal stallion levels. Further work is needed to clarify the mechanism of such contralateral hyperplasia.

Elevated levels of FSH were present in the blood samples taken from the hemicastrate animal with one abdominal testis. This abdominal testis appeared to have a

histological morphology similar to the two inguinal testes already described. The higher circulating plasma FSH concentration in this hemicastrate cryptorchid, may reflect the absence of a contralateral scrotal testis in this case. Hemicastrate cryptorchid stallions have sex steroid concentrations similar to those of normal stallions (table 2.3.) (Cox et al 1973; Cox, 1975b; Crowe et al 1977). Therefore, the higher FSH levels in this hemicastrate abdominal cryptorchid provides some evidence that peripheral FSH concentrations in the stallion are controlled by hormones other than the sex steroids, that are not secreted in the normal way by cryptorchid testes.

More cases are required before definite conclusions can be drawn, but this preliminary study suggests that a cryptorchid stallion with no scrotal testes present may produce blood samples containing FSH concentrations similar to those found in normal geldings. Thus, FSH estimation cannot be used to identify whether or not testicular tissue is present in animals with stallion-like behaviour. However, one result of importance is that in stallions the absence of normal testicular tissue may be accompanied by elevated FSH levels. This suggests that FSH estimation could be used to monitor spermatogenesis, and/or aid the diagnosis of the cause of infertility in stallions. In man an inverse correlation is reported between the histopathology in biopsy samples of testicular parenchyma taken from infertile men, and peripheral FSH concentration

(de Kretser, 1979; Hunter et al 1974; Wu et al 1981). Such studies result from the accumulation of a large amount of data from biopsy samples. Widespread biopsy of normal and infertile stallions has not been carried out, and as few infertile stallions are castrated each year, similar data for the stallion will not become available. The possible use of FSH estimation for the assessment of fertility and infertility in the stallion, must therefore be deduced from more extensive studies using the bilateral cryptorchid as a model of infertility associated with germinal epithelial aplasia, or by comparing other markers of spermatogenesis with FSH levels. Studies in man have suggested an inverse correlation bewteen sperm count, and peripheral FSH concentration in infertile men (Purvis et al 1975; Hunter et al 1974; Rosen and Weintraub, 1970;Wu et al 1981). Therefore, the value of FSH assay of blood samples for identifying spermatogenic function in the stallion, may be assessed from similar studies in the equine.

CHAPTER FIVE

SEMEN CHARACTERISTICS AND PERIPHERAL FSH CONCENTRATION, AND THE EFFECT OF VASECTOMY AND CASTRATION UPON PERIPHERAL FSH CONCENTRATION

CHAPTER FIVE

SEMEN CHARACTERISTICS AND PERIPHERAL FSH CONCENTRATION, AND THE EFFECT OF VASECTOMY AND CASTRATION, UPON PERIPHERAL FSH CONCENTRATION

5.1. INTRODUCTION

Initially an efficient and reliable method of semen collection, and evaluation, was established. This required the construction of a phantom mare, to allow semen collection to take place when an oestrus mare was not available.

Subsequently, semen samples were collected at one week intervals from two stallions. The quantity and quality of the semen ejaculated was compared with peripheral FSH concentrations in these stallions, sixty days prior to the date of semen collection.

Finally the effect of vasectomy, and castration, upon peripheral FSH concentration was investigated.

5.2. MATERIALS AND METHODS.

5.2.1. Semen collection and estimation of peripheral FSH concentration

5.2.1.1. Animals used

Max and Steptoe have already been described (section; 2.2.3.). Both stallions were housed in loose boxes, received a concentrate ration (Spillers, Horse and Pony cubes), and ad lib hay and water. Each morning the stallions were used to tease mares. During December 1983, both stallions were used for natural service. From January until June, semen samples were collected weekly, unless weather conditions prevented access to the phantom mare. Both stallions were turned out to grass in June, and could not be caught for regular collections of blood, or semen during the summer. During the following autumn semen samples were again collected from the stallion Max.

5.2.1.2. Artificial vagina construction

A modified Hauptner artificial vagina (AV) consisted of three components; a permanent water jacket, a semen collection vessel, and a disposable inner liner and cone.

a) Permanent water jacket;

The outer case of the A.V. was a rubber tube, with one open end, and one closed end, perforated by an eccentric orifice (Plate 5.1.). A Hauptner, latex rubber liner was pulled taut through the case and, after checking that the liner was not twisted, the 15cm of excess liner

was folded back over the rigid case and secured in place with two 3cm wide, Hauptner rubber bands. Two leather straps with a carrying handle between them were then fixed around the rigid case, helping to secure the latex liner in place. The handle was orientated so that the eccentric end orifice of the rigid case was ventral when the AV was held in the right hand. Once assembled this structure was not dismantled between collections.

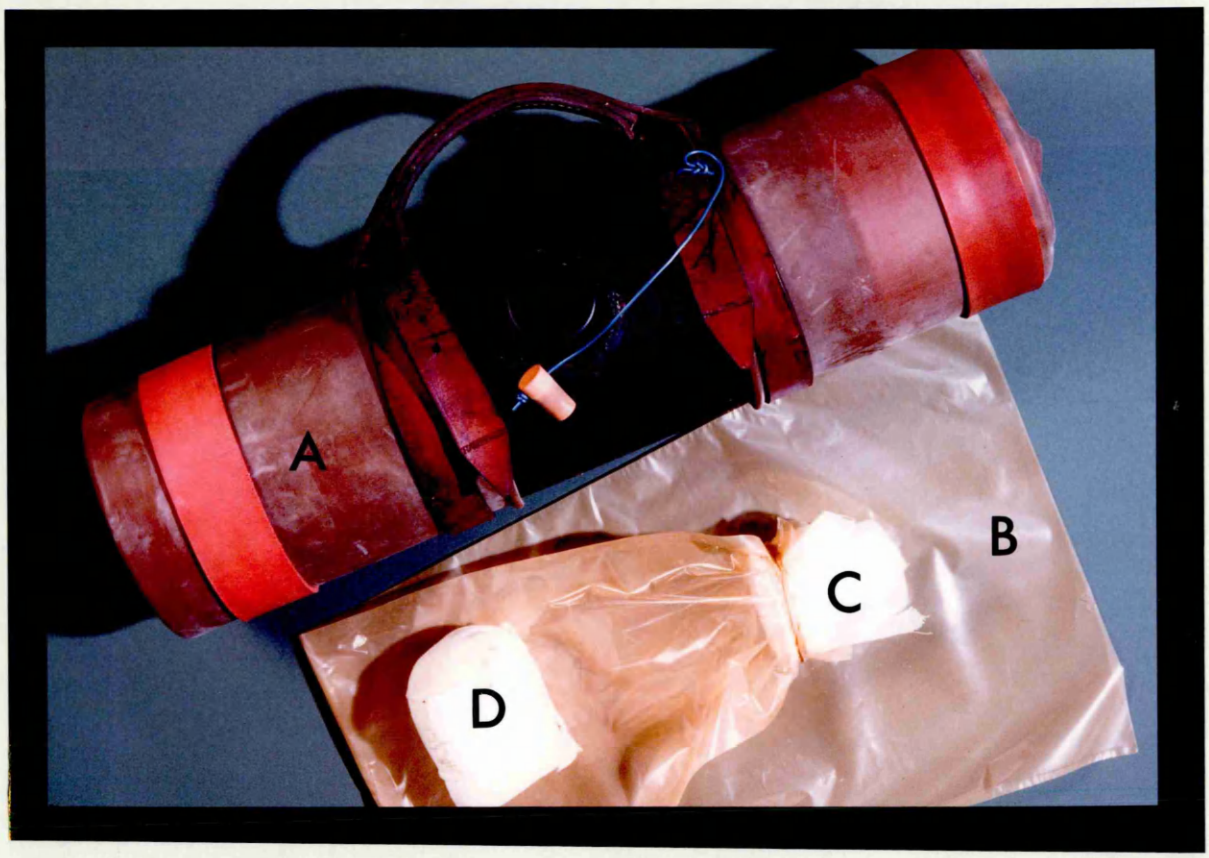


PLATE 5.1.

THE MODIFIED HAUPTNER ARTIFICIAL VAGINA USED FOR SEMEN COLLECTION

A = PERMANENT WATER JACKET

B = DISPOSABLE INNER LINER

C = COLLECTION BEAKER AND FILTER ASSEMBLY

D = MUFF

b) Collection vessel

This was a 100ml plastic beaker (Gesaminenge, 100ml), washed thoroughly with distilled water, and fitted with a gel-excluding filter. This filter consisted of a layer of tissue (Kleenex Medical Wipe), covered by a single layer of 4" surgical bandage (plate 5.1.).

c) Disposable inner liner and cone

A plastic rectalling glove (Supergluv, Polysem) was trimmed to form a combined disposable inner liner and cone (plate 5.1.). This was attached to the collection vessel, drawn through the permanent water jacket, folded back at the open end of the AV, and then secured in place with a Hauptner, 3cm, rubber band.

5.2.1.3. Preparation of the AV for use

The water jacket was filled with water, and the temperature adjusted to 42-44^{o}C. The volume of water within the jacket was regulated by inserting a hand into the open end of the AV, with the water inlet open, and then sealing the inlet. The exact amount of displacement, optimal for each stallion, was arrived at by trial and error. After the jacket was filled, a polyurethane foam muff was pushed over the collection beaker. The inner liner was lubricated with 1ml of Lubrol (Dales Pharmaceuticals), smeared over as much of the liner as possible.

A fresh inner liner was used each time a stallion's penis entered the AV, whether he ejaculated or not.

5.2.1.4. Construction of a phantom mare

An artificial mare was constructed, as illustrated in plate 5.2.. A railway sleeper was rested on two 6" fence posts, and secured with iron brackets. The ends and edges of this sleeper were then covered with sections of steel radial tyres, and then padded out with straw. Once a "mare shape" was achieved, the straw was covered with a skin of sack-cloth, and then a coat of brown carpet.

The height and girth of the phantom were designed for the pony stallions, after reference to the girth and heights of mares successfully covered by them previously.

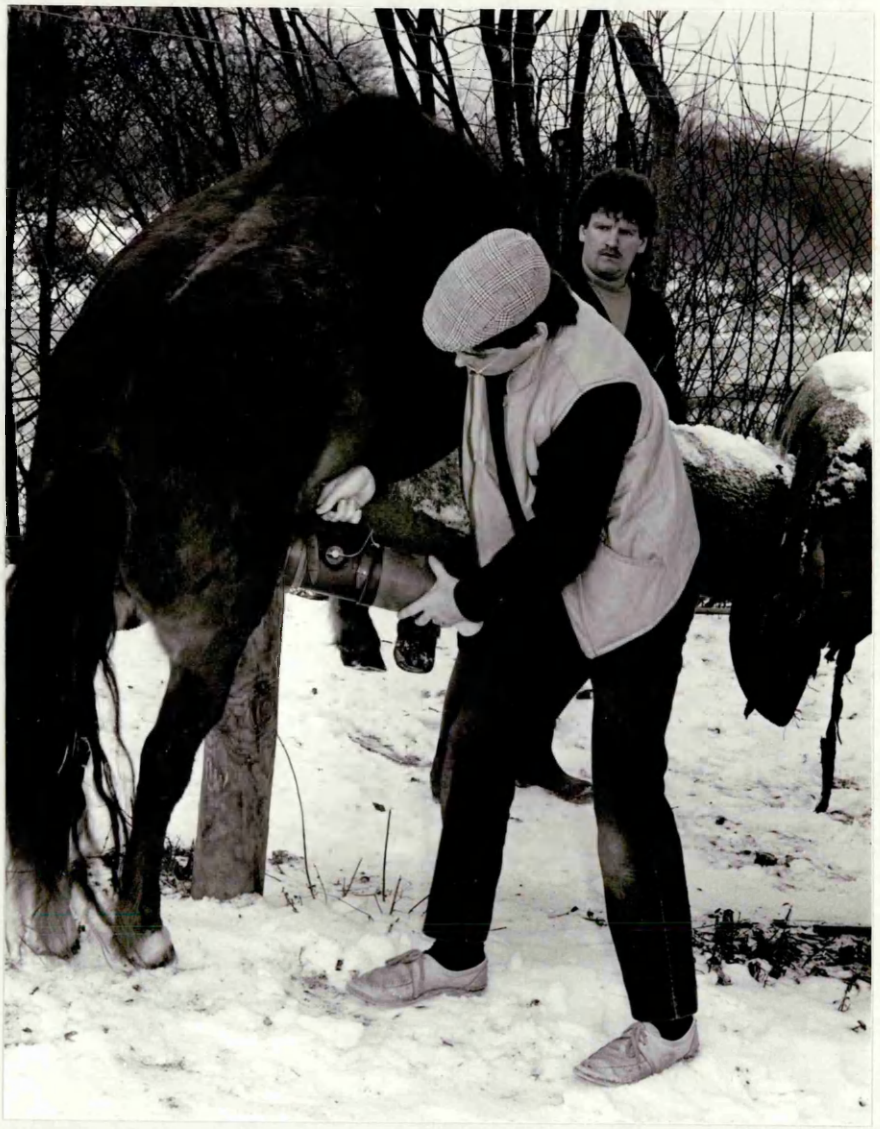


PLATE 5.2.

SEMEN COLLECTION USING THE ARTIFICIAL (PHANTOM) MARE

5.2.1.5. Method of semen collection

The stallion was teased across the "thorax" of the phantom mare, with the mare on the phantom's left, and the stallion on the phantom's right. The teaser mare was either a mare in oestrus (though not necessarily in standing oestrus), or any mare in dioestrus, that had stimulated an erection in the stallion at the morning teasing that day.

Once the stallion gained an erection, the mare was walked forward to a position parallel to the phantom, with her hindquarters at the level of the phantom's "thorax". The stallion was then walked round to the phantom's hindquarters, and walked into its rear. The stallion jumped the phantom, as his brisket contacted the phantom.

If the stallion failed to mount the dummy, the process was repeated until he did so. The number of mounts made by the stallion before ejaculation was recorded.

Once the stallion had mounted the phantom, the AV operator moved in close to the phantom, on the phantom's right side. Holding the AV in the right hand, the left hand guided the stallions penis into the AV. Pulsation of the urethra was detected manually, and once pulsations ceased the glans penis was emptied manually, and keeping the AV vertical, the semen was rushed to the laboratory.

5.2.1.6. Semen analysis

On arrival at the laboratory the AV was hung vertically above a shallow plastic tray. The collection beaker was then removed, and the fractions of the ejaculate

lying above the filter were allowed to drain into the tray. After removal of the filter, the collection beaker was placed in a water bath at 37°C. Subsequent analysis was performed upon the fraction of the ejaculate collected within this vessel. The following assessments were made;

a) Motility;

0.25ml of semen was added to 4.75ml of a 5% dried skim milk, 5% glucose (w/w), extender solution. After mixing a drop of this solution was placed on a warmed glass slide, and covered with a cover slip. The movement of the spermatozoa was observed under low power, and the percentage of spermatozoa demonstrating forward progressive motion estimated.

b) Volume;

The inner liner was removed from the AV, and any semen retained within its folds was "milked" into the collection tray. Similarly the filter was squeezed, and its contents added to the collection tray. The gel was removed from the mixture of first, second, and third, fractions in the tray, using a 10ml plastic syringe (B-D Plastipak). The gel volume was recorded.

The volume of gel-free semen ejaculated was calculated from the volume of gel-free semen in the collection tray, and from the volume of gel-free semen in the collection beaker.

c) Spermatozoan morphology;

Materials;

Nigrosin-Eosin stain;

The following were boiled for 20 minutes;

Nigrosin (Gurr)	1.25g
Eosin (Gurr)	0.05g
Trisodium citrate ($2H_2O$)	0.362g
distilled water	15ml

After filtration through No. 1. filter paper (Whatman) the solution was stored at 5 ^{o}C until use, within one month.

Methods;

A drop of semen was mixed with a drop of the warmed Nigrosin-Eosin stain, on a warmed slide. This was mixed, and smeared using a cover slip. After drying, the smears were fixed with D.P.X.(BDH), and a coverslip applied.

The smear was examined under a x100 magnification, oil immersion objective lens. 100 spermatozoa were counted. Spermatozoa that contained stain were recorded as dead, and spermatozoa that contained no stain were recorded as alive. The number of spermatozoa exhibiting the following abnormalities was also recorded.

Primary abnormalities;

Spermatozoa possessing; microheads, macroheads, mishapen heads, double midpieces.

Secondary abnormalities;

Spermatozoa possessing cytoplasmic droplets.

d) Number of spermatozoa ejaculated in the gel-free volume

The concentration of spermatozoa in the semen extended in dried-skim-milk/glucose extender, was estimated using a Coulter counter, model ZF, with a C1000 Channelyzer (Ferguson, 1976). Batches of extended semen samples were stored at -20^{o}C until counting. Samples were then randomised and counted in batches of 25 samples.

The extended semen was diluted 1 in 100 with Isoton (Coulter). Using an orifice tube of 70µm, a sample volume of 100µl, an aperture current (I) of 16units, and an attenuation setting of 0.5, size distribution curves were prepared for each sample counted. These curves were similar for all samples, with one slightly skewed peak at a relative threshold setting of 20. Threshold settings between 10 and 14 were used for all spermatozoa counts.

The number of spermatozoa per ml of the gel-free semen was calculated, allowing for the 1 in 20 dilution in extender. The total number of spermatozoa in the gel-free ejaculate was estimated from the total gel-free volume.

5.2.1.7. Blood sample collection

Blood samples were collected, and FSH concentrations estimated, as part of the work for the previous chapter (4.2.2.). The concentration of FSH in the peripheral circulation 60 days prior to the date of semen collection, were then compared with semen quality and quantity.

5.2.2. Vasectomy and castration

5.2.2.1. Animals used

Max; this stallion has already been described, in section 2.2.3..

Larry; a six year old, 11h Shetland pony stallion. Larry had not worked at stud prior to purchase. He was in good physical condition throughout the period of study.

5.2.2.2. Maintenance

Both stallions were housed in loose boxes, and received a ration of horse and pony cubes (Spillers), ad lib hay, and ad lib water. Max was used for weekly semen collection as part of the previous investigation.

5.2.2.3. Vasectomy

On 19th October 1984, Larry was vasectomised using the method of Selway et al (1977). Under general anaesthesia, a 2cm skin incision was made over the proximal part of the vas deferens of the right testis. After dissection of the dartus muscle, the common vaginal tunic was incised, and the proximal vas deferens exteriorised. After the mesorchium was incised adjacent to the vas deferens, two ligatures (2-0 silk) were placed around the exteriorised vas deferens at an interval of 2cm, and the section of vas deferens between the ligatures was resected.

The vas deferens of the left testis was treated similarly, through the same skin incision. The vaginal tunic was closed with a simple continuous suture of 2-0, Vycril.The skin incision was closed with simple interrupted

sutures of 2-0, Vycril.

The stallion received tetanus antiserum (Wellcome) immediately post surgery, and penicillin/streptomycin (Depomycin, Mycofarm), and oral meclofenamic acid (Arquel, Parke-Davis) for 5 days post surgery.

5.2.2.4. Castration

On 12th February Larry was castrated using a closed technique, described in section 4.2.5.2.. The ligated sections of vas deferens were excised along with the scrotal contents.

5.2.2.4. Blood sample collection

Blood samples were collected from the control stallion, Max, as described in section 4.2.2.. Blood samples were collected from Larry daily. This schedule was interupted occasionally, due to either the stallion's temperament, or the condition of the stallion's jugular furrows.

5.2.2.4. Pathological and histological examination of the testes

After castration the excised tissues were examined macroscopically, and sections of testicular parenchyma were prepared for histological examination, as described in section 4.2.5.3.

5.3. RESULTS

5.3.1. Semen quality and quantity and peripheral FSH concentration

Figure 5.1. demonstrates that on many occasions this stallion Steptoe ejaculated into the artificial vagina on his first mount of the artificial mare. More mounts before ejaculation were made during the first collections, and after interruption of regular collections in March. The total volume of the first and second fractions of the ejaculate varied more between collections than any of the other semen parameters studied. The motility of the spermatozoa and percentage of live spermatozoa varied little between collections or between months, except for one poor quality sample collected in March. The small variations in motility and live percent, appeared to follow similar patterns.

The number of spermatozoa with primary abnormalities was also relatively constant. The percentage of spermatozoa with secondary abnormalities was more variable, ranging from 10% to 60%. There was no discernable seasonal trend to this variation.

The number of spermatozoa ejaculated was relatively constant, 1 - 5 $x10^9$, and was not correlated with ejaculate volume. Many more spermatozoa were ejaculated in the first ejaculate collected in April,

following an interruption to collections in March.

Peripheral FSH concentrations were plotted so that FSH concentrations 60 days prior to semen collection could be compared with subsequent ejaculate quality and quantity. The shallow troughing of FSH concentration during the January, February, and March, did not correspond with any changes in semen quality in March, April, or May.

FIGURE 5.1.

STEPTOE, 1984

NUMBER OF MOUNTS BEFORE EJACULATION, SEMEN QUALITY AND QUANTITY, AND PERIPHERAL FSH CONCENTRATION

(The X axis of the FSH graph has been displaced 60 days to the right to illustrate the peripheral FSH concentration at the time the semen was produced)

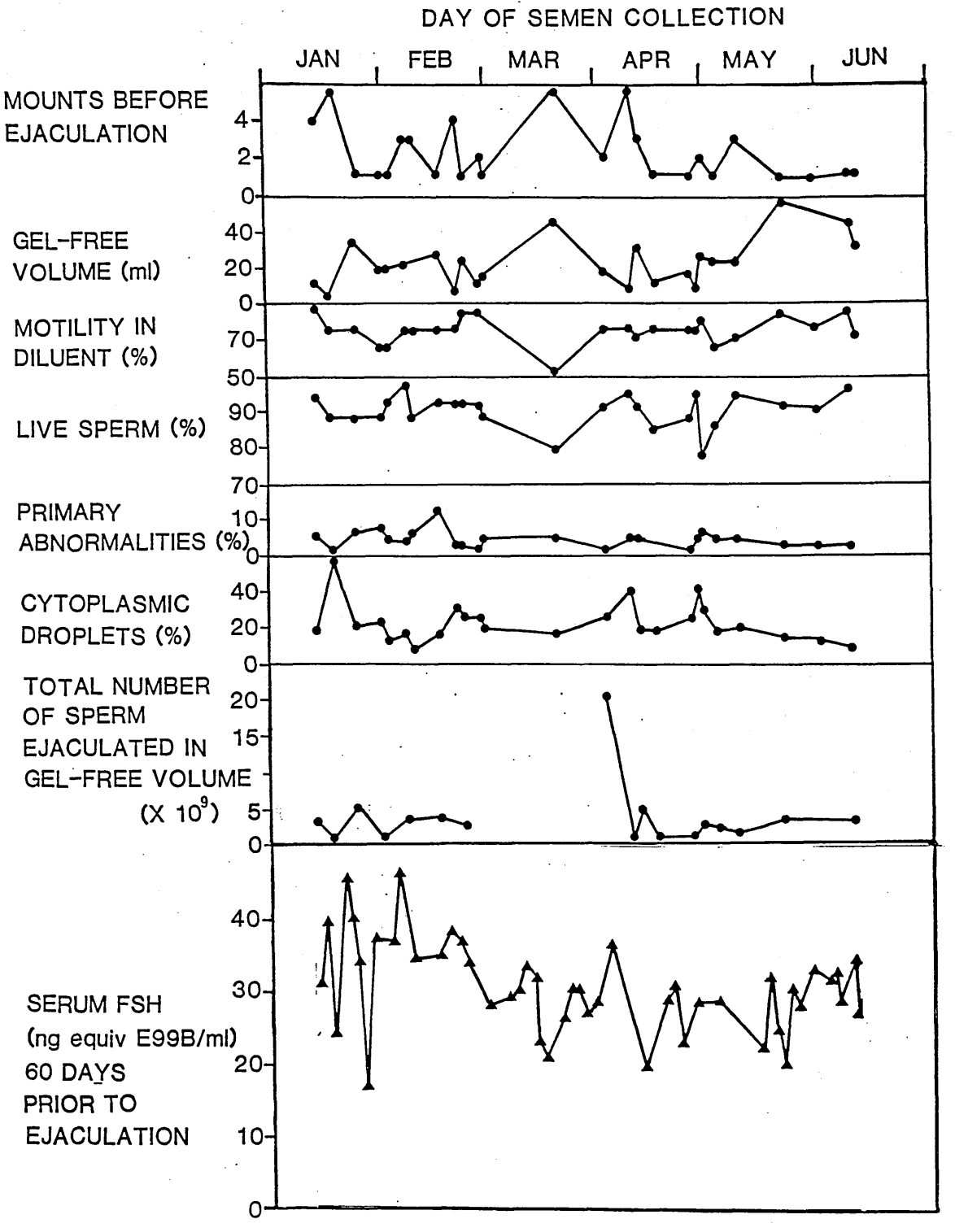


Figure 5.2. shows similar results for the younger stallion Max, collected over a longer period. This stallion persistently required at least two mounts before he ejaculated.

The combined volume of the first and second fractions of the ejaculates, varied considerably between collections, ranging from 5ml to 50ml. This gel-free volume was similar in January and February, in both years. Slightly larger volumes were ejaculated during October and November of the second year of study.

Motility varied between 50% and 90%, but differences in motility appeared not to be associated with the month of semen collection. A consistently high percentage of spermatozoa were unstained on microscopic examination, and deemed alive. Motility was not correlated with the percentage of spermatozoa alive.

A consistently small percentage of spermatozoa possessed primary or secondary abnormalities.

The total number of spermatozoa ejaculated varied considerably. Several long intervals occured between sample collections from this stallion. When collections were resumed large numbers of spermatozoa were ejaculated in the first samples collected. Sperm counts fell in subsequent collections. This effect was especially obvious in November and February.

Peripheral circulating FSH levels were relatively constant throughout the period of study, and appeared not

to be correlated to changes in semen quality.

On average the semen from the younger stallion Max (figure 5.2.), contained a higher percentage of live spermatozoa than the semen collected from the stallion Steptoe (figure 5.1.). The samples from the younger stallion also contained fewer spermatozoa with cytoplasmic droplets present. Max ejaculated more spermatozoa during those periods where collection intervals were similar for both stallions (Max in March, and Steptoe in April/May).

FIGURE 5.2.

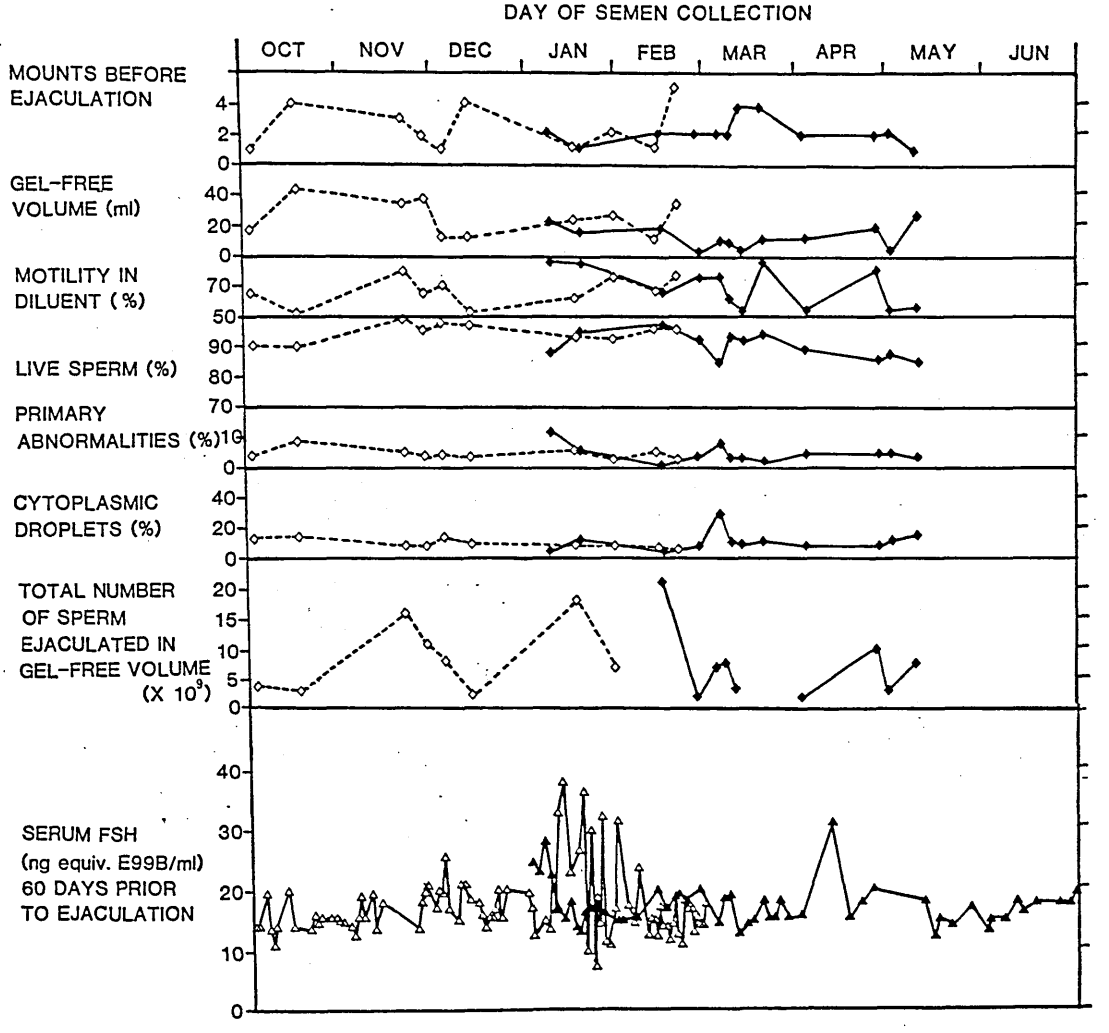
MAX, 1984 AND 1984/85

NUMBER OF MOUNTS BEFORE EJACULATION, SEMEN QUALITY AND QUANTITY, AND PERIPHERAL FSH CONCENTRATION

(The X axis of the FSH graph has been displaced 60 days to the right to illustrate the peripheral FSH concentration at the time the semen was produced)

SEMEN 1984 (◆—◆) AND 1984/1985 (◇- -◇)

FSH 1984 (▲—▲) AND 1984/85 (△—△)



5.3.2. Effect of vasectomy and castration upon peripheral FSH concentrations

The FSH concentration estimated in the blood samples from the control stallion Max, varied little during most of the period of study (figure 5.3.). Episodes of increased between-sample variation occurred especially during November and February.

Peripheral FSH concentrations were slightly more variable in the stallion Larry prior to vasectomy, than in the stallion Max (figure 5.3.). Mean concentrations were similar in both stallions. Vasectomy had no effect upon peripheral FSH levels. Following castration in February FSH levels rose dramatically during the following month, and were maintained at a significantly higher level ($P<0.05$) thereafter. More variation between samples occurred in this post castration period.

5.3.3. Examination of testes removed from Larry

The testes removed from the stallion Larry were of normal gross appearance, and normal consistency. The epididymides of both testes were very turgid, and contained semen filled cysts.

Histologically, the diameters of the seminiferous tubules within the testicular parenchyma, and the number of nuclei lining these tubules, were similar to those observed in the normal stallions studied in the previous chapter (table 5.1).

FIGURE 5.3.

MAX AND LARRY

CONCENTRATION OF FSH IN BLOOD SAMPLES COLLECTED FROM ONE CONTROL STALLION AND ONE STALLION BEFORE AND AFTER VASECTOMY AND CASTRATION

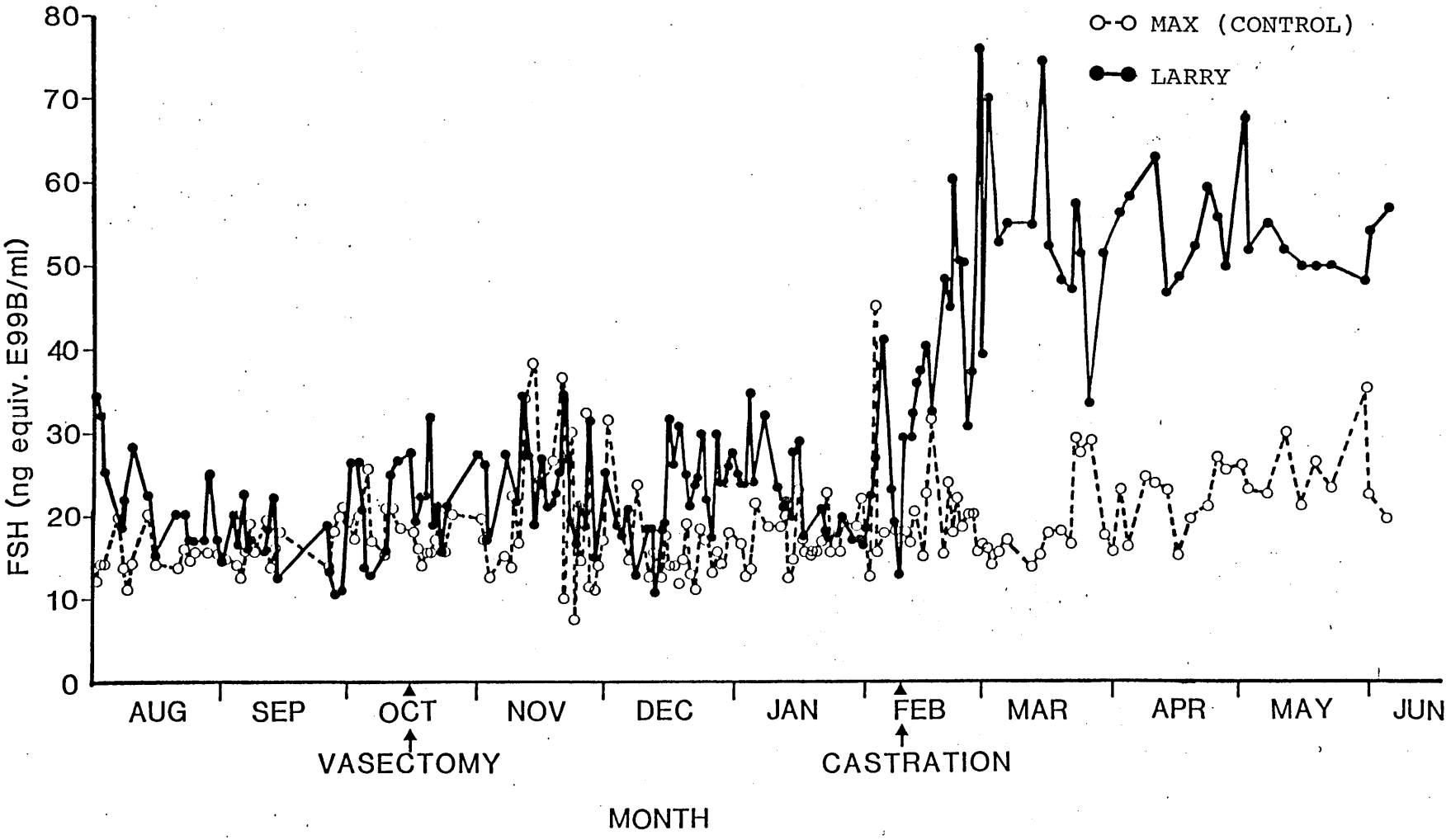


TABLE 5.1.

LARRY

RESULTS OF A HISTOLOGICAL EXAMINATION OF TESTICULAR PARENCHYMA TAKEN FROM A VASECTOMISED STALLION

	TESTIS	
	LEFT	RIGHT
NUMBER OF NUCLEI LINING TUBULES	15	15
SEMINIFEROUS TUBULE DIAMETER; μm	35.4	33
% TUBULES WITH SPERMATOZOA PRESENT	100	90

5.4. DISCUSSION

A variety of factors may influence the characteristics of an ejaculate, and adversely affect the quality of semen collected from a stallion. These factors include; (a) reduced spermatogenic rate, (b) overuse resulting in depletion of sperm reserves, (c) unilateral or bilateral obstruction of the efferent duct or deferent duct, (d) production of abnormal spermatozoa, and (e) the failure of maturation of spermatozoa. In addition the collection technique may itself alter the composition of the ejaculate. An untrained stallion rarely produces a representative semen sample when collected from using an artificial vagina, for the first time.

The characteristics assessed on examination of semen from domestic animals, and man are; initial motility, live/dead count, total sperm count and percentage of abnormalities. However, the relationship between any of these and fertility in the stallion, has yet to be established (Voss, Pickett and Squires, 1981; Voss, Pickett and Loomis, 1982; David, 1982; Pattie and Dowsett, 1982). Indeed, in some studies a high incidence of secondary abnormalities has been associated with higher than average fertility (Dowsett and Pattie, 1982), and stallions with only 60% normal spermatozoa have been reported to have normal fertility (Voss, Pickett and Squires, 1981; Bielanski et al 1982). However, studies relating to artificial insemination suggest that the total number of

motile spermatozoa inseminated is of great importance in determining subsequent conception rates, and this suggests that a minimum number of motile spermatozoa must be ejaculated during natural service, for conception to occur (Pace and Sullivan, 1975; Pickett, Voss and Nelson, 1976). This would suggest that the total number of spermatozoa produced in an ejaculate is perhaps the best indicator available at the moment, from semen examination, of the potential fertility of a stallion. Therefore, although collection and evaluation of semen is an integral part of attempts to assess fertility in stallions, its limitations are recognised.

At present, the establishment of the spermatogenic activity of a stallion can only be carried out in retrospect, using sperm depletion experiments (Swierstra, Gebauer and Pickett, 1975b; Amman et al 1979; Gebauer, Pickett and Swierstra, 1974a). This technique is both time consuming, and detrimental to the stallion. Therefore, if other accurate methods of assessing spermatogenesis in a stallion, that did not require semen collection, were available, they would be of great value to the equine industry.

The Sertoli cells of the seminiferous tubules are responsible both for nursing the developing spermatids, and for the secretion of androgen binding protein (ABP), a protein which concentrates testosterone within the epididymides (Hansson et al 1975). At present it is

derstood that such a concentrating effect is necessary to intain normal epididymal function, and is essential for e normal maturation of spermatozoa. As Sertoli cell tivity affects inhibin secretion, FSH levels in the ripheral circulation may reflect Sertoli cell activity d possibly also, by association, the rate of ermatogenesis within the seminiferous tubules. Similarly ey may also reflect the number of abnormal spermatozoa oduced due to defective nursing, and the competence of turation within the epididymis.

Studies of oligospermic men have shown that ripheral levels of FSH are inversely correlated with erm count (Purvis et al 1975; Hunter et al 1974; Rosen d Weintraub, 1970). As sperm count would appear to be a ry important factor affecting fertility in the stallion, similar correlation in the stallion may be of interest. owever, as spermatozoa are ejaculated approximately sixty ys after the initiation of their spermatogenesis wierstra, Pickett and Gebauer, 1975a; Amman, 1981), the jaculates collected in this study were compared with ripheral circulating FSH levels sixty days prior to the te of each collection.

Several different approaches were investigated for the llection of semen from the two stallions. The collection dismount samples, and the collection of semen from lyurethane tampons inserted into the anterior vagina of strus mares prior to service, did not collect a complete

ejaculate. As this was essential to allow a total sperm count to be estimated, these methods were abandoned. The use of latex condoms proved very hazardous, and frequently resulted in the loss of the ejaculate during dismount. This method was also abandoned in favour of the use of an artificial vagina.

A Hauptner artificial vagina was modified and subsequently used for all collections. A new disposable plastic liner was placed inside the permanent latex water jacket for each collection. This also allowed the liner to be replaced in those instances where the erect penis of a stallion entered the artificial vagina but he failed to ejaculate, thus preventing dilution of the sperm-rich fraction by presperm fractions produced during successive mounts prior to ejaculation. After each collection the thin plastic liner was removed, and any semen collected in its folds was milked into the collection tray. Such complete and rapid emptying was not possible when only the thicker latex jacket employed, in the traditional Hauptner design. This reduced post-collection sperm losses which may otherwise approach 38% of the sperm ejaculated (Pickett et al 1974).

It is well recognised that it is beneficial to remove the gel fraction of the stallion ejaculate prior to assessing motility and spermatozoa concentration (Pickett and Back, 1973; Pickett et al 1973). Although the tissue filter incorporated at the neck of the collection beaker

did not separate all the gel immediately after collection, it did provide an aliquot of gel-free semen for rapid assessment of motility, and for the preparation of stained slides, within minutes of ejaculation. Separation of the gel fraction from the semen retained above the filter, was achieved quickly using a syringe to withdraw the clear translucent gel. If this was not carried out soon after collection the gel may have dissolved or dispersed (Pickett and Back, 1973).

On many occasions it was desirable to collect semen when an oestrous mare was not available. Therefore, the two stallions were trained to mount a phantom mare. This phantom was of simple design and construction (plate 5.2.), but proved a very effective way of collecting semen from these pony stallions. The use of such a technique is well established in the bull, the ram, and the boar. A few reports of its previous use in the stallion are also available (Richardson and Wenkoff, 1976; Pickett, Squires and Voss, 1982). In the investigation reported here, the phantom was initially introduced as a teasing board. When oestrous mares were teased, it took less than one hour to train these stallions to mount the phantom, and for semen to be collected. However, each stallion subsequently behaved differently in his approach to the phantom. Whilst the older stallion, Steptoe, usually gained an erection as soon as he entered the service area, the younger stallion, Max, often required a period of teasing across the phantom

before an erection was achieved. Other workers using a phantom mare for semen collection have recommended blindfolding stallions during the initial training period. This was unnecessary in these studies.

With repeated use, the two stallions lost all respect for the phantom, and showed increased aggression when it was used for teasing. Pickett, Squires and Voss (1982), and Richardson and Wenkoff (1976), have advocated the use of a phantom mare as therapy for aggressiveness in stallions. The studies described here do not endorse this suggestion.

The quality of the semen collected using the phantom was similar to that reported by other authors who use oestrus mares for semen collection (Pickett et al 1974; Van der Holst, 1975; Squires, Pickett and Amman, 1979, Pattie and Dowsett, 1982; Voss, Pickett and Squires, 1981; Harris, Irvine and Evans, 1983; Bielanski et al 1982). Therefore, the use of the phantom was considered to be a suitable method for obtaining semen from the stallions under investigation. Semen collected from some stallions may sediment within a short time after collection (Pickett et al 1975a; Pickett and Voss, 1972). In other species such sedimentation is rare and its occurrence does not affect fertility (Bedford, 1970). In these studies the sperm rich fractions from both stallions sedimented within approximately 5 minutes of collection. This could be seen by the naked eye, and microscopic examination revealed that

during this process adhesion of the heads of the spermatozoa occurred, and clumps of 5-10 spermatozoa were formed. During this phase, clumps of spermatozoa "cartwheeled", due to continued flagellar activity. Sedimentation and the formation of clumps of spermatozoa hampered the assessment of initial motility and attempts to estimate sperm concentration. Therefore, the sperm rich fractions were diluted with a glucose/skimmed milk extender, to prevent clumping from occurring. This diluent was recommended by Pickett and Back (1973), and since it proved effective alternatives were not investigated.

As the estimation of spermatozoa concentration was an essential part of the semen examination, it was important to use as accurate a method as possible. Many methods of determining sperm concentration have been investigated in semen studies, in various species. These include the use of an EEL spectrophotometer, haemocytometer slides, and the Coulter counter (Ferguson, 1976). EEL spectrophotometry was not used in these studies, as the skimmed milk/glucose diluent proved too opaque to allow accurate calibration. Preliminary studies showed close agreement between the results of concentration estimation using the haemocytometer slide, and the Coulter counter. Therefore it was decided that the less time-consuming Coulter counter method should be employed for the determination of sperm concentration. Although the Coulter counter can be used to estimate the concentration of living

spermatozoa in semen, in these studies samples were collected over a period of time, and were then stored at -20^{o}C before analysis. After thawing at room temperature, there was little evidence of tail loss, or disruption of the spermatozoa, which may have affected the analysis. This storage technique allowed batches of semen samples to be randomised before estimation of concentration was carried out.

Two of the factors that are reported to influence normal semen quality and quantity are; (a) the time interval between ejaculations (Sullivan and Pickett, 1975; Pickett, Sullivan and Seidel, 1975b) and (b) the season of the year (Van der Holst, 1975; Harris, Irvine and Evans, 1983; Dowsett and Pattie, 1982; Pickett, Faulkner and Voss, 1975c; Johnson and Thompson, 1983). In this study the length of time since the last collection did appear to influence the number of spermatozoa ejaculated. When semen samples were collected regularly the total number of spermatozoa ejaculated was relatively constant. On a few occasions when regular weekly collections were not carried out, the first ejaculates collected after the interval contained higher total numbers of spermatozoa than when collections were made regularly. However, increasing the time interval between collections had no effect upon; the volume of the ejaculate, the motility of the spermatozoa, the percentages of spermatozoa with abnormalities, or the percentage of spermatozoa that were dead.

The fate of all the spermatozoa produced in the testes is still not established. Some authors believe that all the spermatozoa entering the epididymis are voided during ejaculation at service, or during masturbation, or are voided passively during urination (Swiersta, Pickett and Gebauer, 1975a). Others believe that spermatozoa also undergo autolysis within the epididymis.

The results from two stallions in this study tend to suggest that when the interval between semen collection is increased, more spermatozoa accumulate within the caudae epididymides between ejaculations, and that this leads to increased numbers being ejaculated subsequently. This result is similar to that recorded by Gebauer, Pickett and Swierstra (1974a), and Swierstra, Gebauer and Pickett (1975b), and suggests that in the two stallions studied, ejaculation during semen collection was the main route for spermatozoan release.

Therefore, if semen collection and evaluation is to be employed as part of a fertility examination, semen collection must be integrated into the normal working schedule of the stallion, so that the semen collected is representative of his "working" ejaculates. Similarly if stallions have been rested, subsequent semen collection for fertility assessment should only be carried out after a period of "normal" work.

Another interesting feature of the results of the semen examinations carried out, was that the semen samples

collected at regular intervals throughout the year had similar physical characteristics. Therefore, no seasonal changes were apparent in the semen from either stallion. This aseasonality, in all parameters measured, disagrees with the findings of other workers in various parts of the world (Van der Holst, 1975; Harris, Irvine and Evans, 1983; Dowsett and Pattie, 1982; Pickett, Faulkner and Voss, 1975c) . Many workers report a gradual improvement in semen quality, and quantity, during the spring and summer, and the reverse in the autumn and winter.

In the studies reported in this thesis, various management practices could have influenced the quality of the semen produced by the two stallions. Both animals were exposed to artificial lighting during the night, as part of a photoperiod experiment carried out on mares in adjacent barns. However, although workers in many species consider photoperiod to play a part in the seasonal control of semen quality, all the evidence available for the stallion does not substantiate this hypothesis, and Thompson et al (1977) have demonstrated that artificial lighting does not effect stallion semen quality or quantity.

Other management factors which may have affected semen quality in these two stallions were nutrition and housing. Ample hay and concentrate rations were provided throughout the winter, and both stallions were maintained in sheltered accomodation. Since these animals did not experience nutritional hardship, or severe weather

conditions, metabolic energy may have been available for gonadotrophin secretion and continued spermatogenesis throughout the winter. However, in the studies where seasonal changes were reported, commercial stud management of the stallions, with regard to feeding and housing, was very similar to the management described here (Van der Holst, 1975; Harris, Irvine and Evans, 1983; Pickett, Faulkner and Voss, 1975c). This suggests that nutrition and shelter were not responsible for similar characteristics of the semen collected in the winter and summer months from these stallions.

One unusual feature of the management of these stallions, was their use for natural service and semen collection throughout the whole period of study, including the winter months. Stallions in the U.K. are generally only exposed to mares from January to July, and are kept in isolation for the rest of the year. However, in all the other studies where seasonal changes in semen have been reported, semen collection was necessarily continued throughout the year. Therefore the reason for the production of similar ejaculates throughout the year in these two stallions remains unclear.

As only two animals were involved in this investigation, the implications of these results must be assessed cautiously. The aseasonality recorded here could reflect a breed difference between British ponies and ponies elsewhere in the world. However, although these

findings were observed in pony stallions, they could explain the fertility of the British Thoroughbred, who compares very well with Thoroughbreds elsewhere in the world, despite his enforced use in Britain during what many workers consider to be the nonbreeding season (Merkt et al 1979; Burns et al 1982; Dowsett and Pattie, 1982; Sanderson and Allen, 1984).

Although the study of the semen characteristics of these two stallions were of importance, the main object of this investigation was to relate semen quality and quantity, to the peripheral FSH levels that existed during the production of these samples of semen. Steptoe demonstrated a slight drop in FSH levels during the winter. No subsequent changes in semen quality or quantity were obvious 60 days later. First consideration of these results suggests that there is no relationship between spermatogenesis and circulating FSH levels. However, the drop in circulating FSH levels was small, and these results could indicate that only marked changes in FSH levels result in recognisable changes in semen quality and quantity.

The average peripheral FSH level in each individual stallion was compared with the average semen characteristics of that stallion. Throughout the study the percentages of spermatozoa with primary abnormalities were similar for both stallions. However, the older stallion consistently ejaculated fewer spermatozoa, and more of

these spermatozoa were dead, or had cytoplasmic droplets present. These differences in the semen from the older stallion may have been associated with age. A slight reduction in sperm production in aged stallions has been reported by Johnson and Thompson, (1983). However, reduced sperm numbers can also result from mechanical obstruction of the efferent duct, epididymal duct or ductus deferens of one or both testes (Pickett, Squires and Voss, 1981b). Reduced numbers can also result from a reduced spermatogenic rate within the testes, associated with toxins, drugs (Carson and Thompson, 1979), autoantibodies, local infections, viral disease, and nutrient deficiencies (Swerczek, 1975). The higher incidence of spermatozoa with cytoplasmic droplets present in the older stallion could also be associated with specific obstructive or degenerative changes in the epididymis (Pickett, Squires and Voss, 1981b).

The blood samples collected from this older stallion contained higher levels of FSH than were found in the blood from the younger stallion. The possible relationship between these elevated levels and reduced spermatogenesis, as described in the previous chapter, is one explanation for both the semen picture for this stallion, and the higher levels of FSH in this stallion. In addition to ejaculating fewer spermatozoa the older stallion ejaculated more spermatozoa with cytoplasmic droplets present. Sertoli cells in the seminiferous tubules

of the testes are responsible for secreting ABP into the seminiferous tubules, which is responsible for maintaining epididymal function. Therefore, reduced Sertoli cell metabolism, which could be due to age, may also be associated with reduced epididymal function. This could explain the higher percentage of cytoplasmic droplets and dead spermatozoa seen in the semen of the older stallion, Steptoe, and the higher circulating plasma FSH levels in this stallion. However, only two animals were available for this study, and links between age, spermatogenesis, and FSH levels need to be investigated further, before any firm conclusions can be drawn.

In equine practice it is of importance to be able to differentiate cases of male infertility caused by obstruction of the excurrent ducts of the reproductive tract, from those cases where infertility is caused by primary lesions of the germinal epithelium. In this context the effect of vasectomy upon FSH levels in the stallion Larry are of interest. The vasectomy method employed resulted in a total bilateral occlusion of the ductus deferens. Few adhesions between the vaginal tunics were evident when Larry was eventually castrated, but small semen filled cysts were present within the epididymis. Histological examination of sections from Larry's testes revealed that vasectomy had resulted in slightly increased numbers of nuclei lining the seminiferous tubules of the

testes. No difference in tubule diameter was apparent.

The peripheral FSH concentration in this stallion was apparently unaffected by vasectomy, and this was consistent with the normal seminiferous tubule function observed histologically. This concurs with evidence from other species, which indicates that vasectomy does not affect subsequent spermatogenesis, and therefore, that mechanical obstructive lesions of the excurrent ducts apparently do not affect peripheral FSH levels (Wu et al 1981; Rosen and Weintraub, 1970).

Castration of Larry produced the expected rapid rise in peripheral FSH levels, thought to be associated with reduced pituitary inhibition by gonad secreted steroids and inhibin (Thompson et al 1979a; Wesson and Ginther, 1980; Caraty, 1983; Schanbacher and Ford, 1977). This postcastration rise helped confirm the functional competence of the Larry's pituitary during the post vasectomy period, when no change in FSH levels was observed.

Such findings make reasonable the investigation of the use of FSH estimation as a means of differentiating beween different causes of infertility in the stallion. Obstructive lesions apparently cause no change in peripheral FSH levels. Lesions causing germinal epithelial arrest, similar to that observed in cryptorchidism in the previous chapter, cause an elevation in peripheral levels. This approach to diagnosis is already used in the

investigation of infertility in man (Wu et al 1981; Rosen and Weintraub, 1970; Hunter et al 1974; Purvis et al 1975; de Kretser, 1979). Its use in equine medicine could allow differentiation of those cases with obstructive disease, that may resolve or be operable, from those cases with primary seminiferous epithelial lesions, that are inoperable and generally carry a hopeless prognosis (de Kretser, 1979). Further work is needed to confirm these findings before the general application of such a test may be advocated.

CHAPTER SIX

GENERAL DISCUSSION

CHAPTER SIX

GENERAL DISCUSSION

The study of peripheral circulating testosterone concentrations in cryptorchid stallions was limited by the relatively small throughput of such cases in the surgery department. Therefore, further work is needed to consider whether testosterone levels in cryptorchid stallions vary in the same way as in normal stallions.

An interesting feature of the results of this study was the similarity between testosterone concentrations in unilateral cryptorchid stallions, and in normal stallions. This may have reflected either contralateral hypertrophy of the scrotal testes in these cases, or unaffected Leydig cell function in both testes.

A hemicastrate unilateral cryptorchid stallion also had peripheral testosterone concentrations similar to those of normal stallions, suggesting that in this case compensation for the loss of one testis had occurred. Further research into hemicastrate compensation may reveal the mechanism of such compensation. Possible mechanisms include; an increase in Leydig cell numbers, or increased testosterone synthesis by existing Leydig cells, due to increased sensitivity to LH, or increased LH secretion. Understanding this mechanism may permit the induction of increased Leydig cell function to treat impotent or infertile animals.

More immediately, work is needed to identify the optimum conditions for the HCG stimulation test advocated in this study. The minimum dose of HCG required, and the minimum time taken for stimulation to occur, must be established to reduce the cost of the test. As the test appears to be unable to provide a diagnosis in every case, the risks associated with repeat testing also need to be evaluated.

FSH estimation proved to be of no value for the determination of whether testicular tissue was present in problem animals. It seems unlikely that measurement of blood concentrations of LH, other steroid hormones, inhibin, or ABP would be of more value.

An alternative approach may be to treat all animals that show stallion-like behaviour, as if they possess Leydig cell tissue. All such cases could be vaccinated against Leydig cells, LH receptors, LH, or against testosterone. This would obviate the need for either testing or surgery in such problem cases.

The variation in peripheral FSH concentration between the animals in this study was greater than the variation observed within animals. The cause of this between-animal variation is unclear. More animals must be studied to decide whether an ageing effect is responsible for some of this variation.

The mechanism of any ageing effect may be of

interest if sperm production per gramme of testis, or the total Sertoli cell mass of the testes, is found to fall with advancing age. As FSH is catabolised by the liver, the affect of liver dysfunction upon FSH levels and sperm output in ageing horses may also be worthy of study. The higher FSH levels in older stallions could also result from changes in the sensitivity of the pituitary to inhibin, with age. Isolation and assay of inhibin may demonstrate the mechanism of this effect, and allow inhibin inhibition tests of pituitary function to be undertaken, to investigate changes in pituitary sensitivity. This could lead to specific treatments to improve fertility in the older stallion.

Similarly, by understanding the mechanism of seasonal changes in reproductive potential, if they do exist in the U.K., treatments to improve fertility could be devised. Other workers have suggested that rising FSH concentrations stimulate a seasonal increase in spermatogenesis. This would require a stimulation of FSH secretion without a concomitant increase in inhibin secretion, or would require a reduction in the sensitivity of the pituitary to inhibin. Inducing such a desensitisation to inhibin could be used to increase FSH release, and increase spermatogenesis in subfertile animals.

The failure to identify seasonal changes in semen quality and peripheral FSH concentration in this study was

inconclusive. More animals and more regular blood and semen collections would be required to allow statistical analysis of the results. To identify a correlation between seasonal changes in spermatogenesis and peripheral FSH concentration would require the estimation of the daily sperm output of stallions during different months of the year, calculated using sperm depletion tests.

The failure to detect seasonal changes in semen quality in this study could suggest that a careful study of the management and lighting of these stallions may allow a similar aseasonality to be induced elsewhere.

Semen analysis of infertile stallions would have allowed a direct comparison between peripheral FSH concentration and different daily sperm outputs. Testicular biopsy of such cases and sophisticated morphometric analysis of such samples would also have helped directly link peripheral FSH concentration, spermatogenic rate and Sertoli cell mass in infertile animals. To overcome a lack of infertile stallion cases oligospermia could be induced by treatment with various drugs, and their effect on these parameters assessed.

Further investigations into the mechanism of contralateral compensatory inhibin secretion in unilateral cryptorchids, may suggest how compensatory testicular hyperplasia occurs. Reproducing these conditions artificially could allow treatment of cases of subfertility or infertility in stallions.

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