A Study of the Oestrous Cycle, Artificial Insemination and Embryo Retrieval in Mares

by

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Declaration

The work presented in this thesis is original and has been carried out solely by the author, except where collaboration with others has been acknowledged.

Alison Margaret Dickie

November, 1995

Summary

The reproductive behaviour of eleven mares was studied from October until June using various techniques including daily teasing, rectal palpation, ultrasound scanning, plasma progesterone and LH assays. It was necessary to combine information from all these sources to determine the exact reproductive state of each of the mares.

The average length of oestrus in this study was 6.0 ± 2.4 days and dioestrus 18.9 ± 7.2 days. A wide range of cyclic behaviour and anomalies were seen throughout the study. Double ovulations occurred during 9.6% of oestruses and dioestrous ovulations occurred with an incidence of 7.2%. LH peaks were recorded in conjunction with ovulations during silent oestruses but not dioestrous ovulations. The LH assay was also found to crossreact with eCG in pregnant mares beyond day 35. One mare was particularly prone to silent oestruses as well as displaying erratic oestrous behaviour and shortened dioestrous intervals. 55% of the mares continued to ovulate throughout the year without entering anoestrus. Another mare continued to cycle normally and conceive in the presence of a previously physiologically active granulosa cell tumour.

A comparison was made between a radioimmunoassay and an ELISA kit for the measurement of plasma progesterone concentrations. The accuracy of the ELISA was found to be 61% with a tendency to give higher results than those obtained using the radioimmunoassay. The most reliable way to use it was in a qualitative manner rather than the semi-quantitative way the manufacturers intended, improving the accuracy to 85.5%. One mare consistently gave lower than expected results suggesting some factor in her plasma prevented reaction with the kit.

Mares were inseminated throughout the study using chilled, extended semen of varying age and quality at different intervals before ovulation. An overall pregnancy rate of 61% was achieved, despite the lack of therapeutic interference and operator experience. No difference was found between pregnancy rates obtained using semen less than or greater than 48 hours old, or between insemination less than or more than 3 days before ovulation. The percentage of progressively motile sperm in a sample was found to be a subjective assessment while percentage live spermatozoa was more objective but although both parameters decreased as the semen aged, there was no correlation found between these results and pregnancy rates. The use

of prostaglandin injections to bring mares into oestrus was found to have no effect on their fertility.

14 pregnancies were achieved as part of a study on the early development of the equine eye. Three cases of early embryonic death occurred, all between day 13 and 26 of gestation giving an incidence of 21.5%, which is high when compared to previously reported figures. Two cases, involving the same mare, were associated with vesicles which were considered to be undersized for their gestational age and the other appeared normal until it was no longer detected on ultrasound. All were associated with a prolonged luteal phase. Attempts were made to terminate three pregnancies using prostaglandin. One resulted in a return to oestrus and reconception, one resulted in pseudopregnancy and the other maintained her pregnancy. Two sets of twins occurred but in both cases, one was resorbed before day 23.

Of six pregnant mares, embryos were successfully collected via the transcervical route from four mares at days 14-15, 17, 22 and 25, giving a success rate using this method of 66%. Two further mares were flushed, one on day 12 and the other on day 21 of gestation with no attempt made to retrieve the conceptus. In both cases, the embryo was lost and they returned to oestrus within 3 days.

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Acknowledgements

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General Introduction

Anatomy of the Mares' Reproductive Tract

Ovaries

The mare's ovaries are bean shaped and approximately 5 - 8 cm in length and 3 - 4 cm in width. They are suspended along their convex or attached border by the mesovarium, the ovarian part of the broad ligament, from the sublumbar area at around the level of the fifth lumbar vertebrate and approximately 10 cm caudal to the kidneys. (See Figure 1). The left ovary is situated more caudally than the right and is surrounded by loops of large intestine so care should be taken to avoid confusing it with faecal balls during rectal palpation of the tract. It is also closer to the ipsilateral kidney.

The germinal epithelium from which the oogonia develop is concentrated in the fossa in the concave free border of the ovary and this is the only area from which ovulation can occur (Arthur, 1968), unlike other species in which ovulation can occur from almost any point on the surface of the ovary. The rest of the ovary has a thick covering of tunica albuginea (Stabenfeldt et al., 1975) In the mare, the parenchymatous zone of the ovary is deep to the ovulation fossa and the vascular zone is associated with the poles and the attached border (Budras et al., 1994 ; Ginther, 1992 ; McKinnon and Voss, 1993).

Primordial follicles are present in the ovary at birth. They develop first into primary and then secondary follicles to form a pool of around 100 preantral follicles in the ovary at any one time (Ginther, 1992). The mechanism that causes the follicles to enter this pool is not yet known but it appears to be independent of gonadotrophin stimulation and they can develop up to 15 mm in diameter without hormonal support (Fay and Douglas, 1987). From this pool, gonadotrophin stimulation causes several follicles to develop in waves into preovulatory follicles from which normally only one will be selected to ovulate. Follicular development occurs continuously in the mare's ovary at all stages of her reproductive cycle including pregnancy.

Figure 1 - Anatomy of the Mare's Reproductive Tract

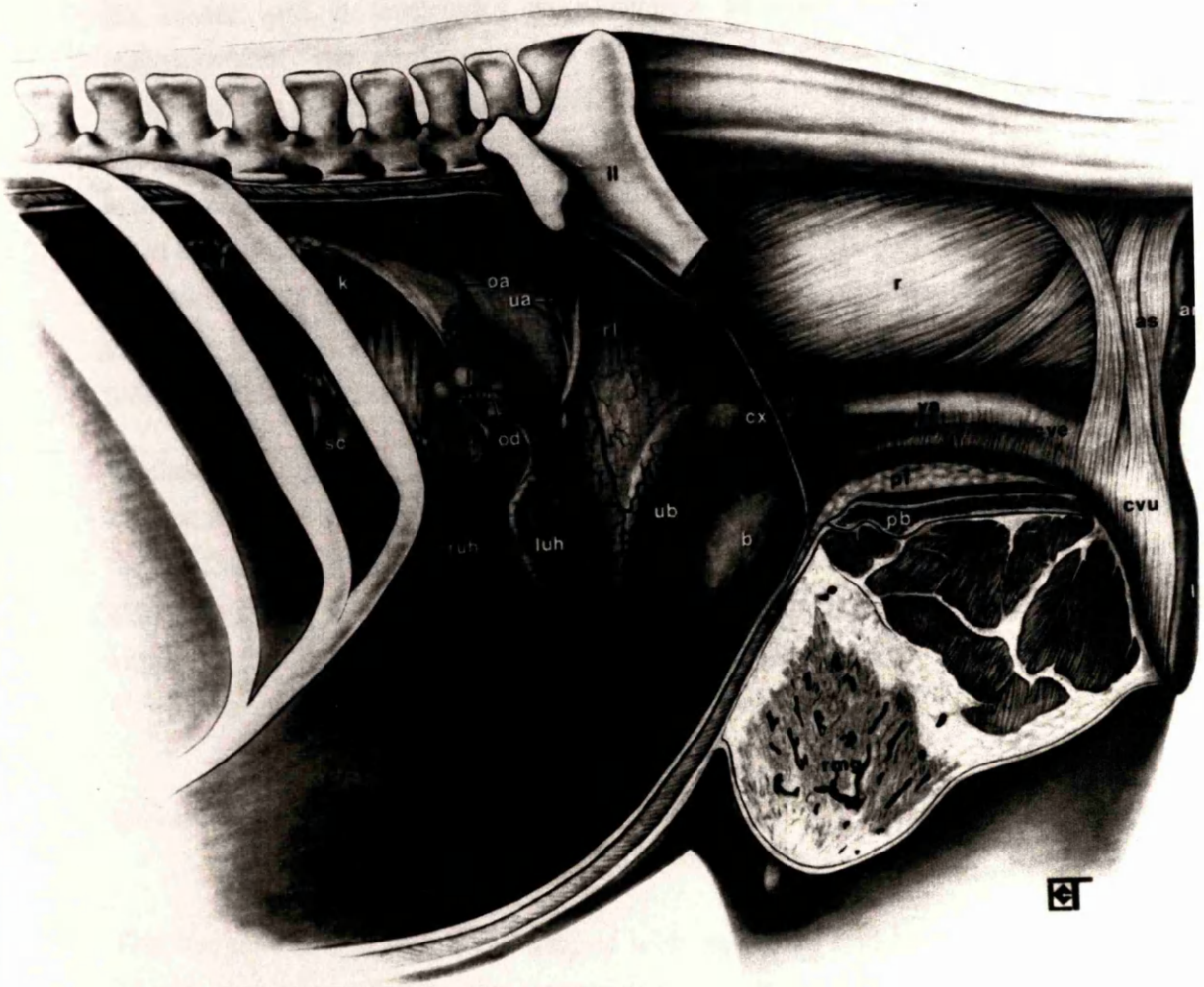


FIGURE **1.2.** Lateral view of reproductive tract. Details of the arrangement of muscles around vulva and anus were adapted from *(1488)*.

an = anus
as = anal sphincter
b = bladder
cve = constrictor vestibuli
cx = cervix
cvu = constrictor vulvae
il = ilium
k = kidney
l = labia
lo = left ovary
luh = left uterine horn
od = oviduct
pb = pubic bone
pf = pelvic fat
r = rectum
rl = round ligament
mg = mammary gland
sc = small colon
ub = uterine body
va = vagina

Ginther (1992)

Uterine Tubes

The uterine tube is a tortuous duct about 20 - 30 cm long which connects the ovary and the uterus and is suspended in a section of the broad ligament known as the mesosalphinx. The uterine tube is divided into 3 parts. The infundibulum is funnel shaped and is closely associated with the ovulation fossa. Its fimbrae serve to trap the extruded oocyte at ovulation and ensure it enters the uterine tube. The follicular fluid, however, is lost into the abdomen (Townsen and Ginther, 1989a). The ampulla is the short and wide central section leading into the long and narrow isthmus which makes up the rest of the uterine tube. It opens into the tip of the ipsilateral uterine horn at a papillum which is thought to help control selective transport of semen into and fertilised ova out of the uterine tubes while preventing the passage of uterine contaminants.

The mucosal lining is of columnar epithelium with intermittent ciliated areas depending on the stage of oestrous cycle, suggesting different functions are required at different times. Complex longitudinal folds and a thin muscle layer are features of the ampulla, thus suggesting a quiescent area for fertilisation to take place while the isthmus has simpler folds and a prominent muscle layer suggesting a role in transport (Budras et al., 1994 ; Ginther, 1992 ; McKinnon and Voss, 1993).

Uterus

The mares uterus is roughly T shaped with the horns and body each measuring around 25 cm. It is suspended by the mesometrium from the sublumbar area and is situated in the abdomen with only the caudal body and cervix located in the pelvic cavity. The tips of the horns are associated with the ipsilateral ovary via the uterine tube and proper ligament and the ovary is located cranial to the uterus, unlike ruminants. The rest of the uterus conforms to the shape of the intestinal mass. (See Figure 1).

The mesometrium contains smooth muscle and blood vessels which are continuous with the outer longitudinal smooth muscle layer of the myometrium. The inner smooth muscle layer of the myometrium condenses caudally to form the cervix and the inner layer or endometrium is thrown into prominent longitudinal folds which are also continuous with the cervix. The endometrium consists of a layer of cuboidal or columnar epithelium over a lamina propria which contains blood and lymph vessels and is rich with branched, tubular, coiled uterine glands. There are prominent histological changes that occur depending on the stage of the oestrous cycle and the dominant

hormone, again reflecting the different functions required of the tract at different times (Budras et al., 1994 ; Ginther, 1992 ; McKinnon and Voss, 1993).

Cervix

The cervix is about 5 - 7 cm long and protrudes from the cranial wall of the vagina, suspended from the dorsal wall by a frenulum. Its longitudinal folds give it a lobed appearance and despite these being a continuation of the uterine folds, there are no uterine glands in the cervix. There are, however, mucous producing glands interspersed with the columnar epithelial cells. The cervix can be easily dilated at all stages of the mares reproductive cycle including pregnancy.

Vagina

The vagina is about 25 cm long and is intra pelvic. It is dorsoventrally compressed which helps prevent contamination ascending to the cranial parts of the tract, especially during dioestrus when sticky cervical secretions cause the walls to stick together. Its longitudinal folds and muscle layer allows enough dilation for the passage of a foal at parturition.

It is divided from the vestibule caudally by a prominent transverse fold on the floor and sides of the vestibulo - vaginal junction just cranial to the external urethral orifice which is the remnant of the hymen. This also provides a good seal against ascending contamination or infection.

Vulva

The vulva is situated ventral to the anus and is a vertical orifice formed by 2 labia which seal the opening. It has a pointed dorsal commisure and a rounded ventral one concealing the clitoris.

Chapter 1 - Study of the Oestrous Cycle of the Mare

1.1 Introduction

1.1.1 The Oestrous Cycle

The oestrous cycle in the mare, as defined by the interval between successive ovulations lasts on average 21.7 days (range 19.1 - 23.7 days) in horses and around 2 days longer in ponies (Ginther, 1979).

The cycle can be conveniently divided into two parts.

Dioestrus is associated with progesterone produced by the corpus luteum (CL) being the dominant hormone causing aggressive behaviour and active rejection of the stallion with ears back, tail switching, biting, kicking etc. There are also physiological changes in the mare's reproductive tract which will be described later.

Dioestrus lasts on average 14.9 days (range 12.1 - 16.3 days) in horses and a day longer in ponies (Ginther, 1979). Luteolysis precedes the start of oestrus by approximately 3 days although this varies (Ginther, 1992) depending on the stage of development which the next wave of follicles have reached at the time of luteolysis. Dioestrus therefore also incorporates proestrus, which is associated with the follicular development between luteolysis and the onset of oestrous behaviour.

Occasionally a day of mixed behavioural signs may accompany the change over from one dominant hormone to the other but this is not usually the case so metoestrus and proestrus are not identifiable clinically in the mare.

Oestrus is associated with a lack of progesterone and oestrogens being produced by maturing follicles causing characteristic behavioural signs. The mare becomes submissive and accepts the advances of the stallion while adopting a urination stance, raising her tail, squirting small amounts of mucous or urine and repeatedly everting her clitoris (winking). The physiological changes will be described later.

Oestrus lasts on average 6.5 days (range 4.5 - 8.9) in horses and a day longer in ponies (Ginther, 1979). The length of oestrus appears to be repeatable with the same mares displaying either short or long periods of oestrous behaviour (Ginther, 1992). Ovulation

occurs in 78% of mares within 48 hours of the end of oestrus (Hughes et al., 1972) so metoestrus, defined as the stage of formation of the corpus luteum, is incorporated into this phase.

There are many factors which can affect the length of the oestrous cycle and these will be discussed elsewhere.

1.1.2 Cyclic Changes in the Reproductive Tract of the Mare

On ultrasound examination, gas and dense tissue such as bone, reflect a large proportion of the sound waves and so appear as bright white or hyperechoic areas. Fluid however, allows most of the waves to pass through it and so appears black or anechoic. The remaining tissues of the body give varying shades of grey or echogenicity depending on their composition and structure.

Ovaries

Because follicles are large, fluid filled structures, they are identified on ultrasound examination of the ovaries as anechoic areas and it is possible to follow their development as well as making measurements (See Photo 1.1). Due to the ovary's mobility, the orientation of the images can vary widely and are seldom the same between examinations (See Figure 1.1).

The dominant follicle is selected more than 6-7 days before ovulation and after this a size difference becomes apparent as one follicle continues to enlarge while the rest become irreversibly atretic and regress (Pierson and Ginther, 1985 ; Pierson and Ginther, 1987). This is shown by a decrease in the number of large follicles at this time. It is impossible to distinguish regressing and developing follicles on ultrasound examination other than by changes in size and up to 50% of follicles in an ovary may be in this state at any one time (Okolski et al., 1990).

The selected follicle develops LH receptors and produces androgens which under the influence of LH are converted into oestrogens and released into the general circulation (Fay and Douglas, 1987). This stimulates further LH release from the pituitary by a positive feedback mechanism. Inhibin and other inhibitory substances are also produced by the dominant follicle and have a negative feedback effect to reduce FSH secretion (Turner and Irvine, 1991). Because the remaining follicles lack LH receptors they are deprived of hormonal support and also cannot convert the androgens to oestrogens, both

Figure 1.1 Orientation of the Transducer During Ultrasound Examination

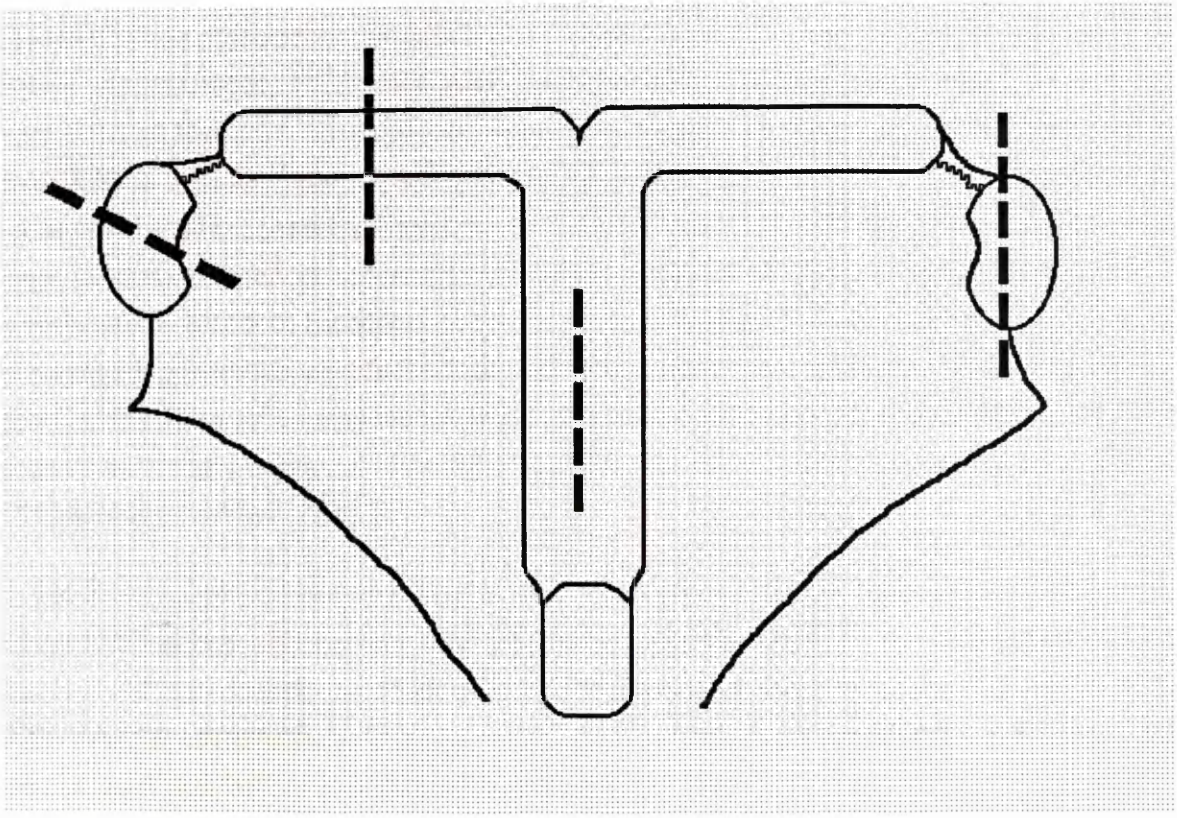
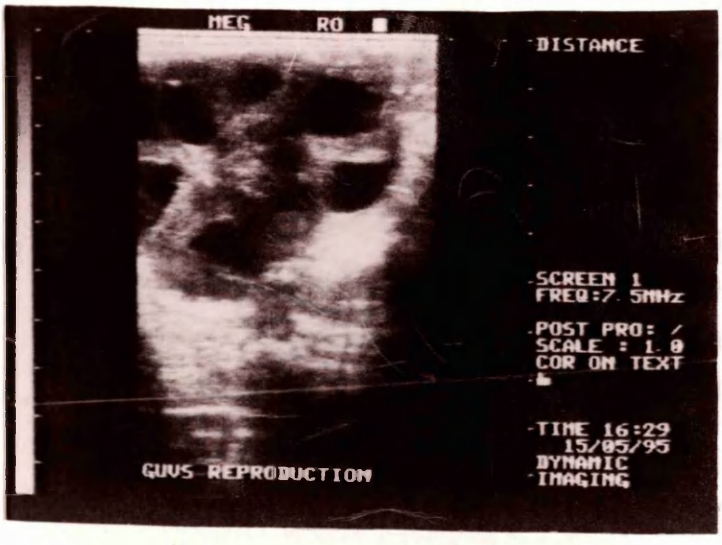


Photo 1.1 - An ovary containing several follicles



of which are thought to contribute to their atresia and regression (Fay and Douglas, 1987).

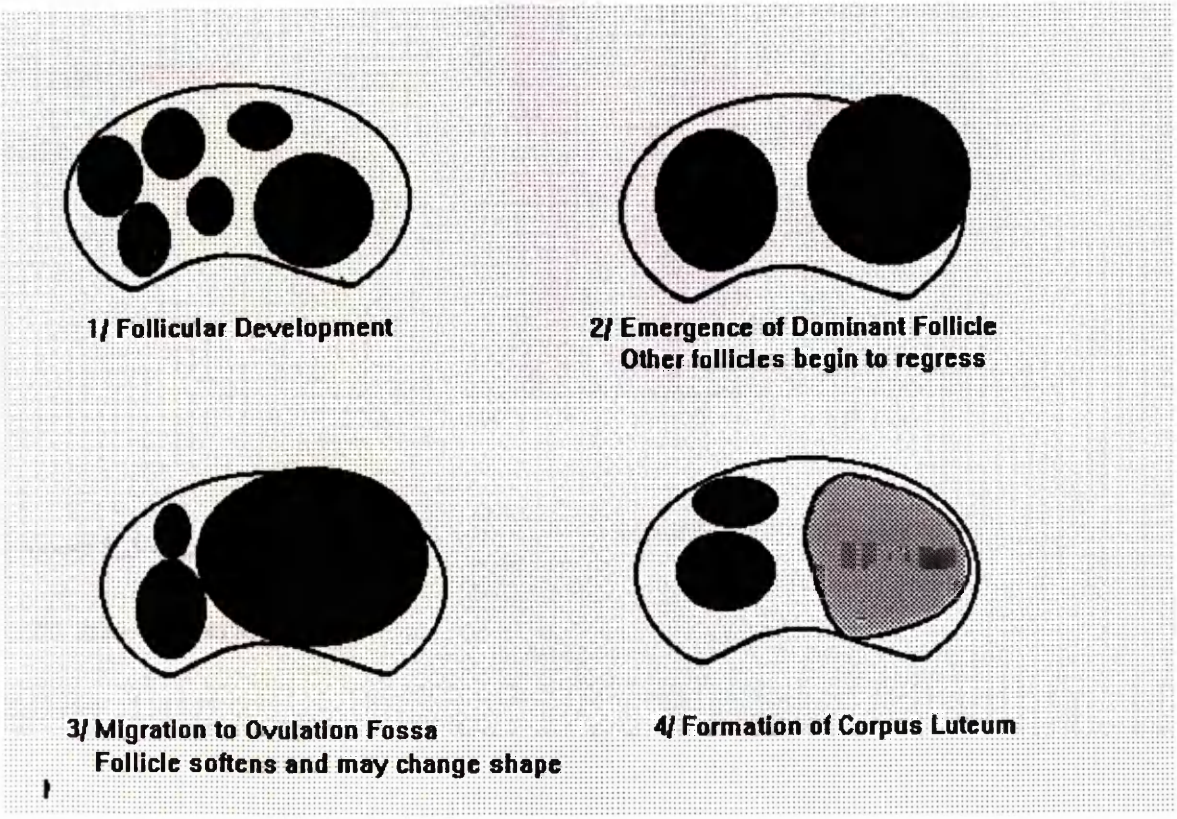
As the follicle approaches ovulation it enlarges at around 3 mm per day to a diameter of 36-66 mm (Ginther, 1986) and once large enough is palpable as a turgid, fluid filled area through the outer layers of the ovary (See Figure 1.2). Palpation studies have shown that 90% of follicles become soft prior to ovulation (Ginther, 1979). This coincides with ultrasound studies showing that 84% of follicles lose their spherical shape and become pear or cone shaped at varying times prior to ovulation (Pierson and Ginther, 1985) which seems likely to be associated with their softening. (See photos 1.2 & 1.3). Many ultrasound properties of preovulatory follicles have been measured in an attempt to predict the time of ovulation such as grey scale and wall thickness, but none have proved to be any more reliable than the changes in size and consistency at this time (Pierson and Ginther, 1985).

It has been suggested that the follicle migrates to the ovulation fossa along the bands of connective tissue that radiate from the fossa into the body of the ovary providing a pathway of least resistance (Walt et al., 1979) but this has not been conclusively proved. However, in some follicles a distinct neck process extending towards the ovulation fossa can be seen just before ovulation (See Photo 1.4).

Ovulation occurs in a matter of minutes with 80% of the antral fluid being evacuated over the space of 90 seconds in one study (Townsen and Ginther, 1989a) accompanied by collapse of the follicular walls. The remaining fluid is either expelled or retained in varying proportions but is less than 5% of the echogenic area. Evacuation should be suspected if the follicle appears reduced in size and irregular in shape during an ultrasound examination (See photo 1.5 - 1.7).

After this, an intense hyperechoic area is seen at the ovulation site in 88% of cases (Ginther and Pierson, 1984a) due to the collapse of the follicular walls. The new corpora lutea tend to be mushroom or gourd-shaped pointing towards the ovulation fossa as they fill the space left by the follicle (Arthur, 1968). (See Figure 1.2 & Photo 1.8). Identification of the occurrence of ovulation by palpation alone can be very misleading in some cases due to the similarity in the consistency and shape of a preovulatory follicle and a new corpus luteum (Hughes et al., 1980). In some studies the detection rate has been as poor as 50% (Michel et al., 1986).

Figure 1.2 Cyclic Ovarian Changes



In about 50% of ovulations, there is an influx of fluid, presumably blood, 20-32 hours post ovulation which is complete by on average 68 hours (Townsen and Ginther, 1988) forming a corpus haemorrhagicum with a non-echogenic centre occupying up to 55% of the total luteal area (Ginther and Pierson, 1985). Because this does not occur in all ovulations, the assumption that the formation of a corpus haemorrhagicum is a necessary stage in the development of a corpus luteum can be disregarded (Townsen and Ginther, 1988). This gives rise to two distinct morphological types of corpus luteum in the mare (See photos 1.9 & 1.10) which occur with equal frequency (Ginther and Pierson, 1985), have no differences in the area or echogenicity of their luteal component (Townsen and Ginther, 1989b) and are functionally identical producing the same progesterone profiles (Townsen et al., 1989).

Both types of CL remain visible on ultrasound for around 16 days (Ginther and Pierson, 1985 ; Ginther and Pierson 1984a) although this is 2-3 days longer than their functional lifespan as indicated by a drop in plasma progesterone concentrations at day 13 -14 (Allen and Hadley, 1974). On palpation, they remain detectable for around 9 days,

Photo 1.2 - Large spherical follicle of approximately 4 cm diameter

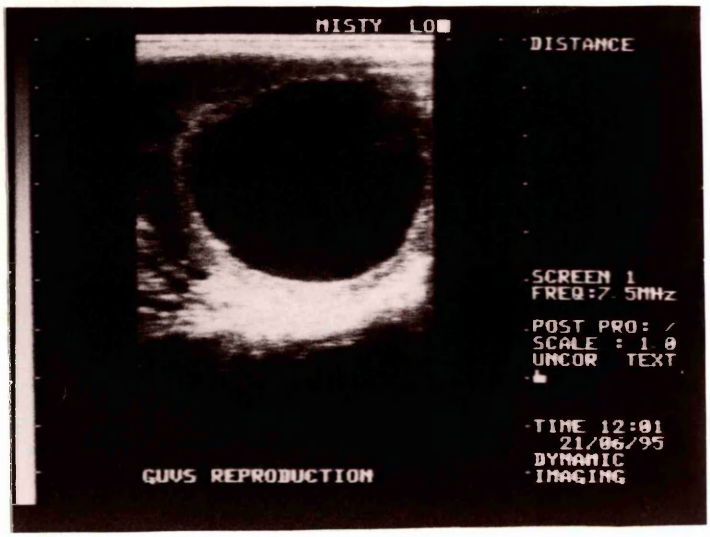


Photo 1.3 - Large, soft, non - spherical follicle

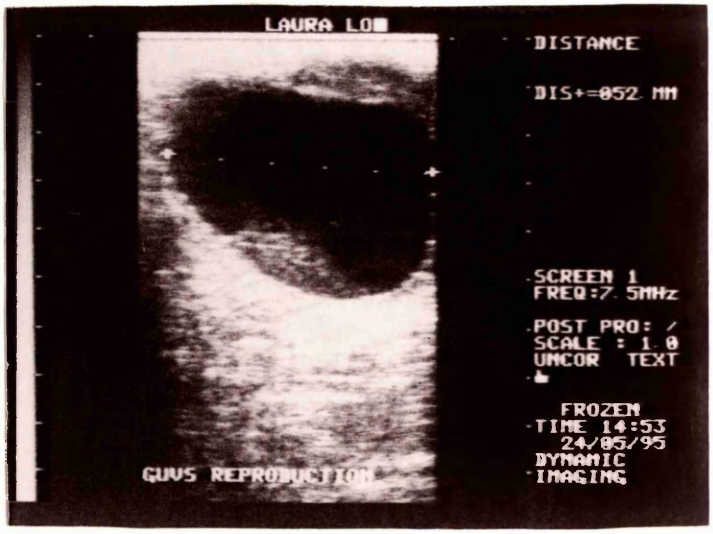
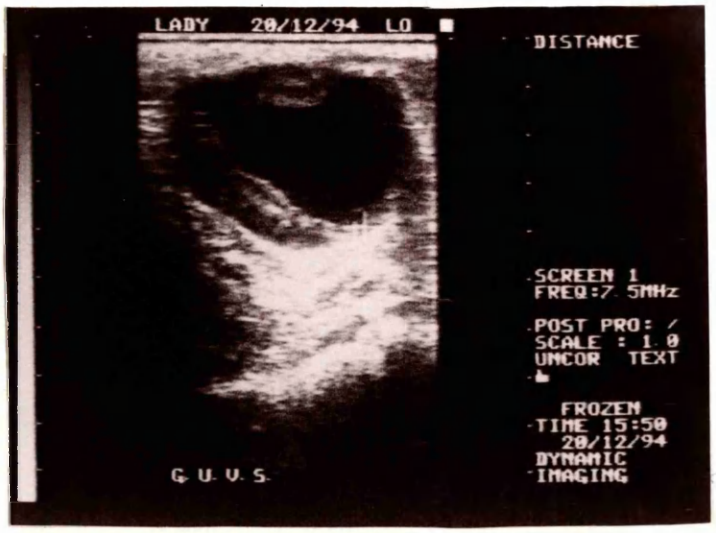
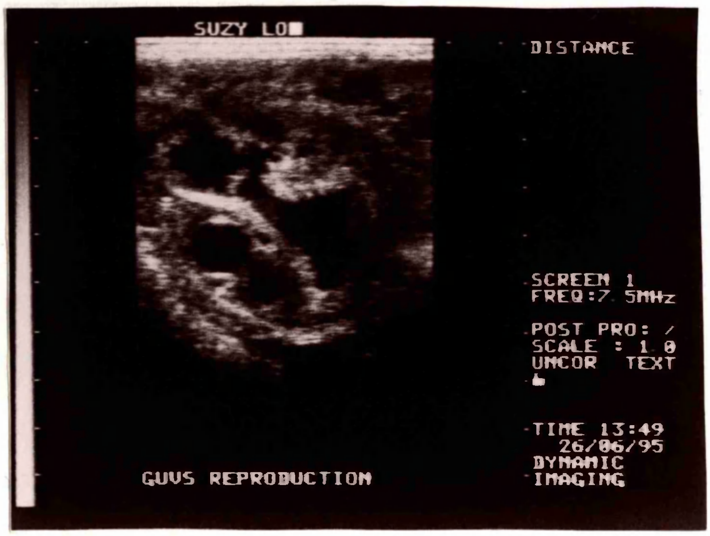


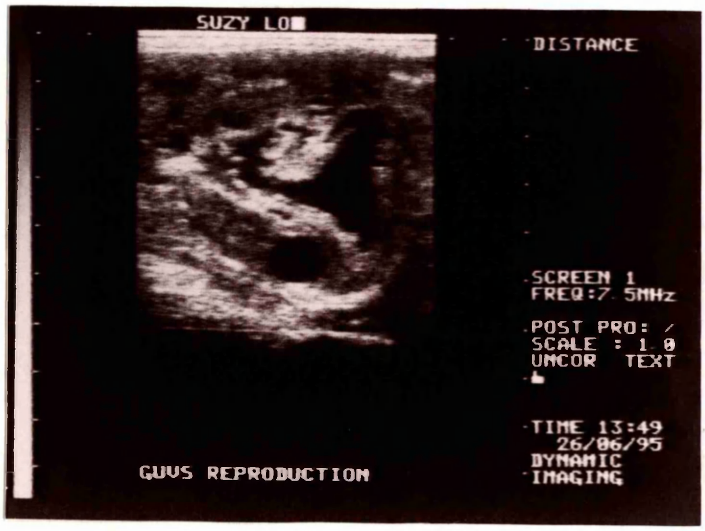
Photo 1.4 - Soft preovulatory follicle with distinct neck process



Photos 1.5 - 1.7 -

Progressive collapse of a follicle at ovulation





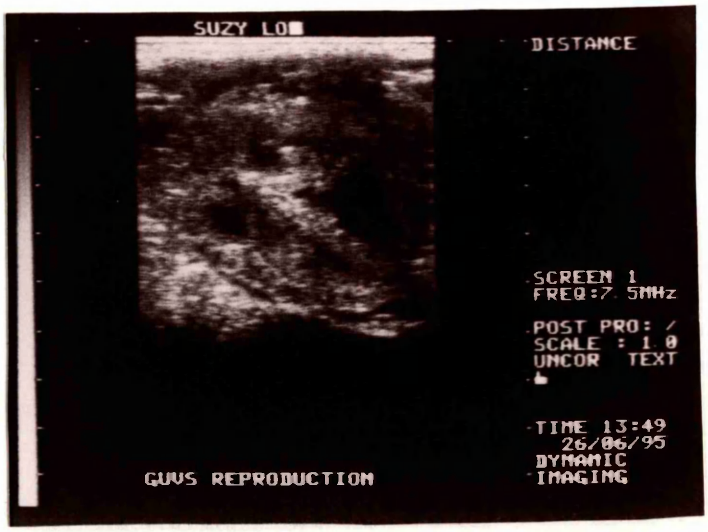


Photo 1.8 - Hyperechoic, gourd-shaped CL pointing towards the ovulation fossa soon after ovulation

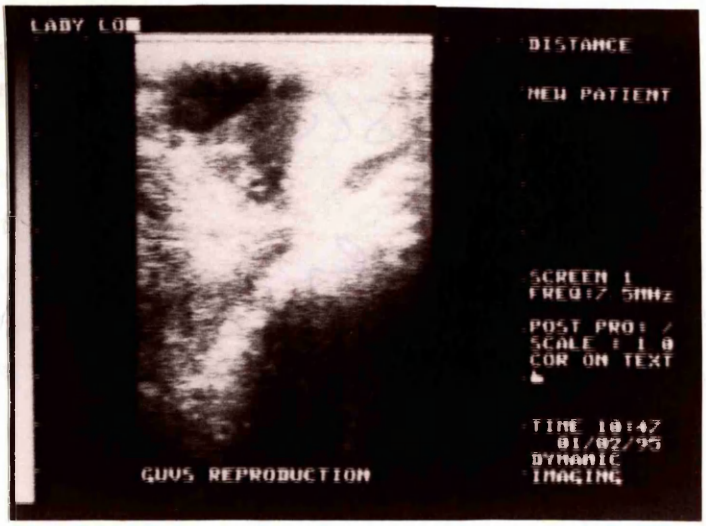


Photo 1.9 - Uniformly echogenic CL

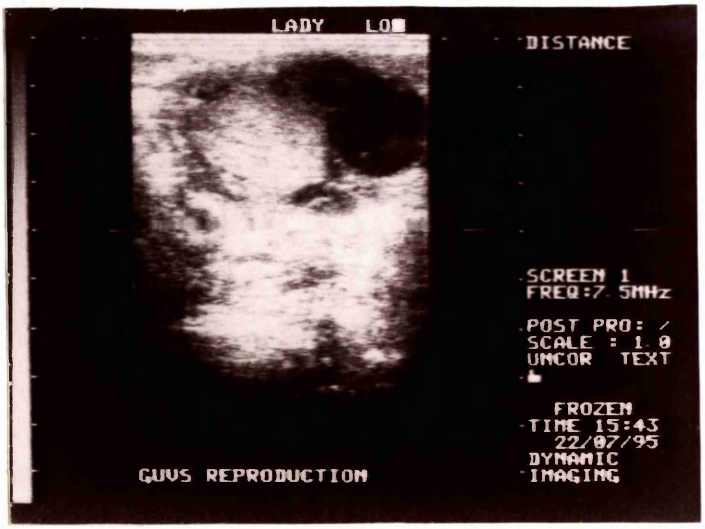
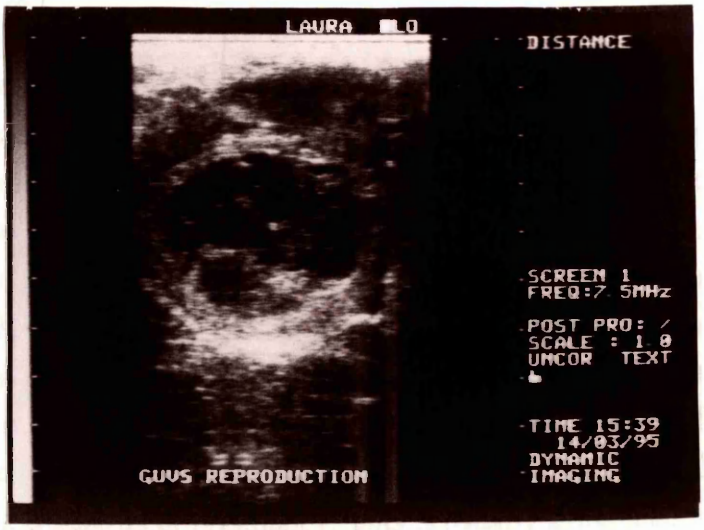


Photo 1.10 - Corpus haemorrhagicum with hypoechoic centre



progressing from a soft mushy structure after ovulation through spongy, rubbery and liver-like consistencies with age (Hughes et al., 1980).

The clots in the centre of the non-echogenic corpora lutea form a honeycomb pattern of echogenic lines representing the fibrinous network that forms as the clot begins to organise soon after formation (Townsen and Ginther, 1988) (See photo 1.11). There is a decrease in the non-echogenic portion of the CL as the central clot resolves to about 10% by the time the CL becomes indiscernible on ultrasound (Ginther and Pierson, 1985) (See photo 1.12).

The hyperechogenicity seen immediately after ovulation lasts for around 2-3 days (Ginther and Pierson, 1984b) and affects the echogenic portions of both types of CL. It then reduces gradually in intensity until around day 8 when it remains constant (Ginther and Pierson, 1985) followed by a return to bright echogenicity in 36% of cases 1-2 days before the CL becomes indiscernible (Ginther and Pierson, 1984a). These changes are thought to reflect the changes in luteal haemodynamics associated with different stages of its lifespan (Ginther and Pierson, 1985).

Follicles continue to develop throughout the lifespan of the CL so at luteolysis the next wave of follicles is ready for the selection process, the stage of development of the follicles and the degree of gonadotrophin stimulation determining the interval to the next ovulation.

Uterus and Cervix

On ultrasound examination, because of the cranio-caudal positioning of the probe in the rectum and the shape of the tract, the body and cervix appear in longitudinal section and the horns in cross section (See Figure 1.1).

During oestrus, the uterus is very relaxed with little tone. There is prominent oedema in the endometrial folds which increases the diameter of the horns but they compress easily on palpation. The folds show up well on ultrasound (See Photos 1.13 & 1.14), the white areas being the tissue-dense central portions of the folds and the dark non-echogenic areas, the oedema in the endometrium. This should be differentiated from free fluid in the uterine lumen (See Photo 1.15), where the endometrial folds are clearly defined by the non-echoic fluid. This is usually indicative of the presence of a pathological process rather than the physiological oedema of oestrus although occasionally small amounts of free fluid are seen at oestrus in otherwise healthy mares. Occasionally a small degree of oestrous oedema may be seen during dioestrus or pregnancy. This is usually associated

Photo 1.11 - Fibrinous network in centre of corpus haemorrhagicum as clot becomes organised

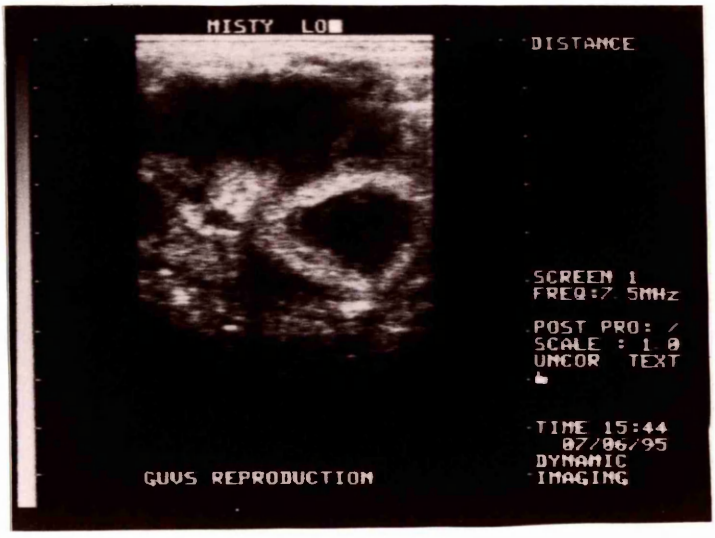


Photo 1.12 - Same CL 10 days later with only small hypoechoic area remaining

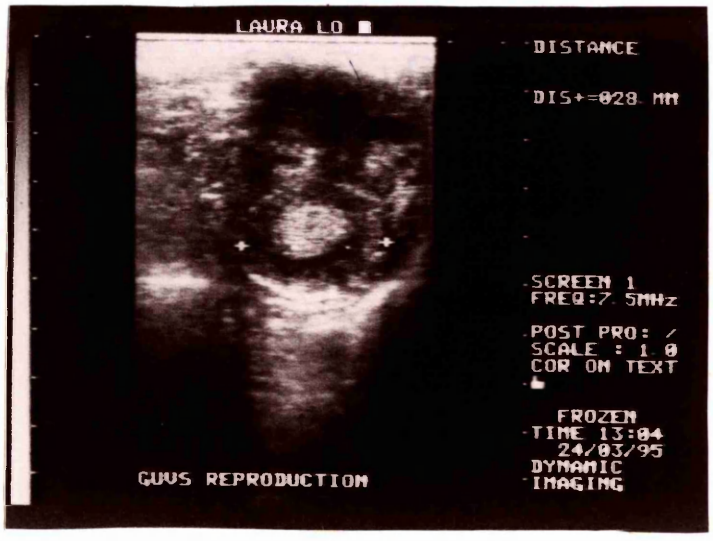


Photo 1.13 - Prominent oestrous oedema in cross section of uterine horn

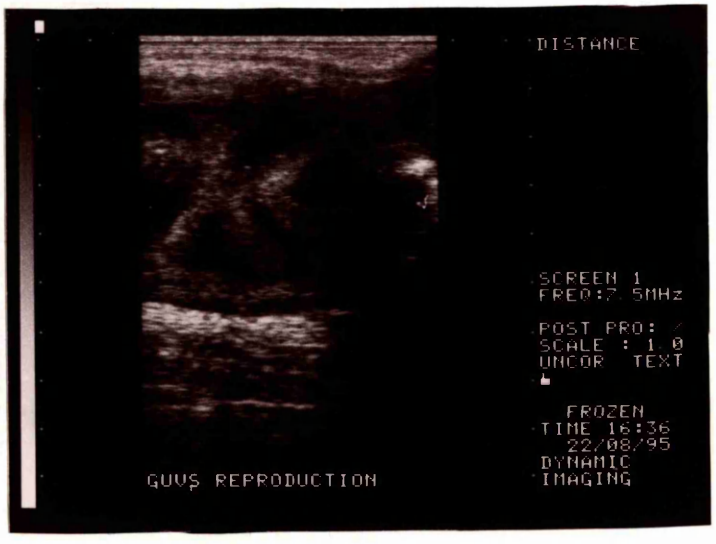


Photo 1.14 - Oestrous oedema in longitudinal section of uterine body

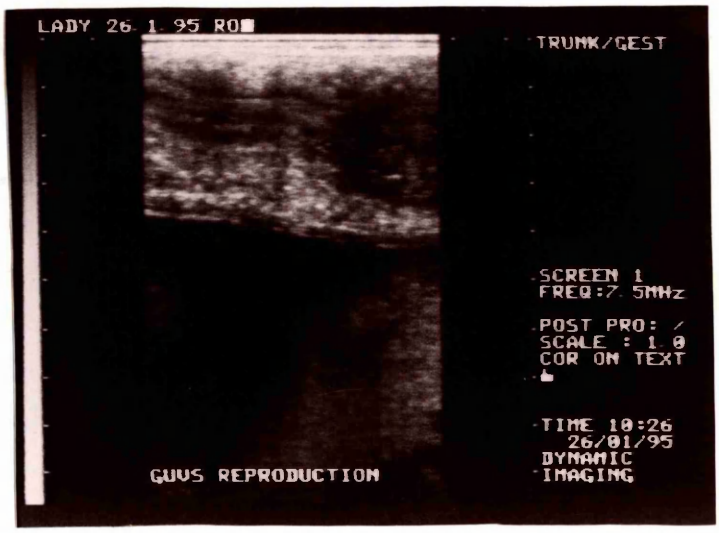


Photo 1.15 - Free fluid in uterine horn with clearly defined uterine folds

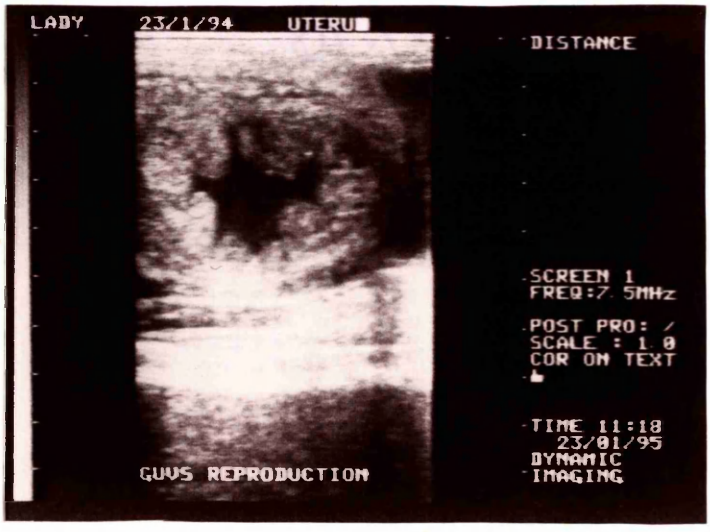
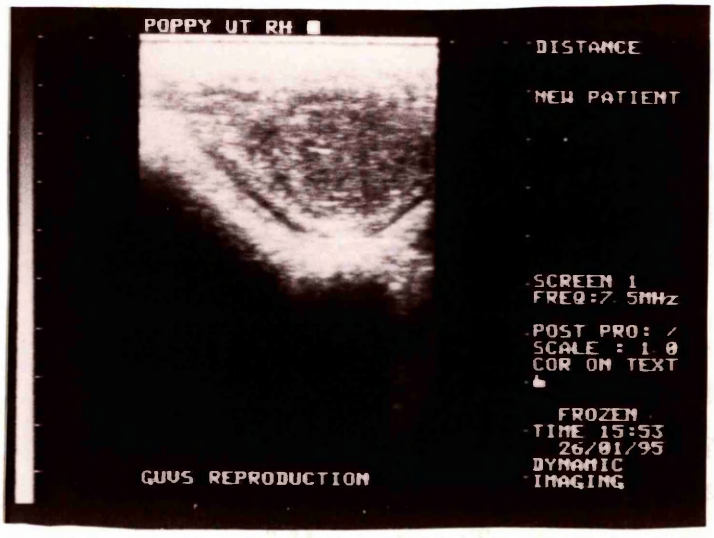


Photo 1.16 - Dense, uniformly echogenic, luteal uterus. Hyperechoic area is artefact caused by apposition of the endometrial folds



with the development of a large follicle and may be localised to the ipsilateral uterine horn.

During dioestrus, there is an increase in tone in the uterine wall and it feels firm and tubular on palpation. The endometrial folds are much less prominent and are pushed together giving a homogenous echotexture on ultrasound with individual folds not being discernible (See Photo 1.16). A bright white line is sometimes seen during this stage of the cycle in the uterine lumen, especially in the longitudinal plane and represents a specular reflection or artefact produced by the close apposition of the folds (Ginther, 1986).

Cervix

During oestrus, the cervix relaxes and drops to the floor of the vagina. The folds become oedematous and glisten due to the increased production of mucous which is thin, clear and has good lubrication properties. It softens markedly and easily admits 3 - 4 digits. The cervix has been described as a 'wilted rose' in this state.

During dioestrus, the cervix becomes constricted, firm and easily palpated. It protrudes prominently into the vagina and the frenulum becomes more obvious. The mucosa is pale and the mucous produced is reduced in quantity, more viscous and sticky. The dioestrous cervix has been likened to a 'rose bud'.

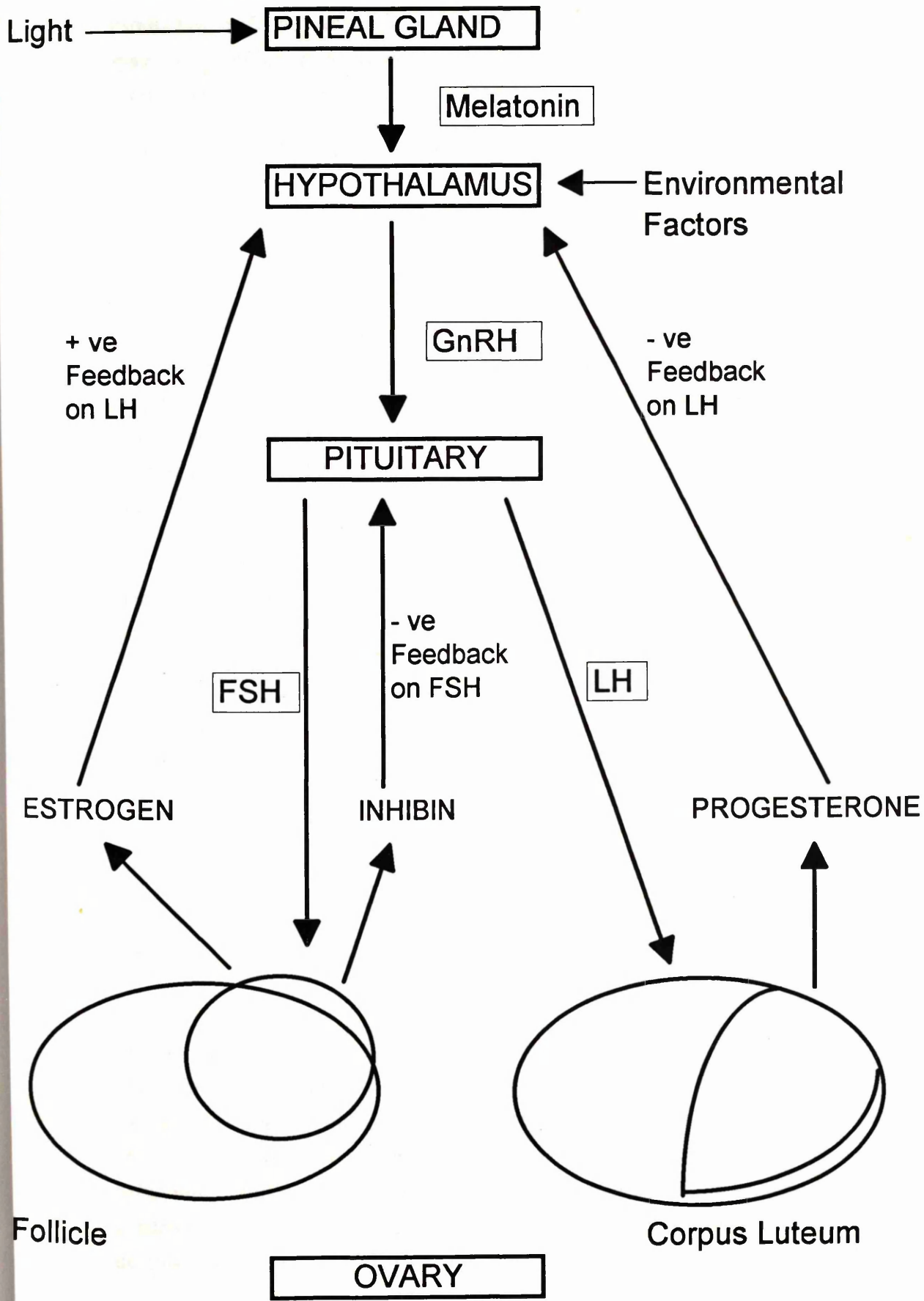
1.1.3 Endocrinology of the Oestrous Cycle

The endocrine pathways involved with regulation of reproductive activity are shown in Figure 1.3.

Reproductive cyclicity in the mare is controlled by the hypothalamus via the production and release of Gonadotrophin Releasing Hormone (GnRH). The hypothalamus is under the influence of many external factors e.g. photoperiod and internal factors e.g. ovarian hormones and level of nutrition which allow the reproductive cycle to function as efficiently and successfully as possible under varying conditions (Garcia et al., 1979).

GnRH is released in pulses from the hypothalamus and travels via the hypothalamo - pituitary portal system to the anterior pituitary where it stimulates the synthesis and release of the gonadotrophins Follicle Stimulating Hormone (FSH) and Luteinising Hormone (LH) (Evans and Irvine, 1976). Low frequency GnRH pulse release causes FSH secretion whereas high frequency pulses cause LH secretion (Turner and Irvine, 1991).

Figure 1.3 Endocrine Control Pathways



The gonadotrophins travel via the systemic circulation to the ovary (Evans and Irvine, 1976) where they have various functions including the stimulation of ovarian hormone production such as oestrogen, progesterone and other substances. These travel via the systemic circulation to feedback on the hypothalamus thus playing a role in the regulation of gonadotrophin release (McKinnon and Voss, 1993).

The hormone profiles associated with the oestrous cycle are shown in Figure 1.4.

FSH

FSH stimulation causes several follicles from the pool of primary follicles to develop up to 2.5-3 cm diameter (Fay and Douglas, 1987). FSH release is not affected by high progesterone concentrations so this development can occur at all stages of dioestrus (Evans and Irvine, 1975). The main FSH wave occurs in mid-dioestrus around day 10 (Evans and Irvine, 1975) producing a group of follicles from which the next ovulatory one will be selected as early as 14 days post ovulation (Fay and Douglas, 1987).

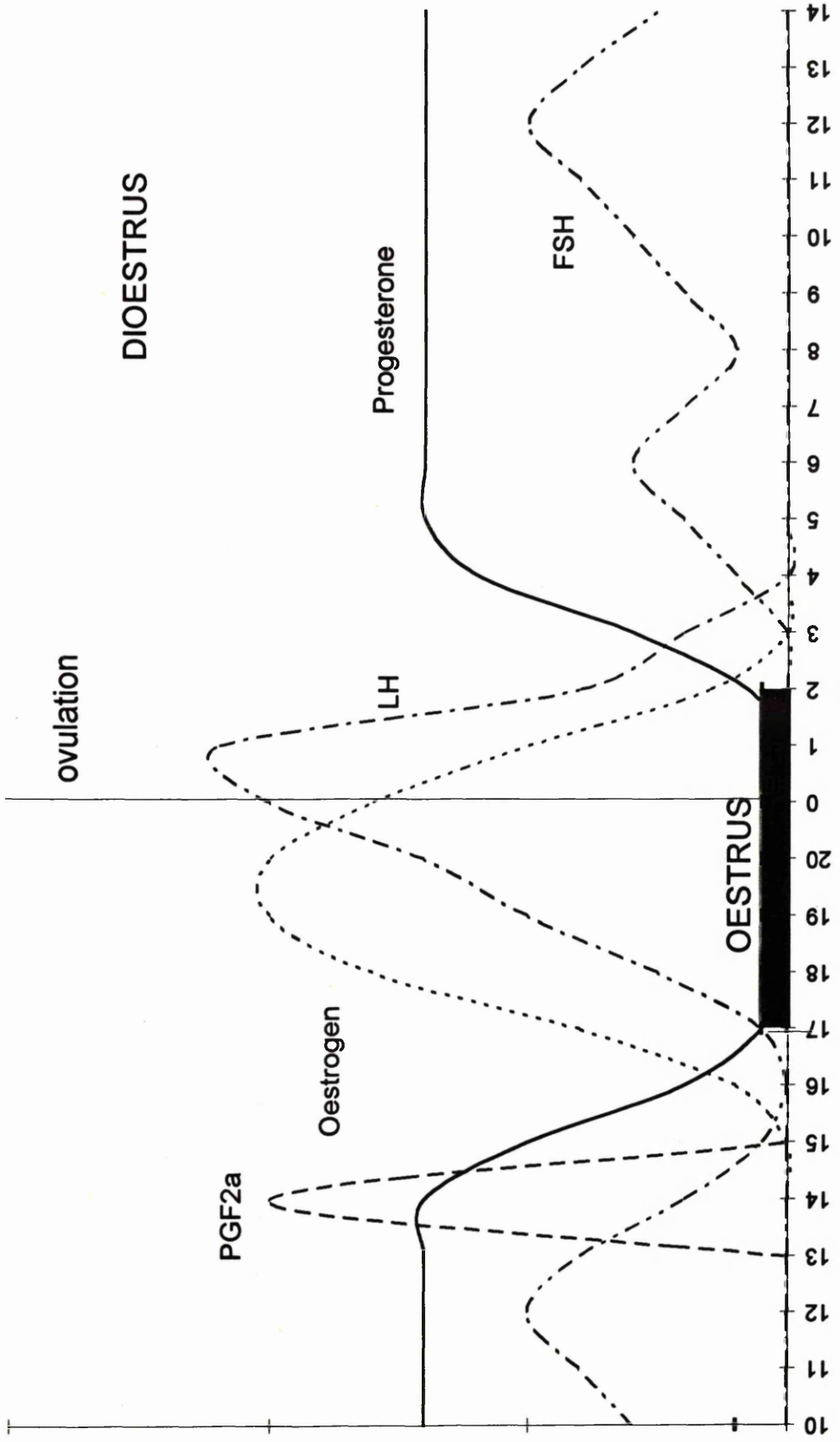
This dominant follicle matures to a stage when it produces oestrogens and inhibin which enter the systemic circulation. Inhibin has a negative feedback effect on the hypothalamus to inhibit FSH secretion and as a result, FSH concentrations in the blood decline (Turner and Irvine, 1991).

In some mares, a second wave of FSH occurs once the negative effects of inhibin have ceased at ovulation but it is very variable in its time of occurrence, length of duration and amplitude (Ginther, 1992). It may be associated with a wave of follicular development but these tend to regress to make way for the main mid-dioestrus wave rather than contributing to the selection pool (Ginther, 1992).

Oestrogen

Oestrogen is produced by the dominant follicle as it matures. Concentrations begin to increase 2-3 days before the onset of oestrus and peak at approximately 2 days before ovulation in association with a significant increase in follicular diameter (Noden et al., 1975). The emergence of oestrogen as the dominant hormone at this time causes the behavioural and physiological changes associated with oestrus as previously mentioned. Oestrogen concentrations are declining at ovulation and drop markedly by the end of oestrus (Noden et al., 1975).

Figure 1.4 Hormone Changes During the Oestrus Cycle of the Mare



There is occasionally a small increase in oestrogen concentrations in early dioestrus but it is not clear if this is due to the second wave of FSH and follicular development or oestrogen production by the CL (Daels et al., 1991). It is not significant enough to cause behavioural changes but it may be associated with some degree of localised uterine oedema, tone alterations etc.

LH

Oestrogen from the dominant follicle has a positive feedback effect on the hypothalamus to cause an increase in LH secretion (Baldwin et al., 1991 ; Garcia et al., 1979). The dominant follicle develops LH receptors which allow it to continue maturing and then ovulate under the influence of LH. The LH rise follows the oestrogen profile by around 1 - 2 days (Fay and Douglas, 1987) and peak values of 40 - 50 ng/ml (Evans and Irvine, 1975) occur from 1 day before to 2 days after ovulation. This is unusual and there has been much speculation as to the significance of the LH peak occurring after ovulation.

The use of an *in vitro* bioassay to measure LH function however, produced a profile with a peak before ovulation (Alexander and Irvine, 1982). This suggests that there is more than one form of LH in the mare and that although the immuno-active one, detected by conventional radioimmunoassays, peaks after ovulation, the bio-active form peaks before and is therefore responsible for inducing ovulation in a manner similar to other species (Alexander and Irvine, 1982).

LH contributes to the early development of the CL but is strongly inhibited by progesterone (Garcia et al., 1979) so concentrations drop sharply as progesterone rises and remains basal throughout dioestrus (Ginther, 1992) until luteolysis occurs. How dioestrous ovulations occur under basal concentrations of LH is not known but may be associated with a peak in bio-active LH concentrations (Alexander and Irvine, 1982) or it may be the case that lower amounts are required for ovulation at this stage of cycle.

A substance produced by the pituitary has been implicated in a luteotrophic role maintaining the corpus luteum throughout dioestrus, but although LH has been suggested, this has not been proved (Pineda et al., 1972).

Progesterone

Progesterone production starts immediately after ovulation with a rise in plasma concentrations being detectable as early as 10 hours post ovulation in some mares (Townsen et al., 1989). By 24-36 hours, concentrations are generally above 1 ng/ml (Ginther, 1992). This emergence of progesterone as the dominant hormone marks the end of oestrous behaviour at this point (Noden et al., 1975) and also feeds back on the hypothalamus to end LH secretion (Garcia et al., 1979).

Progesterone rises in the plasma over 5-7 days to peak at 4-22 ng/ml (Ginther, 1992) and its production continues in an episodic manner (Evans, 1991) to produce an erratic plateau until around day 14 or 15 post ovulation (Allen and Hadley, 1974). This episodic secretion is not associated with LH pulses suggesting that LH does not play a role in maintenance of the CL (Evans, 1991).

Progesterone concentrations then drop back to basal values of well below 1 ng/ml where they remain during oestrus and until the next ovulation.

Prostaglandin

Prostaglandin F2a (PGF2a) is produced by the entire endometrium and acts on the ovary to cause luteolysis (Douglas and Ginther, 1975) via the general circulation rather than the counter current mechanism in ruminants (Ginther and First, 1971). PGFM is its main metabolite which is measurable in plasma and peaks have been recorded in association with CL regression and decreased progesterone concentrations (Kindahl et al., 1982).

Progesterone primes the uterus for prostaglandin release at a specific stage of the cycle but the signal that starts off the cascade is not yet known (Sharp and McDowell, 1985). There is an increase in uterine luminal oestrone and oestradiol secretion which is temporally associated but has not yet been causally related to this (Sharp and McDowell, 1985). However, 3 weeks of progesterone treatment followed by administration of oestrogen gives maximum prostaglandin release from the uterus (Sharp, 1980b).

Newly formed corpora lutea (1-4 days old) are not as susceptible to luteolytic effects of prostaglandins as mature ones.

1.1.4 Seasonal Variations

The mare is generally regarded as being seasonally polyoestrous which suggests the presence of a distinct breeding season during which regular oestrous cycles associated

with ovulation occur and a non-breeding season characterised by sexual quiescence. The function of this limited fertile period is to ensure conception occurs at a time which will produce offspring when environmental conditions are optimal for their survival. Given the 11 month gestation in the mare, the breeding season is usually during spring and summer (April - October) in the northern hemisphere (McKinnon and Voss, 1993). The non-breeding season during the winter months is usually associated with anoestrus but individual mares show varying responses during this time.

Control

The factors controlling seasonal breeding in the mare are not yet fully understood although the main environmental regulator appears to be photoperiod with factors such as body weight, nutrition and climatic conditions also contributing (Ginther, 1992).

The effects of photoperiod are thought to occur via melatonin, a substance produced by the pineal gland during the hours of darkness. Melatonin concentrations were shown to be low throughout the 24 hour period in mares exposed to constant light (Colquhoun, 1984) but in mares exposed to constant dark, melatonin was produced in an episodic manner suggesting the existence of a circadian rhythm (Kilmer et al., 1982). The frequency of the pulses is generally highest during periods of darkness, therefore under natural light conditions, mean concentrations are greatest at night. There are considerable variations between mares in the pattern and concentration produced, suggesting that the photic recognition of night from day is due to the contrast between concentrations rather than absolute values (Guillaume and Palmer, 1994). Other factors such as stress or even sleeping with closed eyes have been shown to directly affect melatonin concentrations (Colquhoun, 1984).

The exact mechanism by which melatonin controls the seasonal variations is not yet known but is thought to occur by the alteration of GnRH production and secretion. Higher mean concentrations of melatonin associated with short daylength during the winter are thought to reduce GnRH release therefore reducing FSH and LH secretion and so suppressing reproductive function. In the summer, long daylength gives lower mean melatonin concentrations and therefore little suppression of reproductive function. In one study, GnRH stores in the hypothalamus on December 22nd, were found to be significantly lower than those during the breeding season but were similar to summer concentrations when measured on January 9th, suggesting the effect of increased daylength on GnRH production in the hypothalamus occurs very rapidly after the winter solstice (Hart et al., 1984 ; Silvia et al., 1986).

There is no correlation, however, between the pattern of melatonin production or the absolute amount produced and the degree of the mare's reproductive activity over the non-breeding season. This suggests that there is considerable variation between mares in their sensitivity to melatonin concentrations which may be influenced by other factors such as plane of nutrition, body weight and environmental conditions (Colquhoun, 1984). Alternatively, melatonin may not be the main regulatory factor in the mare and other substances such as prolactin may also play a role in controlling seasonal variations in reproductive function.

Non - Breeding Season

There is great variation in the behaviour which mares display at this time of the year. Some mares continue to cycle and ovulate normally throughout the year with hormone profiles resembling those of the breeding season (Hughes et al., 1980). These cycles have been proven to be fertile (Colquhoun, 1984).

At the other extreme, some mares go into anoestrus. This is caused by lack of hormonal stimulation as demonstrated by the plasma concentrations of FSH, LH, oestrogen and progesterone being basal at this time. Pituitary stores of FSH and the number of GnRH receptors in the pituitary are constant throughout the year suggesting that lack of GnRH stimulation prevents its release (Hart et al., 1984). As previously mentioned, (Hart et al., 1984 ; Silvia et al., 1986) normal concentrations of GnRH are present in the hypothalamus soon after the winter solstice so it is a reduction in its release that prevents FSH secretion at this time (Sharp and Grubaugh, 1987). LH content of the pituitary is low during the non-breeding season (Hart et al., 1984).

Due to lack of hormonal stimulation, anoestrus is characterised by small, hard, inactive ovaries with little follicular development. The tract has little tone and is non - oedematous and flaccid, conforming to the shape of the abdominal contents while the cervix is pale, dry and may gape open. The mare usually shows behavioural indifference to the stallion during teasing.

During the winter, 0-10% of pony mares will continue to ovulate and 20-50% of horses (Adams and Bosu, 1988). However, another 20-40% of all mares will show oestrous behaviour at this time without ovulation (Adams and Bosu, 1988). This may be due to incomplete suppression of GnRH release allowing some FSH secretion which produces follicular development. Oestrous behaviour may occur due to the background of minimal hormone stimulation making the behavioural centres of the mare more sensitive to small amounts of circulating hormones or hormone-like substances. These may be

produced by the follicles if they reach a sufficient stage of maturity or may come from other sources such as the adrenal (Asa and Ginther, 1982). Because LH stores are depleted, there is no LH secretion and so ovulation does not occur. The follicles become atretic and regress giving erratic and irregular periods of oestrous behaviour and the mare is not fertile.

Vernal Transition

The transition from anoestrus into the regular, fertile cycles associated with the breeding season takes around 40-60 days. This is thought to be due to decreasing melatonin concentrations releasing the suppressive effects on GnRH causing an increase in secretion rate and number of secretory episodes (Sharp and Grubaugh, 1987).

Because FSH stores are present in the pituitary, its release commences immediately and this produces follicular development (Sharp and Grubaugh, 1987). Initially, a lack of androgen precursors means that the early transitional follicles fail to produce oestrogen (Adams and Bosu, 1988). Production starts once the follicles becomes steroidogenically competent and this gives the erratic and irregular oestrous periods associated with the spring transition. These oestruses are not usually associated with ovulation due to the lack of LH stimulation and so are infertile.

LH stores are slowly replenished but release does not occur until a competent preovulatory follicle is present (Alexander and Irvine, 1991) which produces enough oestrogen to cause LH release. LH concentrations associated with the second ovulation of the year are higher than the first suggesting that normal concentrations have not yet been attained at this time (Silvia et al., 1986). There is a decline in FSH concentrations preceding the first ovulation of the year, presumably reflecting the competency of the follicle in producing inhibin (Turner et al., 1979).

The first ovulation of the year has been reported as occurring, on average around the start of April in horses and a month later in ponies (McKinnon and Voss, 1993).

Breeding Season

Once the first ovulation of the year has occurred, a mare will usually continue to cycle until she either becomes pregnant or once again enters the non-breeding season.

The luteal phase of the cycle is relatively consistent but the follicular phase is more variable giving alterations in cycle length throughout the breeding season. In general,

oestrus is longer in the early and late stages and shortest in the middle of the summer (Hughes et al., 1972). This is presumably due to follicular development and maturation becoming more efficient as the breeding season commences (Ginther, 1979). The occurrence of the second wave of FSH release during dioestrus tends to decline towards the end of the breeding season (Turner et al., 1979).

At the end of the summer, decreasing daylength once again begins to inhibit GnRH release and the mare undergoes transition into the non-breeding season. This is associated with decreased LH production causing failure of ovulation as shown by LH concentrations being higher on the day of the penultimate ovulation of the year than that of the last one (Snyder et al., 1979). This is followed by a progressive decrease in FSH concentrations reducing follicular development although this is not well defined so follicular development can occur into and sometimes right through the winter

1.2 Materials and Methods

Introduction

The reproductive cycles of a group of mares were studied over a nine month period using daily teasing, rectal palpation and transrectal ultrasound examination. Plasma LH and progesterone concentrations were determined using radioimmunoassays.

1.2.1 Animals

11 mares as described in Figure 1.5 were used for the study. During the winter, all were fed hay and concentrates (Horse and Pony Cubes, Spillers Horsefeeds, Milton Keynes, UK). 2 mares losing condition were also fed Original Mix (Spillers Horsefeeds, Milton Keynes, UK). During the summer, all the mares were kept at grass with 2 receiving supplementary feeding and 4 receiving hay.

3 stallions were involved in the study. Max was used throughout the whole study while Dandy was available for the first 2 months and Teddy for the last 2 months only. All the animals were kept in a unit which was situated in the city and so there was extra artificial light throughout the night.

Figure 1.5 Mares Used in the Study

MARE	BREED	HEIGHT	AGE	MAINTENANCE		CONDITION	STATUS
				WINTER	SUMMER		
CORRIE	TB X	15.2 hh	17	IN	IN AT NIGHT	POOR - GOOD	Barren
FILLY	New Forest	13.2hh	17	OUT	OUT	FAT	Weaned OCT
GEMMA	TB	16.2hh	13	IN	OUT	GOOD	Maiden
GREEDY	Irish Draught X	17.3hh	13	IN	OUT	FAT	Barren
JENNY	X	15hh	16	IN	OUT	GOOD	Maiden
LADY	Welsh	13hh	21	IN	OUT	GOOD	Barren
LAURA	TB	17hh	4	IN	IN AT NIGHT	GOOD	Maiden
MEG	TB X	17hh	17	IN	OUT	GOOD	Barren
MISTY	Highland X TB	14.2hh	15	OUT	OUT	POOR	Weaned JAN
POPPY	Anglo Arab	15hh	16	IN	OUT	GOOD	Weaned SEPT
SUZY	X	13.2hh	23	OUT	OUT	FAT	Barren

Horse teasing records 1994/5

Figure 1.6

MARE_____. **Week starting Mon.**_____.

Score: one or two negative or positive points

MARE		Monday	Tuesday	Wednesday	Thursday	Friday	Sat	Sun
Squeal	(-ve)							
Kicking	(-ve)							
Flattening ears	(-ve)							
switching tail	(-ve)							
Urination stance	+ve							
everting clitoris	+ve							
tail lifted	+ve							
passing urine/ mucus	+ve							
TOTAL SCORE								

STALLION								
Interest								
Flehmen								
Erection								
TOTAL SCORE								

CLINICAL FINDINGS								

Reproduction Department, University of Glasgow Vet School

The mares were also used for veterinary undergraduate teaching which affected the frequency with which the various procedures involved in this study could be performed and therefore accounts for any missing information.

1.2.2 Teasing

Mares were teased individually at a teasing board during the winter. When 2 stallions were available at the start of the study they were used on alternate days. During the summer, the mares were group teased in the field, half with one stallion and the rest with the other. Any mare considered not to be displaying signs properly was individually teased at a board.

The frequency of teasing was daily when availability of personnel allowed and as often as possible when it did not. Teasing was performed by a different group of veterinary undergraduates each week and in an attempt to standardise the results obtained, each mare's behaviour was recorded using the procedure shown in Figure 1.6. A range of total scores from -8 to +8 was possible with a positive total score indicating the mare was in oestrus and a negative total suggesting she was not. The scores for each mare were then plotted on a chart against the date.

The length of an oestrous period was determined as the number of days a mare received a positive total behavioural score and the length of dioestrus the number of days she received a negative total.

1.2.3 Rectal Palpation and Ultrasound Examination

These two procedures were carried out concurrently. The frequency of examinations was determined by the availability of the mares when they were not being used for undergraduate teaching but was at intervals of less than one week where possible.

Mares were restrained in stocks, their tails bandaged and held to one side. A Concept L Ultrasound Scanner (Dynamic Imaging, Livingston, Scotland) with a 7.5 MHz linear array transducer was used.

The ovaries were examined for

1/ size, shape and palpable structures

2/ number, size, shape and consistency of follicles

3/ number, size, shape and consistency of corpora lutea

The uterus and cervix were examined for

4/ position, consistency and tone

5/ appearance

6/ presence of fluid, cysts etc.

This information was used to determine the mare's reproductive state and stage and whether ovulation had occurred. Later in the study, it was used in an attempt to predict the timing of ovulation in association with artificial insemination. Criterion assessed were the size of the dominant follicle and its growth rate between examinations, alterations in its shape in association with migration towards the ovulation fossa and an accompanying loss of turgidity on palpation

1.2.4 Plasma Hormone Assays

Blood was collected by jugular venepuncture using a 19 gauge 1.5 inch needle (Microlance, Dublin, Eire) into a 10 ml Monovette syringe containing lithium heparin (Sarstedt, Nümbrecht, West Germany). It was then centrifuged at 2000 RPM for 5 minutes and the plasma decanted into labelled vials which were stored at -20°C until assayed.

The frequency of collection of the plasma samples was at a minimum interval of one week and most corresponded with a reproductive examination. However, this varied with mare and personnel availability during term and holiday times respectively.

Progesterone Assay

The Coat-A-Count Progesterone Assay (Diagnostic Products Corporation, Los Angeles, CA, USA) was used according to the manufacturer's instructions as described in Appendix A. This was a radioimmunoassay using I^{125} labelled progesterone and was read using a gamma counter to give a quantitative result. All samples and controls were run in duplicate.

The manufacturer's quality control data reported the approximate sensitivity of the assay to be 0.02ng/ml, the intra-assay coefficient of variation as <6.4% and the inter-assay coefficient of variation as <10%. Their results demonstrated that it was

highly specific for progesterone with a particularly low crossreactivity to other naturally occurring steroids or therapeutic drugs which may have been present in the unknown samples. They also confirmed there was no significant drift associated with a samples position within the run and the results from spiking recovery confirmed that the assay gave an accurate measure of progesterone concentrations throughout its working range.

3 control samples of low, intermediate and high progesterone values were produced by reconstituting known quantities of lyophilised progesterone solutions (ICN Pharmaceuticals Ltd., Oxfordshire, UK) with distilled water. They were stored at -20°C and incorporated into the assays as samples. The manufacturer's values for these control samples when used in an antibody coated tube radioimmunoassay kit such as this were low 1.29 ng/ml (range 0.93 - 1.65), medium 6.18 (range 5.08 - 7.28) and high 24.2 (range 20.1 - 28.3).

The intra or within assay coefficient of variation was calculated using each of the ICN control samples. Due to financial constraints and the desire to run as many samples as possible from individual mares in the same assay, only 6 of each ICN control were included in the one assay. Also, because of the number of samples to be assayed, only 5 runs of the kit were required. The inter or between assay coefficient of variation was calculated from the average of 2 samples of each ICN control across the 5 assays.

Any samples which gave unexpected results were reassayed. Also, when the results from an individual mare were obtained from more than one assay, a high and low sample were included in the subsequent runs to provide some continuity between them. The results were then plotted on a chart against the mare's reproductive behaviour on the date the sample was taken.

LH Assay

The double antibody radioimmunoassay used by Paramo Ramirez (1994) was performed with some modifications, as described in Appendix A. This was a double antibody radioimmunoassay using I^{125} labelled equine LH and was read using a gamma counter to give a quantitative result. All samples and controls were run in duplicate.

Plasma samples from a donor horse of unknown status (ICN Pharmaceuticals Ltd, Oxfordshire, UK), a mare at ovulation and a mid-dioestrous mare were collected

and stored as previously described. They were used as unknown, high and low controls respectively and run as samples at the start, middle and end of each assay.

The intra-assay coefficient of variation was calculated from 6 samples of each of the controls in one run due to the desire to run as many samples from individual mares as possible in the same assay. Again, because of the limited number of runs performed, the inter-assay coefficient of variation was calculated from paired samples of each control over 4 assays.

The LH results were also plotted on a chart against the mare's reproductive behaviour on the date the sample was taken. Due to time being limited, LH concentrations were measured for only eight of the eleven mares in the present study.

1.2.5 Prostaglandin Administration

On 13 occasions throughout the course of the study, prostaglandin was administered to mares at different stages of the luteal phase to bring them into oestrus. This was mainly to provide an oestrous mare for semen collection as part of another study but later in the present study it was also used to allow artificial insemination or on one occasion, in an attempt to produce oestrus at a known time in a mare who continually underwent silent oestruses.

A single dose of either 5mg Dinoprost (Lutalyse, Upjohn, Crawley, UK) or 250mcg Cloprostenol (Estrumate, Coopers, Crewe, UK) was administered intramuscularly.

1.3 Results

Figures 1.7 to 1.17 show the teasing scores together with the plasma progesterone and LH results for each mare.

The key for these figures is as follows:

Lower case letters - event is annotated

A+ - Successful A. I.

A- - Unsuccessful A. I.

Arrow - Prostaglandin administered.

F - Mare was flushed

Coat-A-Count Progesterone Assay

Calculation of inter-assay coefficient of variation for the low progesterone ICN standard gave a result of 5.9%, the medium standard 4.6% and the high standard 4.6%. These corresponded well with the manufacturer's results of <6.4% and suggested that within each run, the samples were relatively consistent across the range of values.

The low standard gave an inter-assay coefficient of variation of 8.3%, the medium standard 6.6% and the high standard 20.6%. The low and medium standards corresponded well to the manufacturer's results of < 10% suggesting that samples up to 6.18 ng/ml (range 5.08 - 7.28) are consistent between assays. However, samples with higher values may give more variable results.

The results from the samples which were reassayed either due to the result being unexpected or to provide continuity between assays are shown in Appendix B. Although there was some variation between the high values, on the whole they appeared relatively consistent. This confirmed that any unexpected results were in fact real and not errors in the assay.

Measurement of plasma progesterone concentrations indicated the presence or absence of functional luteal tissue and correlated well with the teasing and ultrasound results. Concentrations below 1 ng/ml were associated with the absence of functional luteal tissue and therefore indicated that the mare was either in oestrus or anoestrus. Values greater than 1 ng/ml indicated that the mare had a functional corpus luteum but could not be used to differentiate between the various circumstances associated with this such as dioestrus, pregnancy, prolonged dioestrus and the occurrence of spontaneously luteinised follicles.

Progesterone concentrations were useful to indicate whether ovulation had occurred or not under circumstances where there was insufficient information available using the other procedures. Examples were the prolonged oestrus associated with the first ovulation of the year (Misty d and e), silent oestruses (Lady j) and oestruses missed due to teasing not being performed (Meg f). However, on two occasions luteal tissue was identified on ultrasound examination in association with basal or low

progesterone concentrations. These CLs were presumed to be either non or poorly functional.

LH Assay

The mid-dioestrous mare used as the low LH control sample gave an average result of 0.96 ng/ml with a standard deviation of ± 0.18 which was expected. The high control was a mare at ovulation and gave an average result of 2.9 ng/ml ± 0.38 ng/ml which was much lower than the peak concentrations of 40-50 ng/ml that have been reported (Evans and Irvine, 1975) and suggests her LH was just starting to rise prior to the peak at the time of sampling. However, it was decided to continue using this sample as a control due to the small number of assays being run. The donor horse gave an average result of 3.47 ng/ml with a standard deviation of 0.31 which was actually higher than the 'high' control sample. There was therefore no real high control run in this assay.

The donor horse gave an intra-assay coefficient of variation of 8.97%, the mare at ovulation 13.3% and the mid-dioestrous mare 19%. This suggested that there was a relatively large variation between the low results which was consistent with the flat nature of the standard curve of the assay at these concentrations. However, this did not cause a problem in clinical interpretation due to the wide difference between high and low concentrations in the blood.

The donor horse gave an inter-assay coefficient of variation of 20.8%, the mare at ovulation 24.2% and the mid-dioestrous mare 19.5%. This indicated that the amount of variation associated with low results was similar both within and between assays but the intermediate samples gave far more variable results between assays. This meant that a comparison of values between runs would be open to misinterpretation but since all the samples from an individual mare were run in the same assay this was not considered to be a problem.

The sensitivity and specificity of this assay were not determined. However, high values were observed during pregnancy in two mares at stages where LH concentrations have been shown to be basal. These were attributed to cross reaction of the assay with eCG and this suggests that the specificity of the assay was not restricted to LH.

Increases in LH were recorded in association with three of four silent oestruses but not at any dioestrous ovulations. However, Greedy had raised LH concentrations during a prolonged interoestrous interval which was not associated with a dioestrous ovulation (Greedy j). No explanation for this could be found. Filly also

demonstrated unexplained rises (Filly h & j), although the latter could have been due to an undetected ovulation during the holidays or to feedback from oestrogen producing follicles prior to the first ovulation of the year.

Ultrasound Examination

The photos presented throughout this study highlight the variability in appearance of the various structures which occurred in the ovaries and uterus of the mares. With experience it became easier to gain better images and interpret these findings. However, this technique did not give any indication of the functional state of the structures which were present. For example, the CLs present at two consecutive dioestruses in Lady (f and g) both appeared the same on ultrasound despite one producing progesterone and the other being non-functional.

An attempt to predict the timing of ovulation using ultrasound examination was unsuccessful when the criterion described in the materials and methods was used. Although it indicated that ovulation would occur in the next few days, an exact prediction could not be made.

Average Cycle Lengths

In all of the following figures, only the events for which there was sufficient information were included. Oestrous periods not associated with ovulation, dioestrous intervals following an oestrus during which AI was carried out and dioestruses artificially shortened by prostaglandin were omitted.

The average length of oestrus was 6 days ± 2.4 and the distribution of oestrous lengths are shown in Figure 1.18.

Figure 1.19 demonstrates the distribution of oestrous length in relation to month. The small number of oestruses over the winter months were due to some of the mares entering anoestrus and not displaying oestrous behaviour in association with ovulation. It appears that the average length of oestrus increased during the winter months, being longest in December and January and shortened again as summer approached with May and June having the shortest oestruses.

The greatest standard deviations and therefore variations in length appeared to be during January and February and this decreased towards the summer suggesting oestrus became more uniform as the breeding season commenced. Statistical analyses was not performed due to the small group sizes so it is not known whether this observation was significant or not. The average length of oestrus in April would

have been longer if the three periods of greater than seven days could have been included.

The average oestrous length for each individual mare is shown in Figure 1.20 and this demonstrates that while some mares such as Jenny and Suzy appeared to have oestruses of relatively constant length, others such as Greedy, Lady and Poppy displayed a wide variation.

The range in lengths of oestrous behaviour and the variable relationship between oestrous behaviour and ovulation both between and within mares, are demonstrated in Figures 1.7-1.17. Examples are Corrie (a), Filly (d), Lady (g), Misty (b) and Poppy (a). This indicated that the information gained by observing oestrous behaviour in the mare is limited and is unlikely to be useful in the accurate prediction of ovulation.

Figure 1.21 shows the distribution of dioestrous length for all the mares in the study. For the sake of this study, dioestrus was taken as the number of days of negative behavioural signs between periods of oestrous behaviour. The average dioestrous length was 18.9 days with a standard deviation of 7.2. This includes all the cyclic anomalies displayed in the study that were not associated with pregnancy.

Variations Associated with Oestrous Behaviour

At housing in October, some of the mares displayed variations in their oestrous behaviour. Corrie was a particularly nervous mare and was stabled in a different box from previous years with a new stallion being used on alternate days for teasing. She displayed a short 2 day oestrus (Corrie a) with ovulation not occurring until 2-5 days after the end. Her next oestrus was completely silent but then she cycled normally until becoming pregnant.

In contrast, Poppy displayed an 11 day oestrus at housing which commenced before luteolysis was complete (Poppy a). However, other mares did not display alterations in their expression of oestrous behaviour despite being exposed to other potentially stressful situations Misty was in poor body condition, was separated from her foal for teasing and was later weaned without any changes in behaviour.

Filly displayed days of negative behaviour during her first three oestrous periods after housing (Filly a). These days were all ones on which the new stallion was used for teasing indicating a stallion preference. They occurred near the start and end of oestrus but not in the middle which suggested that as Filly approached ovulation she has less control over her behaviour.

When group teasing was carried out towards the end of the study Poppy, as well as displaying the normal signs of oestrus, also 'mouth clapped'. This involved lowering the head and opening the mouth with the lips covering the teeth. She did not display this sign when teased individually at the teasing board during the winter but did during the summer where she was second bottom in the dominance hierarchy in the field, although she did still approached the stallion to be teased. Jenny who was bottom of the hierarchy did not display this sign but also would not come forward to be teased even when in oestrus and had to be teased separately.

Four silent oestruses, which occurred in the absence of positive behavioural signs were detected throughout the study with Corrie displaying one (Corrie b) and Lady three (g, h and j). Repeated examinations were required to identify replacement of the original CL with a new one on ultrasound and also the return to basal progesterone concentrations associated with the follicular phase.

Silent oestruses are associated with an absence of positive oestrous behavioural signs but in Lady's case a mixture of actively negative signs such as kicking and squealing were seen in association with more positive signs such as winking and squirting of urine. This gave her an overall negative teasing score. In fact, her oestrous behaviour was erratic throughout the entire study with few instances of strong positive signs being displayed. Many split oestruses and one day oestrous periods were observed either in association with ovulation or with ovulation occurring a few days later. However, the stallion appeared to know when she was in oestrus despite her active rejection of him as shown by an increased interest in her at this time.

Lady's silent and erratic oestruses were not associated with any change in management. LH peaks were recorded in association with two of Lady's silent oestruses and many of her erratic ones. No LH results were available for her third silent oestrus and no LH rise was detected in association with Corrie's silent oestrus.

As well as her erratic oestrous behaviour, luteolysis seemed to occur after a shorter time than usual in most of Lady's cycles. Her average dioestrous interval was 14.6 ±1.4 days, although this figure excluded the prolonged dioestrus at Lady (i). The reason her dioestrous intervals seemed normal was because ovulation did not occur until after the onset of negative behaviour in many cases.

On a number of occasions fluid accumulations were seen on ultrasound in Lady's uterus although no polymorphonuclear leucocytes were detected in smears. Her cervix was damaged and incompetent due to a previous late-term abortion and she

had a tendency to pool urine in her cranial vagina (See photo 1.30). She also had numerous endometrial cysts (See photo 1.31) although despite her being aged and multiparous, her perineal conformation was good as defined by Pascoe (1979).

Variations Associated with Dioestrus

Seven prolonged dioestrous intervals occurred in the present study unassociated with pregnancy (Gemma a, Greedy d, Jenny a, Jenny b, Laura d, Suzy c & Poppy d).

Four cases (Greedy d, Jenny b, Laura d & Poppy d), were associated with maintenance of the original CL. In the cases of Greedy (d) and Laura (d), these intervals followed an oestrus at which an insemination was performed. However, since no conceptus was detected at the time when luteolysis should have occurred, maternal recognition could not have been responsible for the maintenance of the CL.

No other abnormalities were detected in any of these mares which could provide a reason for this occurrence so they were termed idiopathic. The lengths of these intervals were 39, 64 and 34-36 days. Follicular development occurred at the expected time of 14-16 days in all of these mares but none of them returned to oestrus until the CL had regressed.

The gradually declining progesterone concentrations in Jenny (b) suggested natural ageing and regression of the CL occurred while the sudden reduction in concentrations seen in Greedy (d) and Laura (d) suggested that lysis was induced. In Poppy (d), a prolonged interval was initiated involving one CL but prostaglandin was administered on day 24 so the length it would have lasted is not known.

The other three cases (Gemma a, Jenny a & Suzy c) were associated with dioestrous ovulations detected on ultrasound.

Dioestrous ovulations were detected in six out of 83 luteal phases in the present study giving a 7.2% incidence, with half occurring in the same ovary as the original CL. Of these six, only three were associated with prolonged luteal phases as previously mentioned and all were associated with ovulations occurring late in dioestrus around day 11. Another two (Gemma e & Suzy a), were associated with normal dioestrous length and ovulations which occurred early in dioestrus, around day 6. In the final case (Suzy h), prostaglandin was administered on day 15 before it was known whether a prolonged dioestrus would have resulted.

In the case of Gemma (a), the progesterone profile suggested that the second CL was poorly functional during the second half of the interval and it is likely that it was partially lysed along with the original CL at the usual time of luteolysis. On ultrasound, the appearance of the second CL altered around the period associated with lysis of the original one, becoming smaller and denser which supported this theory.

The lengths of prolonged dioestrus associated with the dioestrous ovulations in this study are 37-39 days and 35 days while the exact length of Jenny (a) beyond 37 days is not known due to it commencing before the start of the study. The two dioestrous ovulations associated with normal dioestrous interval would have remained undetected if ultrasound examination had not been performed at that time.

There were no detected increases in LH associated with Gemma's dioestrous ovulations (Gemma g & h) but since there are no LH results available for the other two mares who also had them, it was not known whether this is significant or not.

An apparently prolonged dioestrous interval occurred in Meg (f) over the holidays but progesterone results confirmed that this was caused by a missed oestrus due to teasing not being performed.

Seasonal Variations

25% of the ponies (1 of 4) and 71% of the horses (5 of 7) continued to cycle throughout the winter, giving a total of 55% (6 of 11). Corrie became pregnant in January and had cycled until then so was included in the ovulatory group.

Lady, the one pony mare who continued to cycle was housed during the winter while the other three ponies were kept outside. All the horse mares were housed under the same conditions, however Gemma and Meg were the only two that entered anoestrus. All the mares were in good body condition throughout the study except for Misty who was in poor condition due to being kept outside and also feeding a foal.

Four of the mares which entered anoestrus had their last oestrus associated with ovulation in November (Filly b, Meg b, Misty a, Suzy b). In the case of Meg, despite the resulting CL still being visible on ultrasound ten days later, the low progesterone concentrations indicate that it was no longer functional. There are no teasing results for the period around the 15th December as she was lame and her dressing did not allow her to be walked to the teasing area (Meg c). However, she did display positive signs when other mares were walked past her box, indicating she was in oestrus, although it is not known whether she ovulated then or not. No

increase in LH was detected at this time (Meg j) so the follicles that had developed probably became atretic and regressed (See photo 1.24).

Misty also followed a similar course of events at the start of December with follicular development and oestrous behaviour but no ovulation (Misty b). Filly displayed one day of oestrous behaviour before the holidays on December 16th (Filly c), but it is not known if she displayed signs for any longer, if it was associated with follicular development or if she ovulated.

Gemma was unusual in that she did not have her final ovulation before entering anoestrus until January 24th (Gemma b). Her stable was outside the shed and so she may have received additional light initially which delayed her entry into anoestrus. She went into anoestrus straight after luteolysis, without displaying any follicular development or oestrous behaviour.

Suzy had her final oestrus associated with ovulation of the breeding season in November (Suzy b) at a similar time to the other pony mares. However, although she did not display any further signs of oestrus until the following spring, she did not go straight into anoestrus. She had a dioestrous ovulation in mid November (Suzy c), which produced a luteal phase of 35 days and once her progesterone concentrations had returned to zero following regression of these CLs, there was another increase to 7 ng/ml for less than 14 days (Suzy d). Progesterone concentrations then remained basal until an increase to 1.2 ng/ml occurred in January for a short period of time (Suzy e) before concentrations returned to zero for the rest of the winter.

On both occasions, a luteinised structure was identified in the ovary on ultrasound (See photo 1.28b) and was therefore the most likely the source of the progesterone. However, the origin of these structures was not clear since she did not display any signs of oestrus which would have been expected to have preceded an ovulation. If ovulation did occur, it must have been associated with a silent oestrus.

The structures were of a similar shape and size to preovulatory follicles but with the development of areas of increased echogenicity within their centres. The lack of the collapse of the follicles and the absence of a tract between the follicles and the ovulation fossa, which both accompany ovulation and help to give the CL its distinctive appearance on ultrasound suggested these may have been spontaneously luteinised follicles.

Suzy's teasing scores ranged on average from 1-4 throughout her anoestrous period and no obvious behavioural differences were noted between the periods when the

luteinised follicles were present and those where her progesterone concentrations were basal.

As well as these two instances of luteal tissue of undetermined origin, Suzy also displayed a similar incident prior to the start of this study. A large, soft mass was present in her left ovary over several weeks, totally obscuring it. It's echotexture on ultrasound examination was originally hypoechoic but became more echogenic over time and a trabecular pattern emerged across the structure which appeared to be the development of a fibrin network and suggested the organisation of a blood clot. Suzy continued to cycle normally despite its presence and it had regressed completely by the start of the study.

On a couple of occasions, structures resembling luteal tissue, such as the one in photo 1.28, were recorded on ultrasound examination of the five anoestrous mares by undergraduates. However, the corresponding progesterone results were basal indicating the structures were non-functional. They could have represented incompetent CLs due to lack of hormonal support but were more likely to have been areas of dense stroma which became visible due to the lack of follicles in the ovary.

Gemma, Meg and Suzy displayed few signs of oestrous behaviour over the winter months in association with small, featureless ovaries and few small inactive follicles on ultrasound examination. Filly and Misty however, displayed erratic, irregular periods of oestrous behaviour and waves of follicular development up to 4 cm diameter throughout the winter. Their basal progesterone concentrations for this time confirmed that the follicles were anovulatory.

All three of the ponies had their first ovulation of the year on the 23rd April (Filly e, Misty e and Suzy f) after an oestrous period that was at least seven days long. Due to the holidays the exact length of time this behaviour was displayed for is not known.

Meg's first oestrus after the winter period occurred on March 17th and was five days long (Meg e) so she did not display a prolonged oestrus as the pony mares did. Although there is not enough information to determine Gemma's behaviour at her first ovulation of the year (Gemma d), it occurred on May 2nd which is relatively late in the breeding season.

Greedy displayed an interoestrous interval of 22-27 days in December (Greedy b) and lack of information makes it unclear as to whether she entered a brief period of anoestrus or had indeed ovulated after the preceding oestrus. However, progesterone concentrations were low nine days before the next oestrus, which means any CL

must have lysed by then. This suggests there was a long follicular phase associated with the next oestrus which could reflect the lower than usual concentrations of FSH in the circulation at this time of year causing a reduction in the rate at which the follicles develop and mature. This also corresponds with her subsequent oestrus being 13 days in length (Greedy c), reflecting the longer period necessary before oestrogen production was sufficient to give a high enough LH increase to produce ovulation (Greedy i). This could have occurred whether she had entered anoestrus or not.

Multiple Ovulations

The incidence of double ovulation in this study was 8 of 83 ovulations or 9.6% with no triple ovulations occurring. They were associated with only three of the mares with Misty, Highland pony cross Thoroughbred having four, Meg, a Thoroughbred having three and Poppy, a part Thoroughbred having one.

Misty's first ovulation of the year (Misty e) was a multiple ovulation.

Both unilateral and bilateral double ovulations occurred with the same frequency. Photos 1.23a and b demonstrate a bilateral double ovulation and photos 1.26 a and b a unilateral one. In this study, 50% of the double ovulations occurred within 24 hours of each other and were termed synchronous with the rest occurring greater than 24 hours apart and being termed asynchronous. 3 of the 4 synchronous ovulations occurred in the same ovary.

Of the 69 single ovulations in the present study, 54% occurred from the left ovary and 46% from the right. Filly and Misty who both had foals last year ovulated from the left ovary in 87.5% and 82% of cases respectively. Poppy also had a foal last year and had left-sided ovulations 57% of the time, while the three maiden mares Gemma, Jenny and Laura ovulated from the left ovary in 50%, 66% and 50% of cases respectively. The rest of the mares demonstrated left sided ovulations in 25% to 58% of cases.

Prostaglandin Administration

The outcome associated with each of the prostaglandin administrations is shown in Figure 1.22 although only the cases for which there was adequate information were included. In Greedy's case, the prostaglandin was used to end a pseudopregnancy (Greedy g), in Poppy to terminate a pregnancy at day 18-20 (Poppy b) and in Jenny following the occurrence of early embryonic death (Jenny d). These aspects of the treatment will be discussed in Chapter Four.

The mares injected with prostaglandin all entered oestrus within 2-3 days following treatment with the exception of Gemma (f), who took more than six days. In this instance, the prostaglandin was administered on day 5-7 of dioestrus and the CL seemed to take longer to lyse than expected possibly due to it not being fully mature at the time of treatment.

Poppy was administered prostaglandin on day five of dioestrus and came into oestrus within two days (Poppy e). However, it took more than nine days for a follicle greater than 3cm in diameter to develop which suggests that at the time the CL was lysed, there was not a follicle at a sufficiently advanced stage to become dominant and go on to ovulate. A prolonged follicular phase therefore occurred. It can be seen from Figure 1.22 that the length of oestrus occurring after prostaglandin administration was quite variable between but relatively constant within mares and since Poppy tends to have relatively long oestrous periods anyway, this may not have been significant.

Suzy also received prostaglandin at a similar stage of dioestrus (day six) but came into oestrus within the usual two days and displayed a normal length of oestrus (Suzy g). In addition, at her next dioestrous interval (Suzy h), prostaglandin was administered at day 15. A dioestrous ovulation had occurred at day 11 meaning this resulting CL was therefore only four days old at treatment yet she still came into oestrus three days later.

Because Lady did not show signs of oestrus well, prostaglandin was used at a time when a CL was visible on ultrasound and progesterone concentrations were high, in order to induce oestrus at a known time (Lady j). Following treatment, the appearance of her ovaries and reproductive tract on ultrasound confirmed she was in oestrus, as did her basal progesterone concentrations but still she did not show behavioural signs of oestrus. None of the other mares displayed a silent oestrus following prostaglandin treatment therefore confirming Lady's predisposition to this.

Ovarian Abnormalities

Information regarding Greedy's ovulations have been excluded from this calculation as she has a granulosa cell tumour in her right ovary and therefore her left ovary is the only functional one. Although Greedy's left ovary is smaller than expected for a mare of her size, it continued to function normally throughout the study with follicular development, maturation, ovulation and CL formation (Greedy a). Photos 1.32a & b show a follicle in her left ovary before ovulation and the CL that formed from it. Her oestrous periods occurred at regular intervals associated with basal

progesterone concentrations followed by values of over 1 ng/ml during the luteal phase.

Greedy's right ovary was so large that only the caudal area could be palpated or scanned. It sat in the ventral midline in the caudal abdomen, pulling the left ovary into a more ventral and medial position than expected. On ultrasound, most of the tumour consisted of large fluid filled areas surrounded by dense stroma, the fluid containing echogenic particles suggestive of debris (See photos 1.33a & b and photo 3.3). This appearance was different from that of an ultrasound photo taken a number of years age when most of the tumour had a honeycomb texture (See photo 1.33c). At that time, Greedy was displaying continuous oestrus or nymphomaniac behaviour possibly in association with high oestrogen concentrations. It was possible that the change in appearance of the tumour was related to an alteration in its activity since it was clearly no longer producing substances which caused behavioural or cyclic abnormalities. Greedy had therefore returned to normal cyclic function.

Throughout the study, a 2 cm fluid filled structure was palpable in the area between Jenny's left ovary and uterine horn. (See photo 1.34). Its size, shape and position did not alter and it was considered to be a peri-ovarian cyst.

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Figure 1.7 - CORRIE

Progesterone

a/ Short 2 day cycle at housing. Ovulation detected on ultrasound 2 - 5 days after end of oestrus. Poor progesterone rise.

b/ Silent oestrus. CL in right ovary and 4 days after no longer visible on ultrasound, ovulation from left ovary occurred.

c/ Fertile in January. AI achieved pregnancy.

d/ Prostaglandin administered on day 41 and 46 of gestation. Progesterone concentrations fell to 2.5 ng/ml.

e/ Still pregnant. Rise in progesterone concentrations to maximum of 24.4 ng/ml at day 109.

f/ Still pregnant. Steady fall in plasma progesterone to 4.6 ng/ml on day 192 at the end of study.

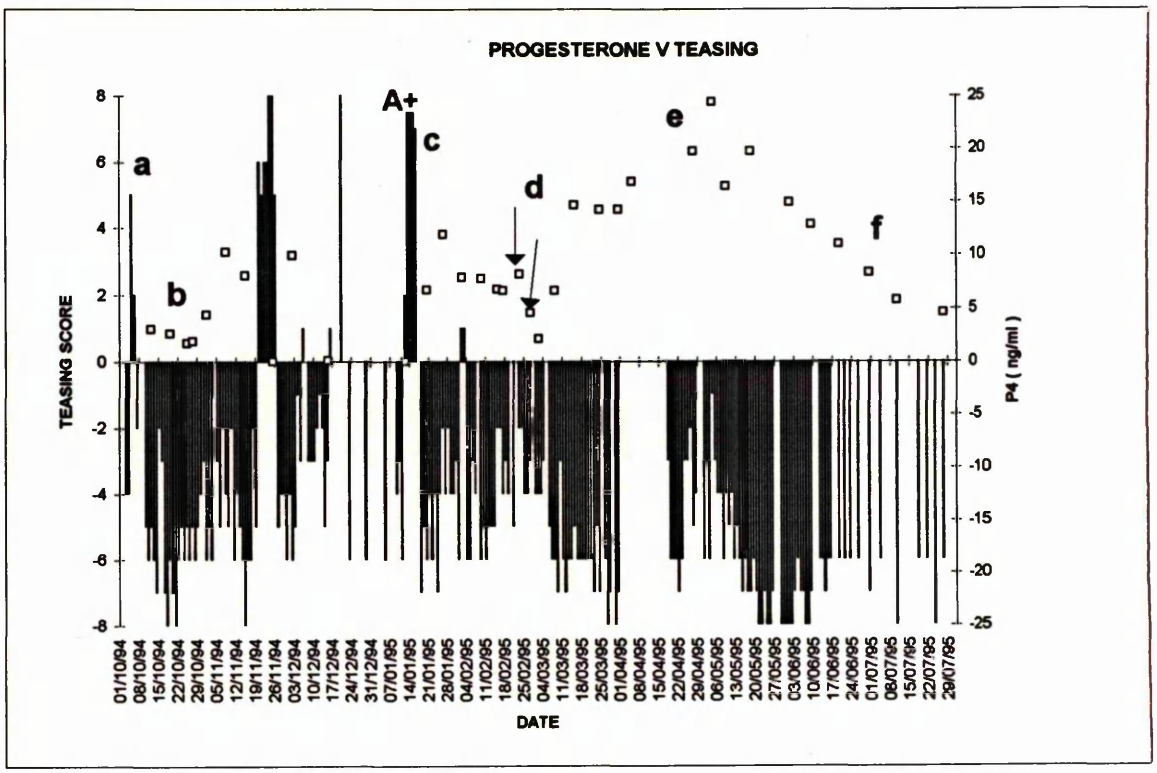
LH

g/ No LH surge associated with silent oestrus.

h/ High LH results due to cross reaction with eCG.

i/ Wide fluctuations in results.

Figure 1.7 CORRIE'S RESULTS



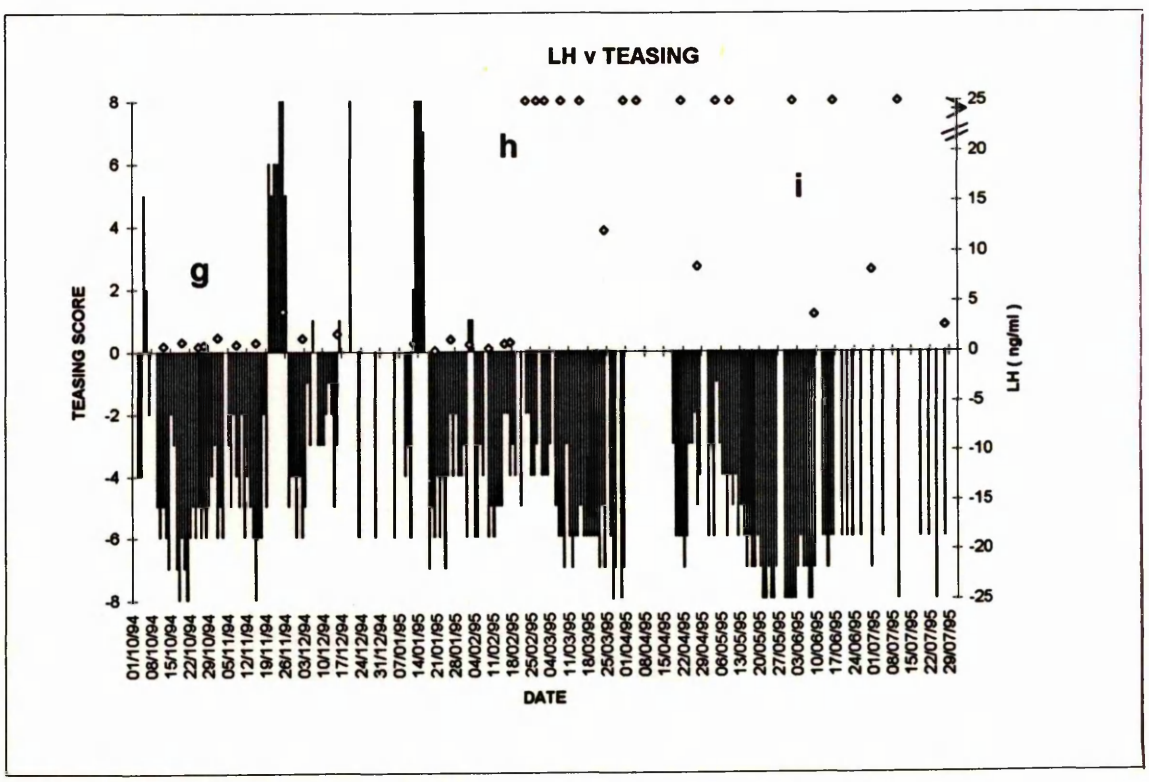


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Figure 1.8 - FILLY

Progesterone

a/ Days of negative behaviour during 3 oestruses after housing. Associated with new stallion.

b/ Last ovulation of the year at end of November.

c/ One day oestrous behaviour before holidays. Not known if returned to oestrus or entered anoestrus

d/ Anoestrus followed by erratic, irregular periods of oestrous behaviour not associated with ovulation.

e/ 1st ovulation of the year on 23rd April. Oestrus more than 7 days long so excluded from calculations as exact length not known..

f/ Pregnant. Flushed on day 14 - 15. Spontaneous oestrus 3 days later.

g/ Series of prostaglandin injections to bring into oestrus for A.I. and semen collection.

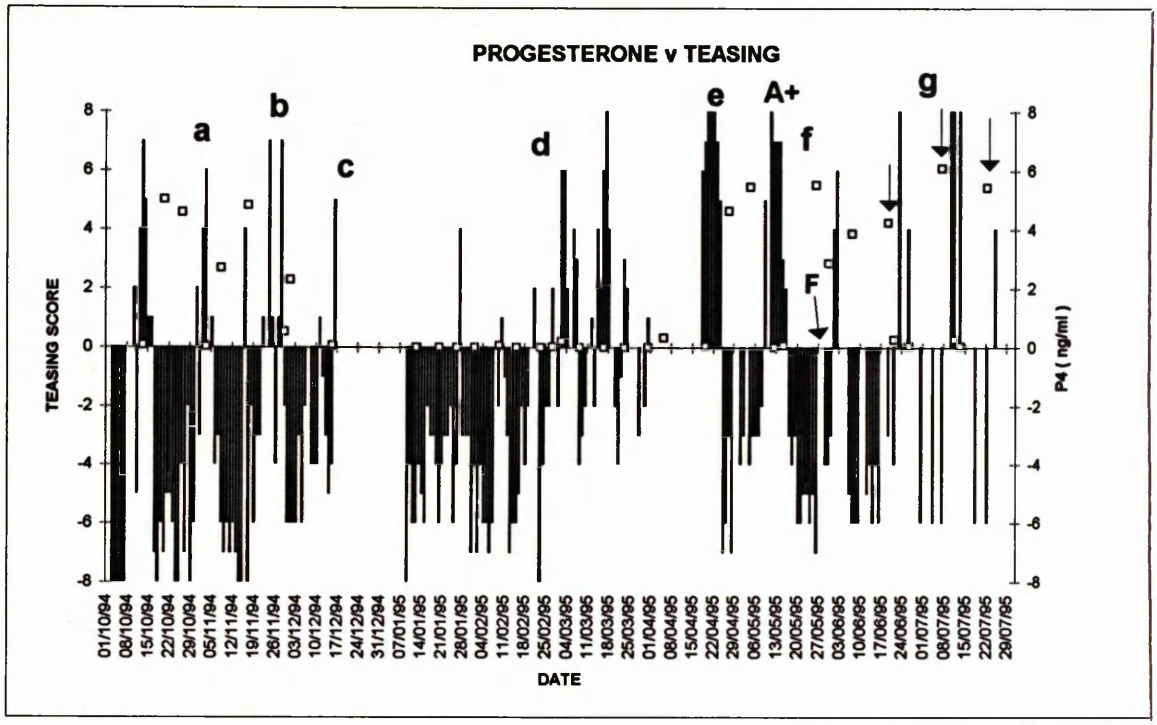
LH

h/ Unexplained high LH result.

i/ LH peak associated with last ovulation of the year.

j/ LH peak during holidays so no other information. May be due to oestrogen feedback from competent follicles. Short luteal phase if ovulation did occur.

Figure 1.8 FILLY'S RESULTS



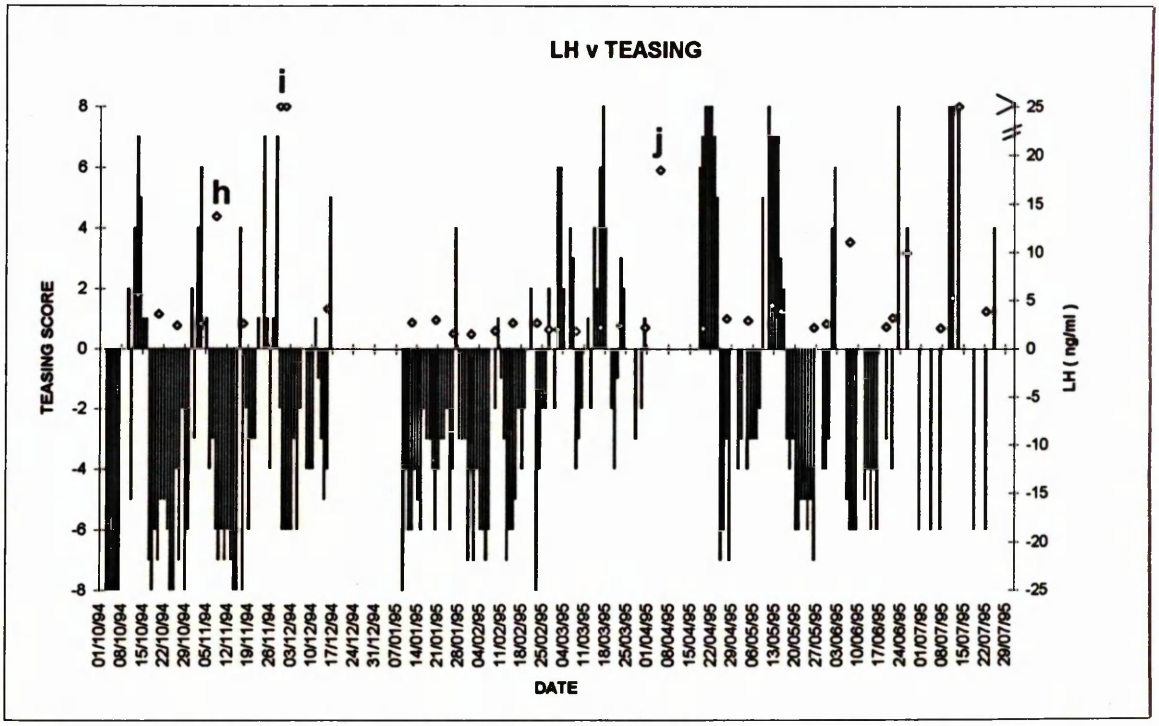


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Figure 1.9 - GEMMA

Progesterone

a/ Prolonged interoestrous interval (37-39 days) due to dioestrous ovulation before day 11 detected on ultrasound. Relatively low progesterone for second part, suggests partial lysis of new CL along with original one at expected time of luteolysis.

b/ Last ovulation on 24th January associated with 9 day oestrus. Normal luteal length. No return to oestrus.

c/ Anoestrus. Few behavioural signs.

d/ 1st ovulation after anoestrus on 2nd May.

e/ Early dioestrous ovulation detected on ultrasound. Associated with normal interoestrous interval. Photo 1.17a shows the pear shaped follicle in Gemma's left ovary prior to ovulation, photo 1.17b shows the original CL that formed from it and photo 1.17c shows the second CL which was present in the right ovary by day six

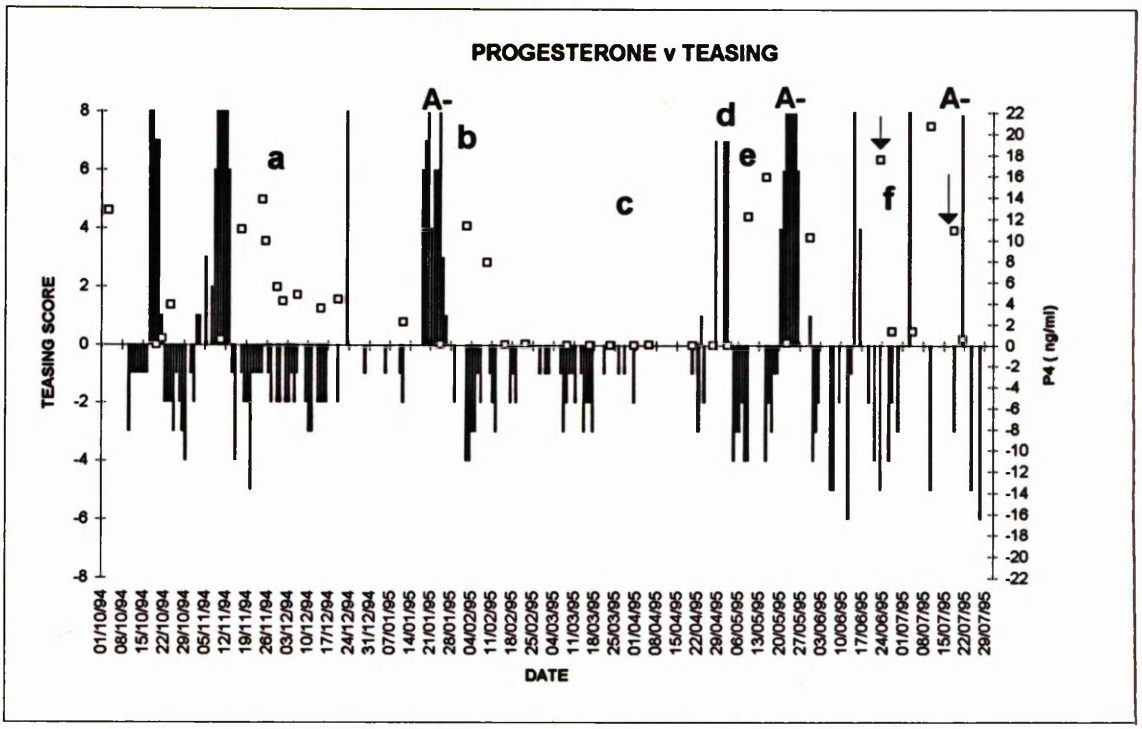
f/ More then 6 days between prostaglandin administration and return to oestrus.

LH

g/ No LH peak associated with dioestrous ovulation.

h/ No LH peak associated with dioestrous ovulation.

Figure 1.9 GEMMA'S RESULTS



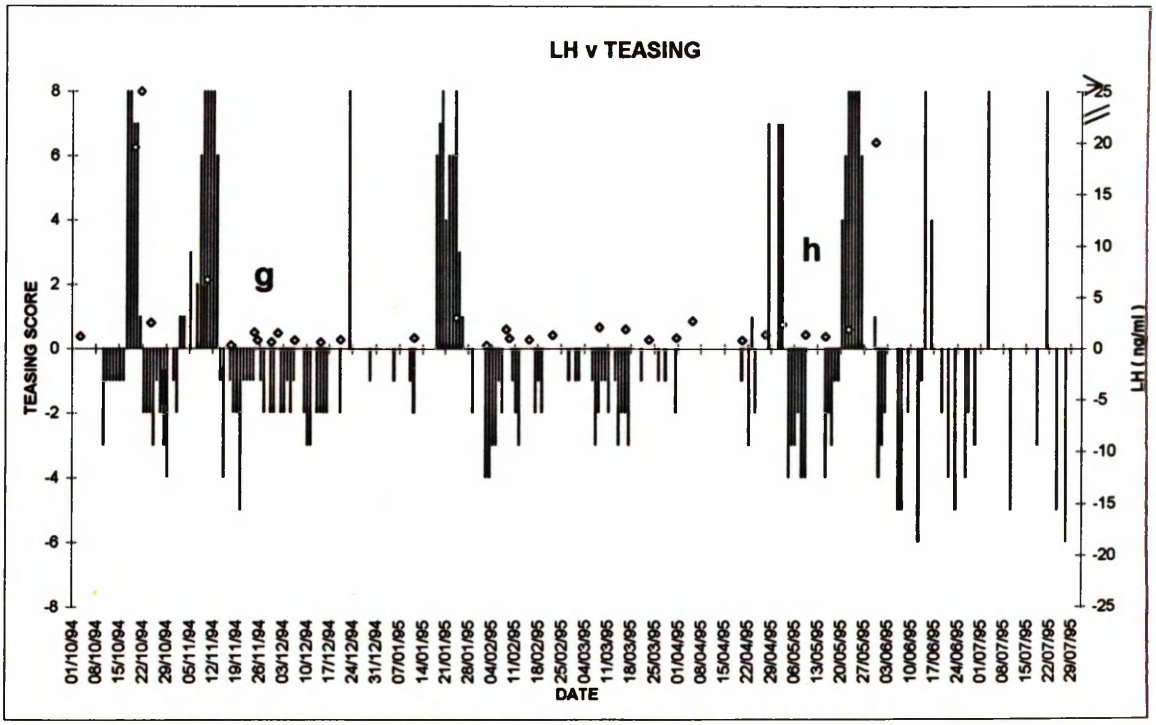


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Figure 1.10 - GREEDY
This mare has a granulosa cell tumour in her right ovary therefore her left ovary is the only functional one.

Progesterone

a/ Normal cyclic behaviour, follicular development and CL formation (See photos 1.18a & b).

b/ 22-27 day anovulatory period or normal ovulatory cycle with prolonged follicular phase before subsequent oestrus.

c/ 13 day oestrus associated with ovulation.

d/ 39 day prolonged interoestrous interval with one CL visible on ultrasound. Poor progesterone rise.

e/ Pregnant. Prostaglandin administered days 39, 40 and 41.

f/ Foetus dead (no heartbeat) but visible for 10 days on ultrasound. Resorbed in next 6 days. Progesterone rose slightly then fell to low concentrations (1.2 ng/ml).Entered Pseudopregnancy Type II.

g/ Prostaglandin administered to terminate pseudopregnancy. Prolonged 31 day oestrus 2 days after administration not associated with follicular development.

LH

h/ LH peak at end of normal oestrus. Associated with ovulation.

i/ Low LH concentrations at end of oestrus then high result 4 days into dioestrus.

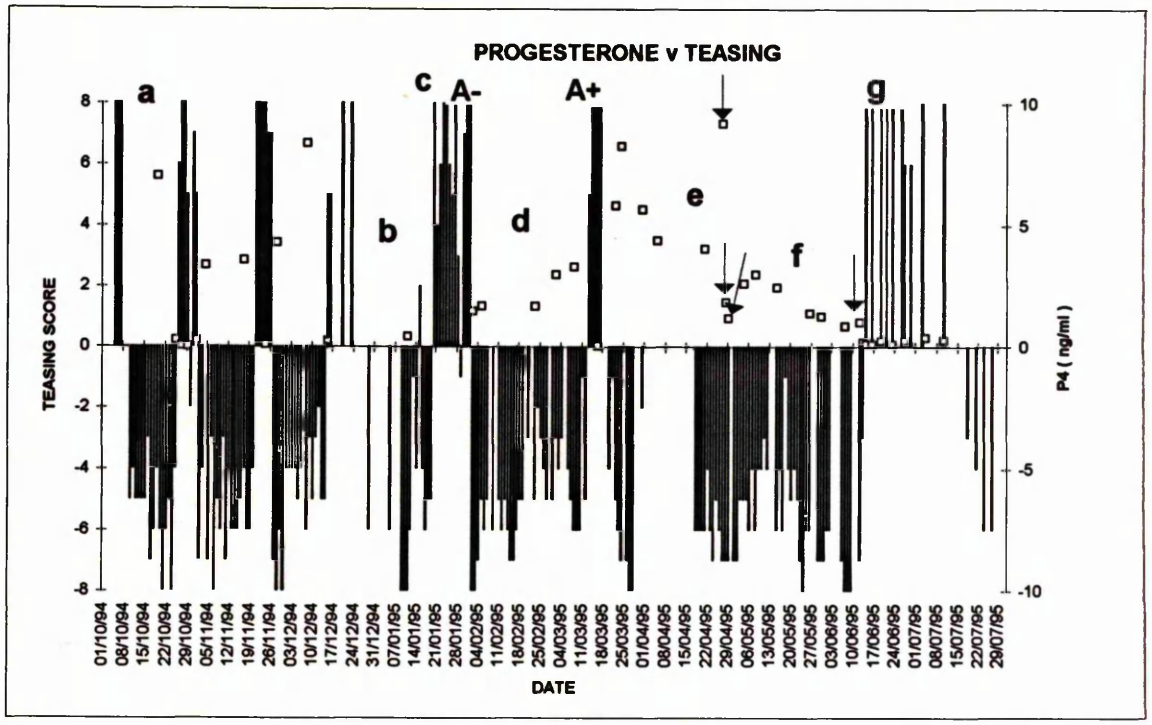
j/ LH concentrations raised during prolonged interoestrous interval. No dioestrous ovulation detected on ultrasound though.

k/ High LH results due to cross reaction with eCG. Concentrations elevated before prostaglandin administration and rose again after.

l/ Continuing eCG production. Wide fluctuations so unclear if concentrations declining or not.

m/ High concentrations at start of prolonged oestrus following prostaglandin administration. No suppression of oestrous behaviour. No further results available.

Figure 1.10 GREEDY'S RESULTS



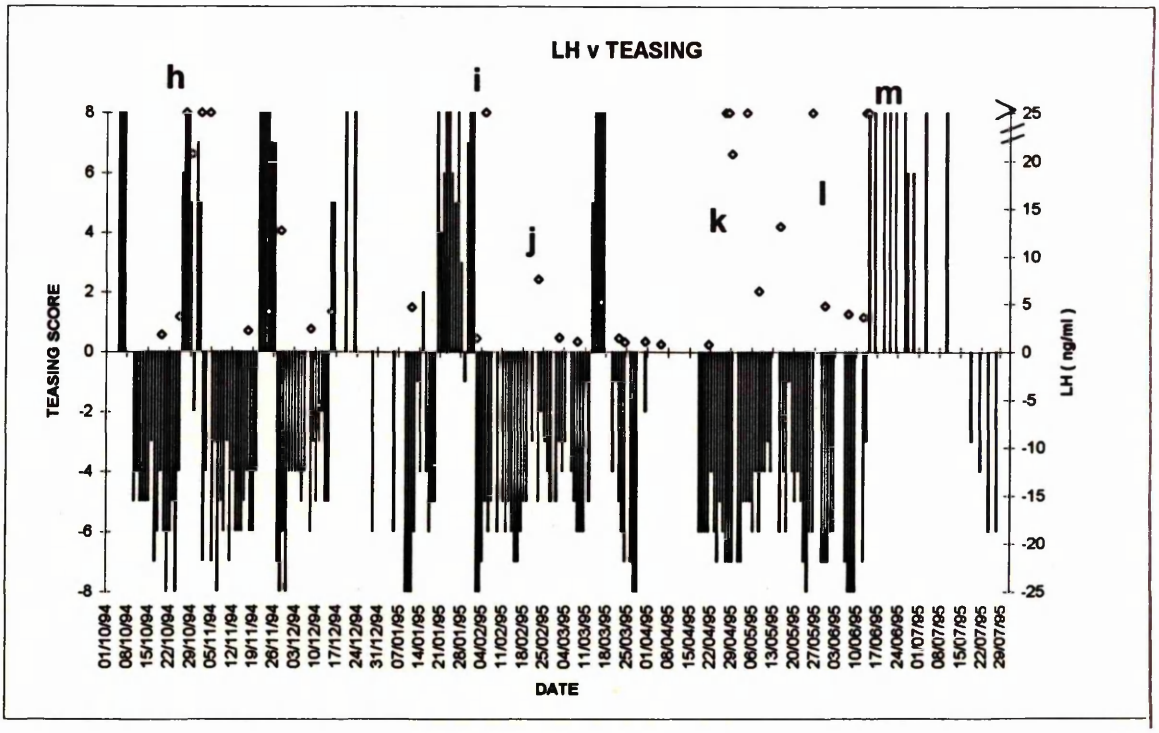


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Figure 1.11 - JENNY

Progesterone

a/ Prolonged interoestrous interval >37 days commencing before start of study. 1 small and dense CL, shown in Photo 1.19a present initially but no longer visible towards the end of the interval. Second CL clearly visible on ultrasound, shown in Photo 1.19, suggesting dioestrous ovulation.

b/ Spontaneous 64 day prolonged interoestrous interval with one CL on ultrasound. Photos 1.20a-c show the progression of the CL from centrally hypoechoic in the early stages, to a uniformly dense structure once the central clot had resolved and then becoming hyperechoic as it regressed in association with declining progesterone concentrations.

c/ Early embryonic death occurred between d 13 and 18. Single CL maintained giving 61 day prolonged interoestrous interval. Pseudopregnancy Type I.

d/ Early embryonic death day 19 - 20. Prostaglandin administered day 20.

e/ Anovulatory follicle present as well as CL.

f/ Pregnant. Flushed day 17. Spontaneous return to oestrus 5 - 7 days later.

Figure 1.11 JENNY'S RESULTS

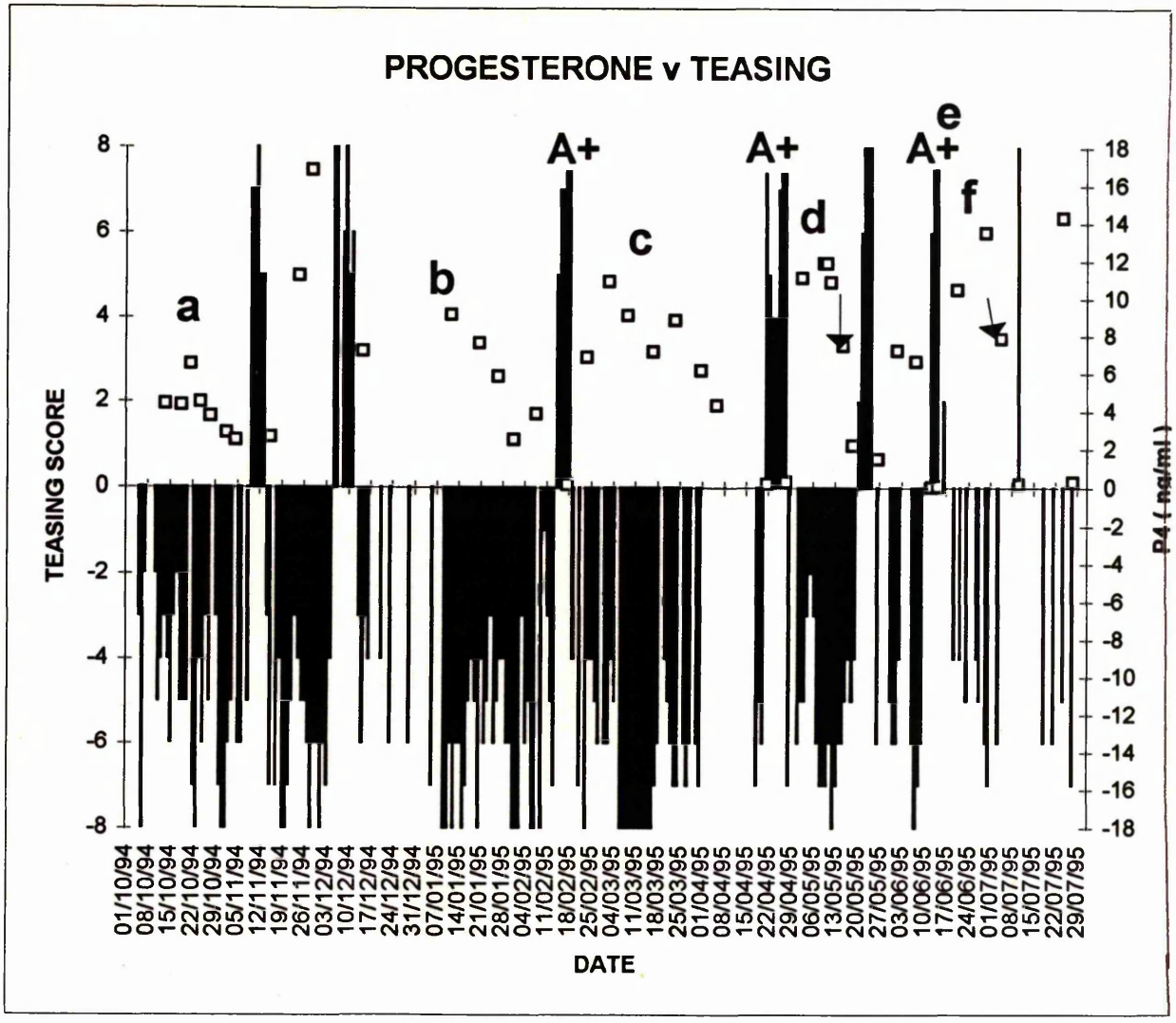

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Figure 1.12 - LADY

Progesterone

a/ 10 day oestrus. Progesterone high by 2 days before end.

b/ Ovulation after end of oestrus. Poor progesterone rise. CL not evident by 10 - 11 days post ovulation on ultrasound.

c/ CL present but short interval and no progesterone results to confirm if functional.

d/ Single day of oestrous behaviour. Ovulation 3 - 5 days later. Short luteal phase although interval between oestruses appears normal.

e/ Holidays - no information if ovulated or not.

f/ Single day of oestrous behaviour. CL formed non-functional shown by low progesterone.

g/ Silent oestrus. Ovulation and formation of new functional CL shown in photo 1.21.

h/ Premature luteolysis of previous CL by day 13 shown by low progesterone. Silent oestrus associated with little follicular development. Ovulation occurred within 5 days and new CL detected on ultrasound. (See photos 1.22a &b)

i/ Approximately 35 day prolonged luteal phase following unsuccessful AI. Terminated by prostaglandin

j/ Silent oestrus following prostaglandin administration. Associated with ovulation and CL formation on ultrasound.

LH

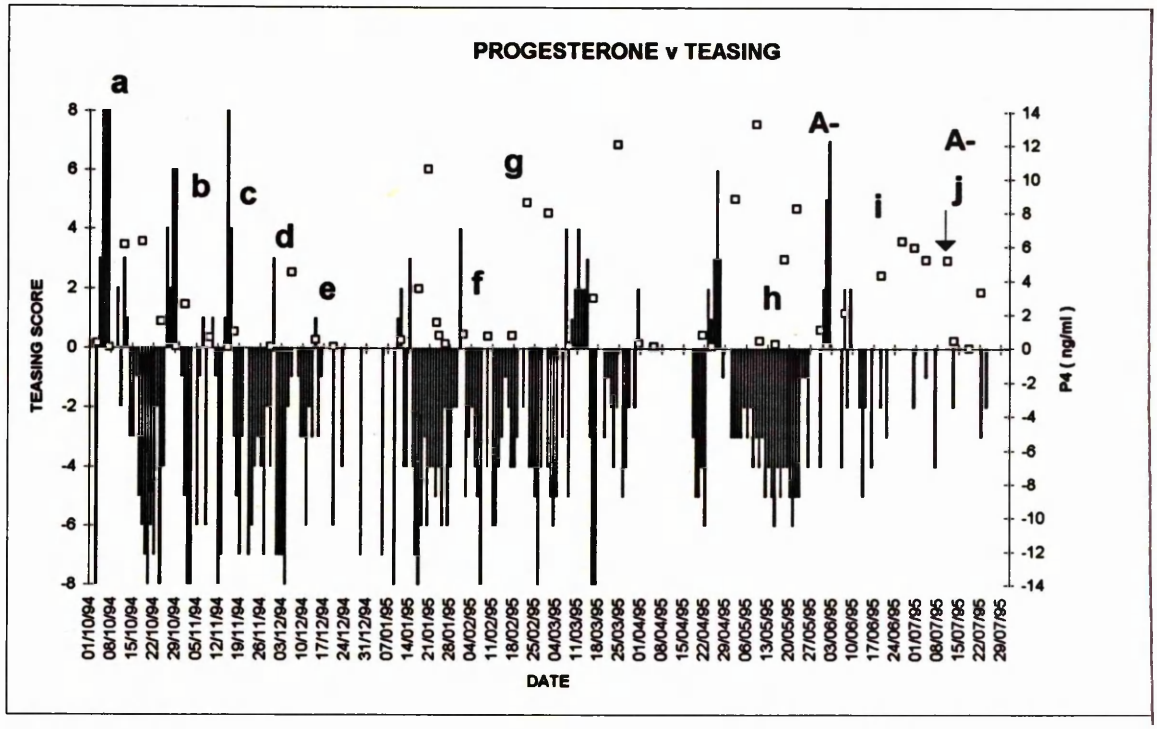
k/ LH peak associated with ovulation before end of 10 day oestrus.

l/ LH peak suggests ovulation did occur at Lady e despite lack of information to confirm it.

m/ LH peak associated with a silent oestrus (Lady g).

n/ LH peak associated with a silent oestrus (Lady h).

Figure 1.12 LADY'S RESULTS



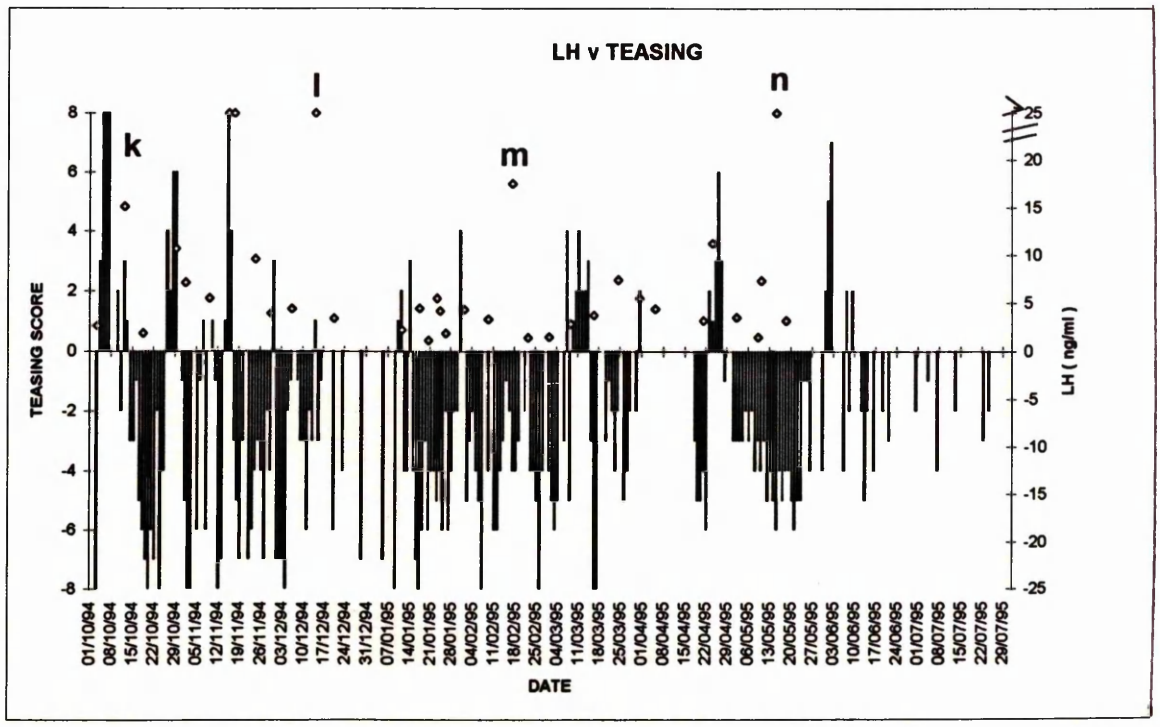


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Figure 1.13 - LAURA

Progesterone

a/ 4 day oestrus. Next oestrus 8 days long.

b/ Early embryonic death 19-24 days and original CL maintained giving 75 day prolonged interoestrous interval. Pseudopregnancy Type I. Return to oestrus spontaneous.

c/ 4 unsuccessful A.I.s.

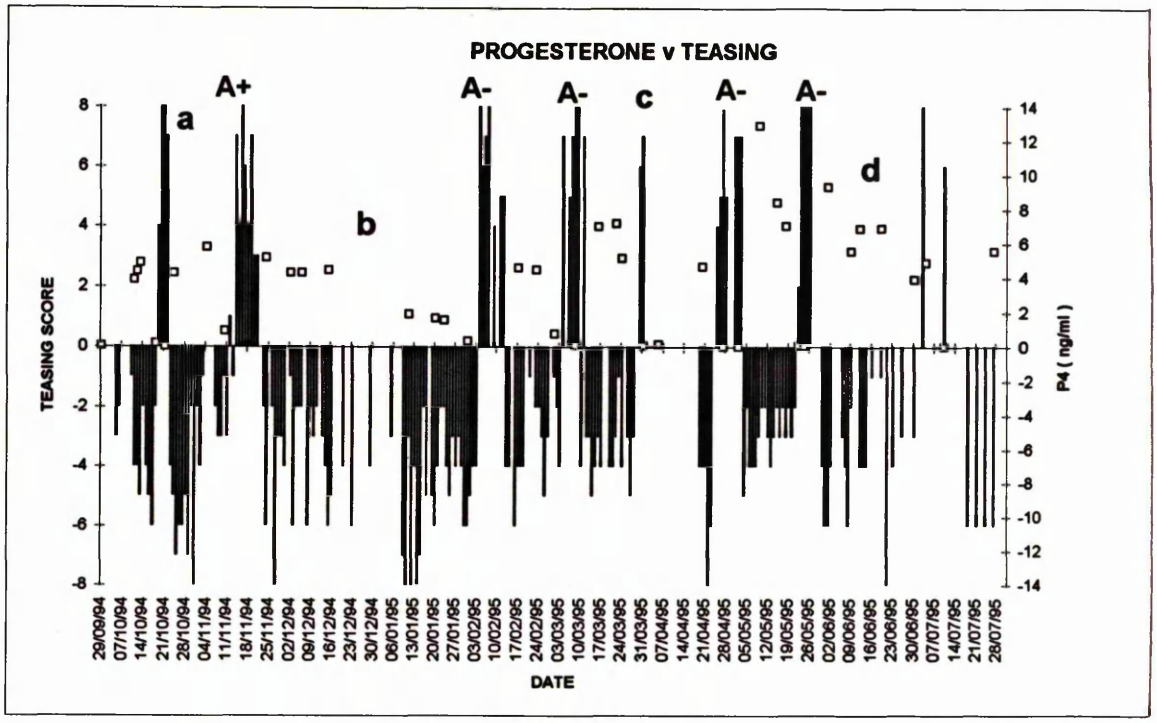
d/ 34 - 36 day prolonged interoestrous interval. One CL visible on ultrasound.

LH

e/ No LH peak associated with ovulation. Probably due to infrequency of sampling.

f/ LH peak associated with ovulation. Only start of oestrus detected due to holidays.

Figure 1.13 LAURA'S RESULTS



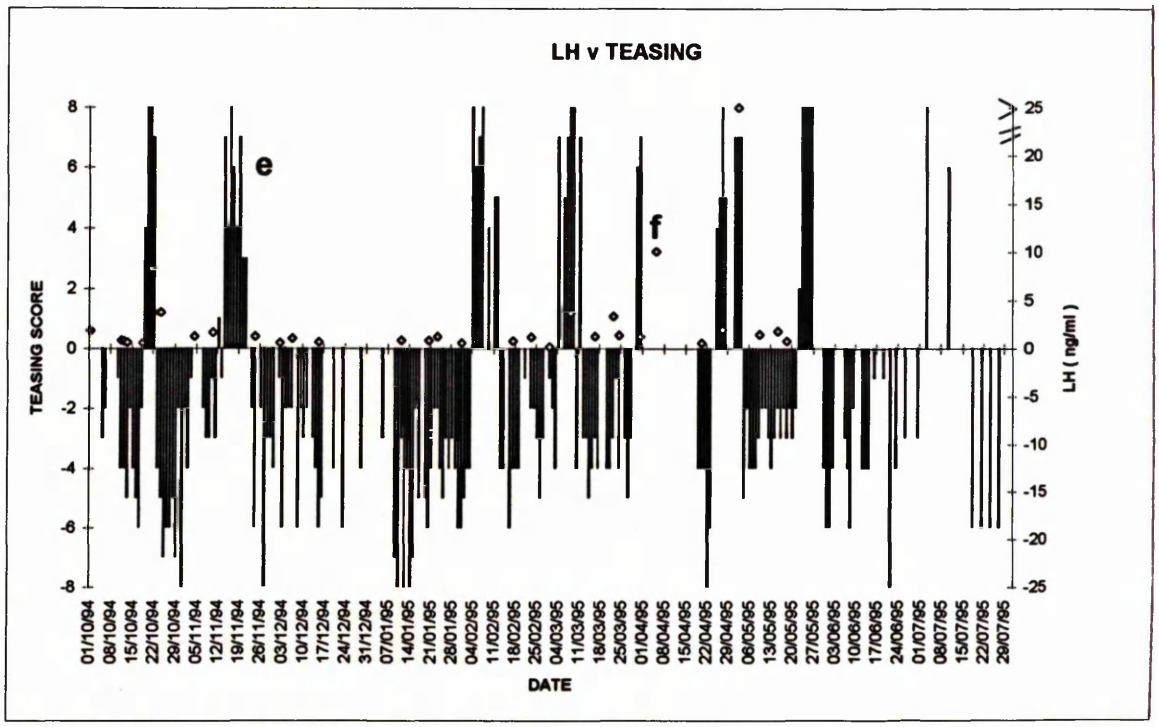


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Figure 1.14 - MEG

Progesterone

a/ Double ovulation left and right ovaries (See photos 1.23a & b).

b/ Last ovulation of year on 25th November. CL still visible 10 days later on ultrasound but progesterone concentrations low so not functional.

c/ In oestrus but not teased as lame. Development of large follicles (Photo 1.24) but no ovulation so follicles became atretic and regressed.

d/ Anoestrus. Indifferent behaviour.

e/ 1st ovulation of year on 17th March.

f/ Missed oestrus due to teasing not being performed during the holidays.

g/ Unsuccessful A.I. in presence of corpus haemorrhagicum < 24 hours post ovulation.

h/ Successful A.I. in presence of follicles <30 mm diameter.

i/ Pregnant. Flushed day 21 - 23. Prostaglandin administered.

LH

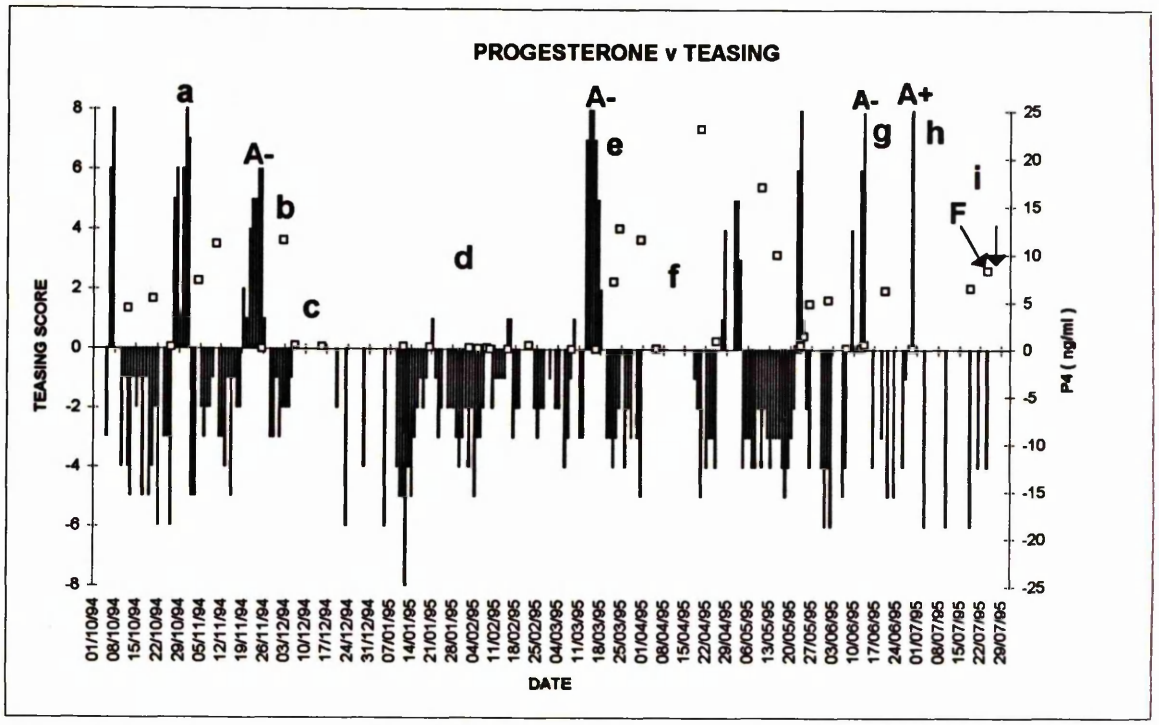
j/ No detectable LH rise associated with the last oestrus of year. Follicles did not ovulate but became atretic.

k/ Concentrations very low during anoestrus except for unexplained small increase not associated with marked follicular development.

l/ Small rise associated with first ovulation of the year.

m/ Peaks higher as progress into breeding season.

Figure 1.14 MEG'S RESULTS



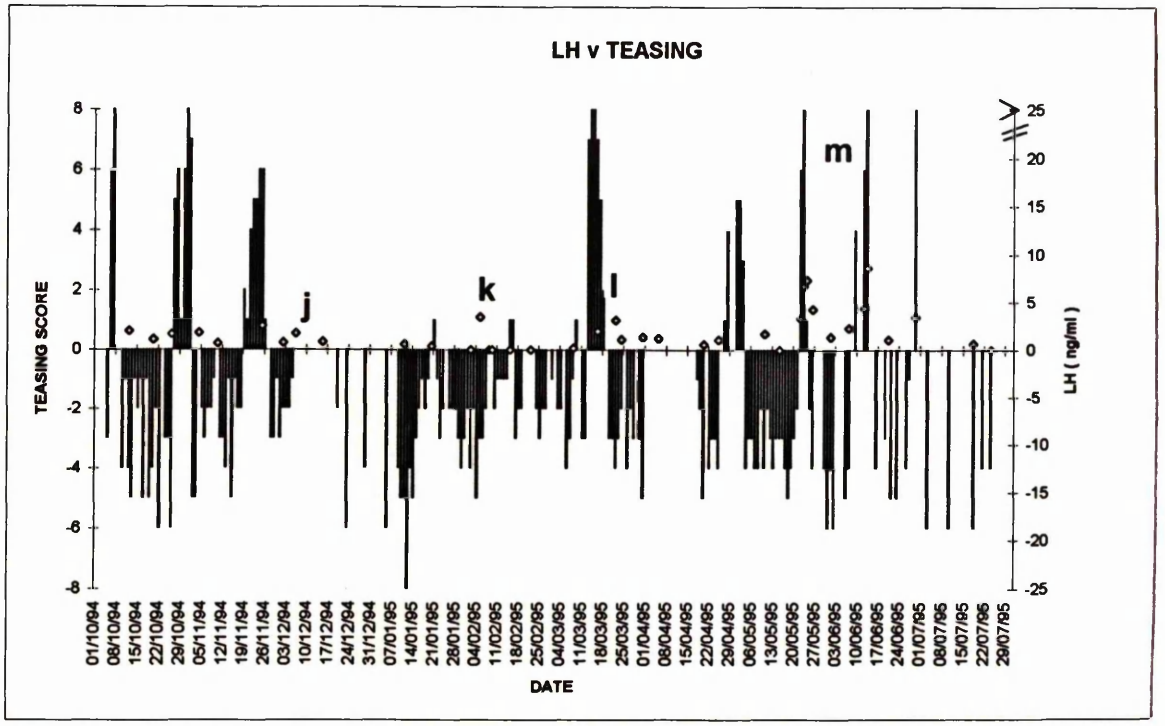


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Figure 1.15 - MISTY

Progesterone

a/ Last ovulation of year in mid November associated with 2 day oestrus.

b/ Oestrus associated with follicular development but no ovulation occurred and follicles regressed.

c/ High progesterone value (15.6 ng/ml) during anoestrus for short time. Possibly due to luteinised follicles.

d/ Anoestrus. Periods of oestrous behaviour not associated with ovulation.

e/ 1st ovulation of year on 23rd April. Associated with at least 7 days oestrous behaviour. (Not included in calculations as exact length not known). 3 large follicles in left ovary. Double ovulation and remaining one regressed (See photos 1.25a-c).

f/ Double ovulation left ovary. Single pregnancy. Flushed day 12. Spontaneous oestrus 5 days after flushing.

g/ Double ovulation same ovary (See photos 1.26a & b)

h/ Asynchronous double ovulation different ovaries. Twins same size day 12 - 14. One resorbed by day 18 - 20. Flushed day 25. Prostaglandin administered.

Figure 1.15 MISTY'S RESULTS

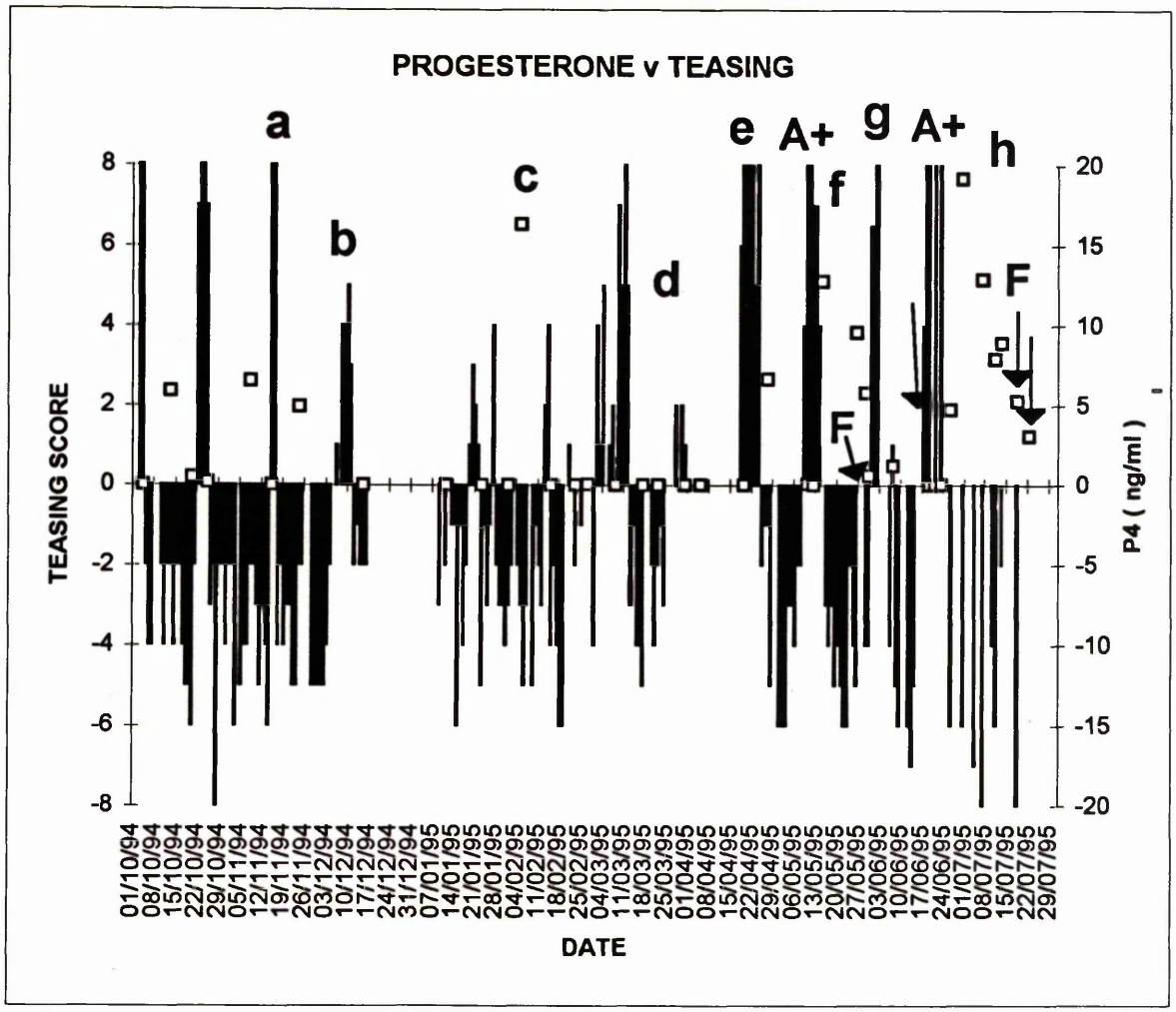

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Figure 1.16 - POPPY

Progesterone

a/ Oestrous behaviour in presence of raised progesterone concentrations before luteolysis complete. 11 day oestrus.

b/ Pregnant. Terminated at day 18-20 by prostaglandin administration. Returned to oestrus 3 days later.

c/ Pregnant. Flushed day 21. Spontaneous return to oestrus.

d/ 2 day oestrus. Possibly ovulated after end of oestrus. Prolonged interoestrous interval associated with one CL on ultrasound. Prostaglandin administered on day 24.

e/ Prostaglandin administered on day 5. Entered oestrus 12 days later and took more than 9 days for a follicle >3cm to develop.

f/ Double ovulation right ovary. Twins. Flushed day 22.

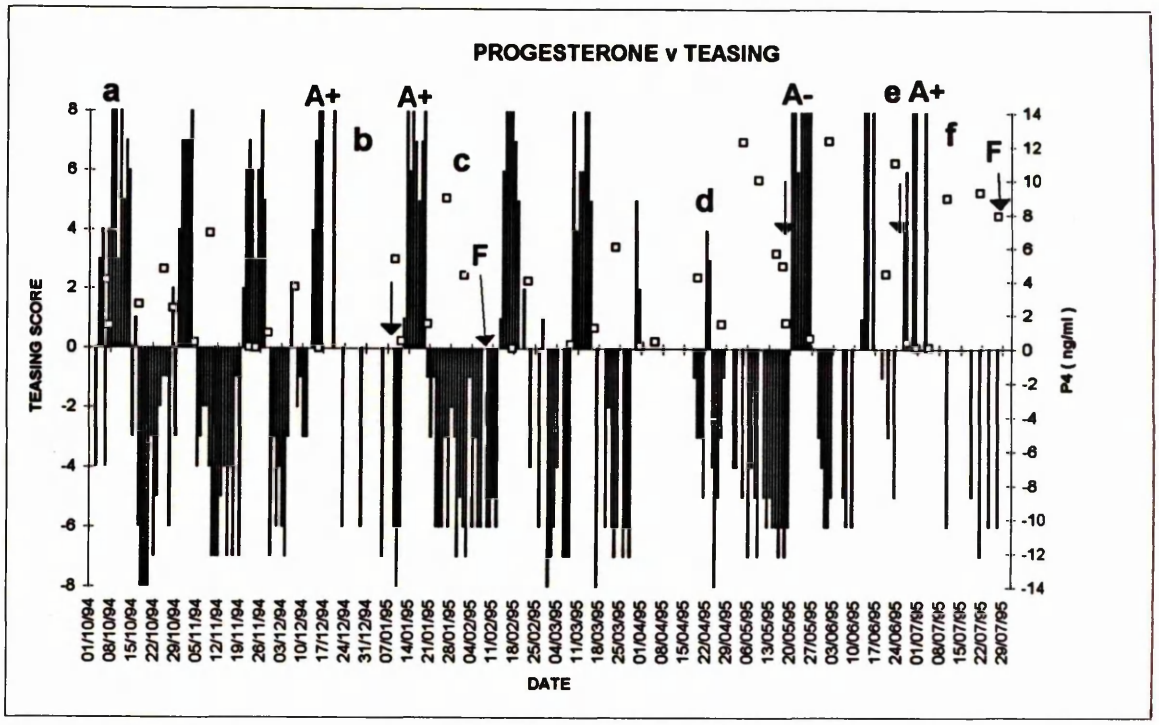
LH

g/ Generally higher resting and dioestrus LH concentrations than most of the other mares.

h/ Raised LH. Probably associated with ovulation at end of oestrus. Start of oestrus only detected due to holidays.

i/ Raised LH supports suggestion that ovulation occurred after end of short oestrus.

Figure 1.16 POPPY'S RESULTS



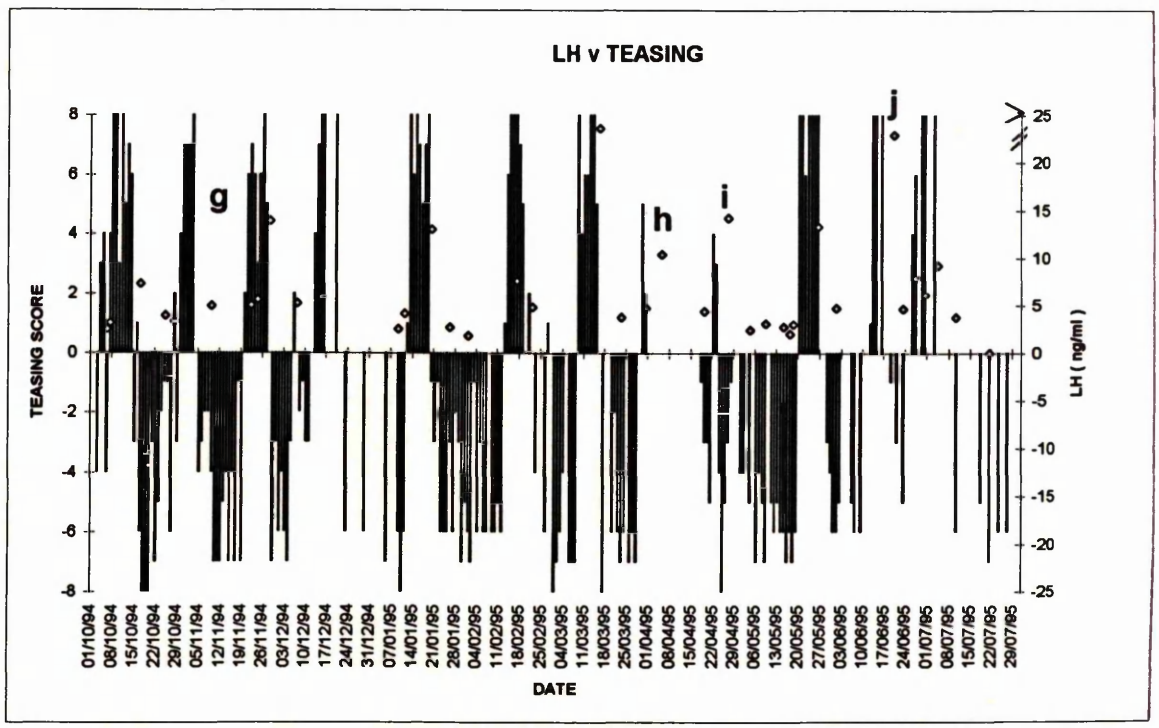


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Figure 1.17 - SUZY

Progesterone

a/ Dioestrous ovulation before day 6 detected on ultrasound. Associated with normal interoestrous interval.

b/ Last ovulation of the year, associated with oestrus, occurred at start of November.

c/ Dioestrous ovulation between day 7 and 23 on ultrasound associated with 35 day prolonged dioestrus. Last ovulation of the year. Photo 1.27a shows the original CL and photo 1.27b shows the second CL while the first one regresses

d/ Progesterone rise to 7 ng/ml. Lasted less than 14 days and was not preceded by oestrous behaviour. CLs from previous ovulations present but not functional (See photo 1.28a). Either spontaneously luteinised follicle or silent oestrus and incompetent CL formed (See photos 1.28b & c).

e/ Rise in progesterone to 1.5 ng/ml. Associated with presence of luteal-like tissue on ultrasound. (See photo 1.29).

f/ 1st ovulation of year on 23rd April. More than 7 days oestrous behaviour. Not included in calculations as exact length not known.

g/ Prostaglandin administration on day 6. Returned to oestrus in 2 days.

h/ CL and dioestrous ovulation on day 11. May have produced prolonged dioestrus if prostaglandin not administered day 15.

i/ Pregnant. Flushed day 22.

Figure 1.17 SUZY'S RESULTS

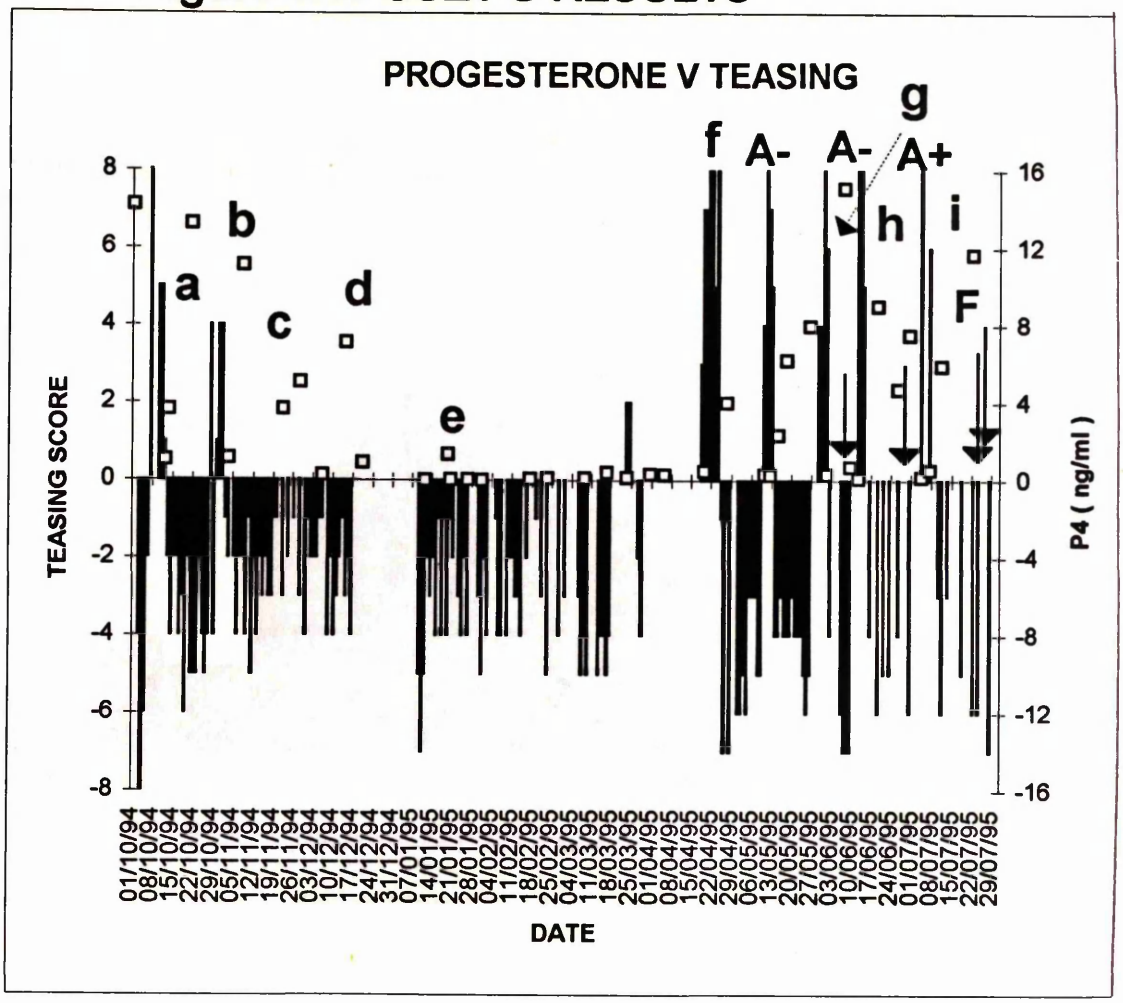


Figure 1.18 Distribution of Length of Oestrus

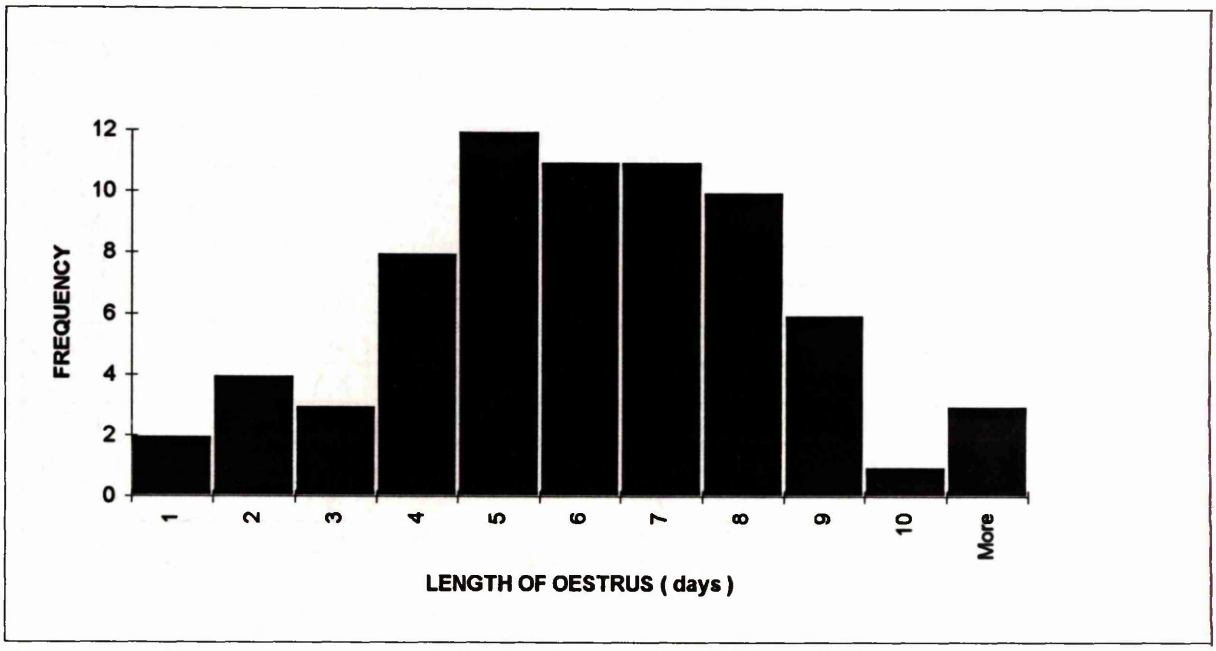


Figure 1.19 Distribution of Oestrous Lengths In Relation to Month.

MONTH	AVERAGE LENGTH OF OESTRUS	STANDARD DEVATION	NUMBER OF OESTRUSES	COMMENTS
OCT	5.8	2.7	13	
NOV	5.8	2.5	13	
DEC	7.3	1.5	3	
JAN	7.4	4.1	5	
FEB	6	3.8	4	
MARCH	7	1.2	5	
APRIL	6	2.4	6	*
MAY	5.6	1.9	10	
JUNE	5.4	1.2	10	

* 3 oestruses more than 7 days long excluded as exact length not known.

Figure 1.20 Oestrous Length Associated With Each Mare.

MARE	AVERAGE LENGTH OF OESTRUS	STANDARD DEVIATION	COEFFICIENT OF VARIATIO	NUMBER OF OESTRUSES
CORRIE	5 (2 - 7)	2.2	44	4
FILLY	6.2 (4 - 8)*	1.5	24.2	6
GEMMA	6.8 (4 - 9)	2.1	30	6
GREEDY	7.75 (5 - 13)	3.6	46.5	6
JENNY	5.8 (5 - 7)	1	17.2	4
LADY	4(1 - 8)	2.5	25	6
LAURA	7.4 (4 - 9)	1.8	24.3	9
MEG	5.6 (3 - 8)	2	36	7
MISTY	4.8 (2 - 8)*	2.3	48	5
POPPY	7.6 (2 - 13)	2.8	36.8	10
SUZY	5.2 (4 - 6)*	1	19.2	6

* 3 oestruses more than 7 days long excluded as exact length not known.

Figure 1.21 Distribution of Length of Dioestrus

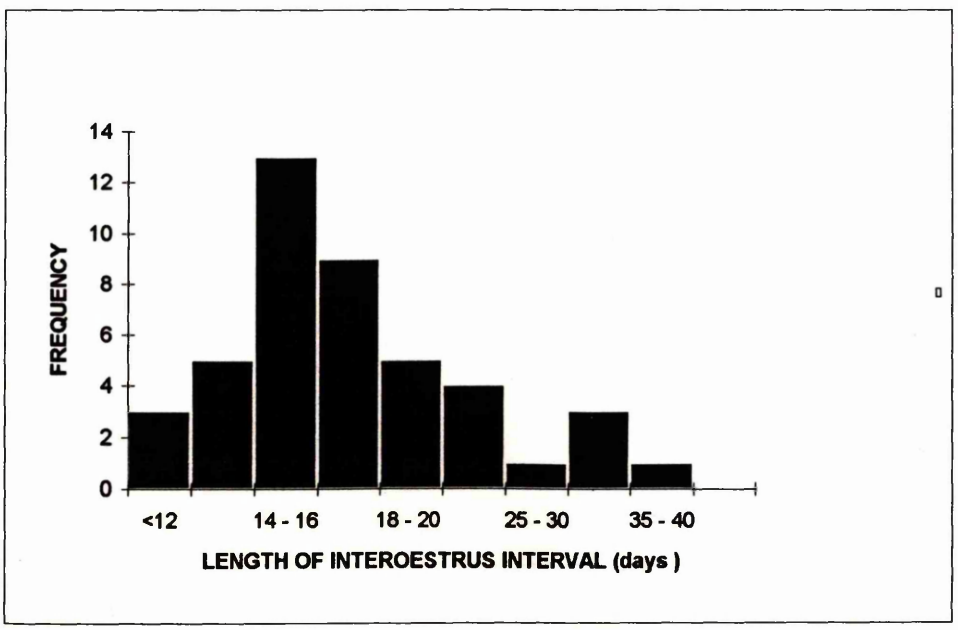


Figure 1.22 Outcome of Prostaglandin Treatment

MARE	DAY OF DIOESTRUS ADMINISTERED	TIME TILL IN OESTRUS (DAYS)	DURATION OF OESTRUS (DAYS)
FILLY	16 - 19	2	5
FILLY	10	3	4
GEMMA	5 - 7	6 - 8	NOT KNOWN
GEMMA	9	3	NOT KNOWN
GREEDY	87	2	35
JENNY	20	3	5
LADY	7	2	SILENT
MISTY	9 - 12	3	7 - 9
POPPY	18 - 20	3	8
POPPY	23	3	8
POPPY	5 - 7	3	>9
SUZY	6	3	6
SUZY	15	3	5 - 7

Photo 1.17a - Gemma d. Pear shaped follicle in left ovary prior to ovulation.

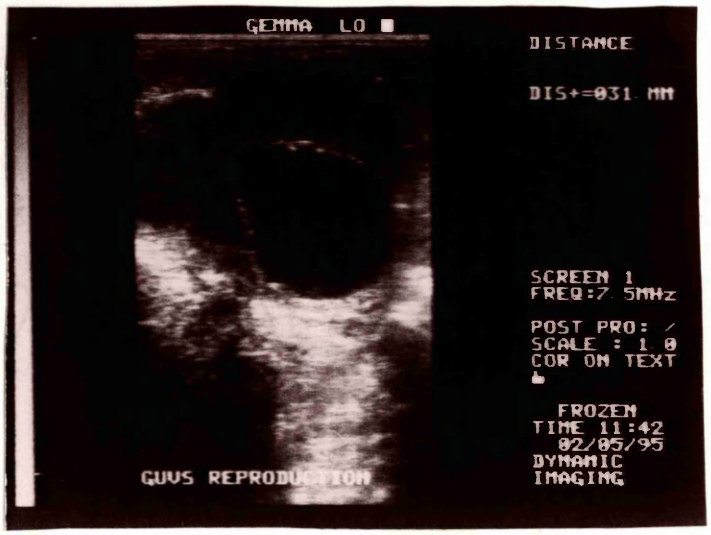


Photo 1.17b - Gemma d. CL in left ovary formed from ovulation of follicle in 1.17a.

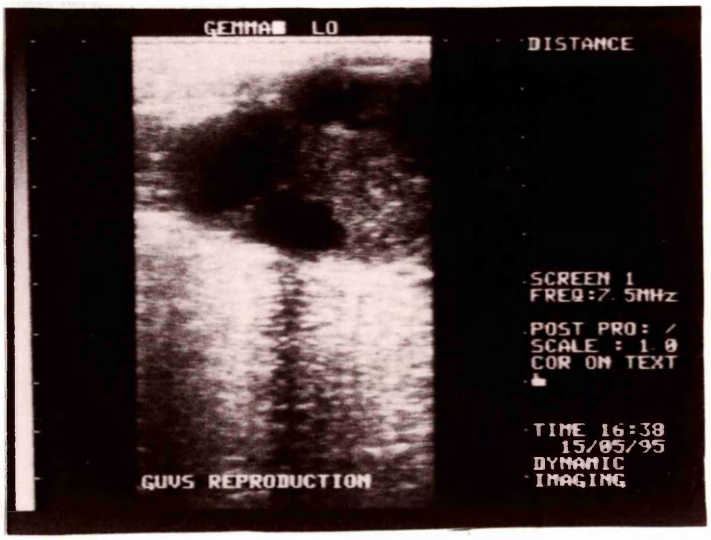
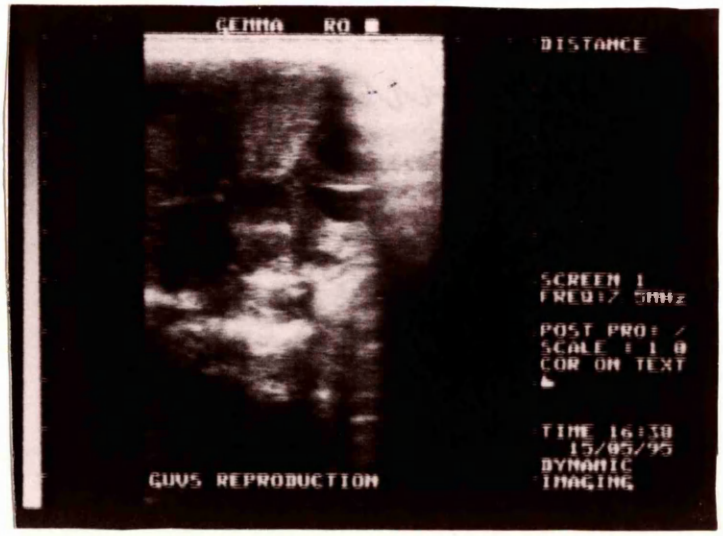


Photo 1.17c - Gemma e. CL in right ovary formed from dioestrous ovulation. Present at same time as CL in 1.17b.



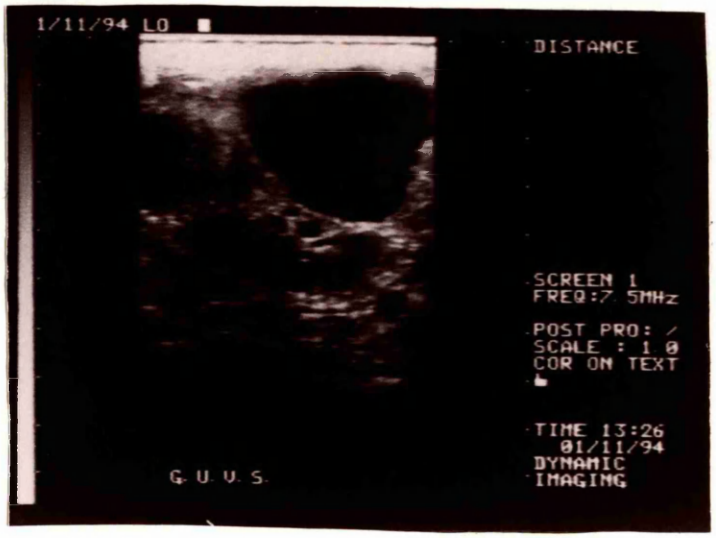


Photo 1.18a & b - Greedy a. Follicular development and CL formation in the left ovary of a mare with a granulosa cell tumour occupying her right ovary. (The markers indicate the position of the CL).

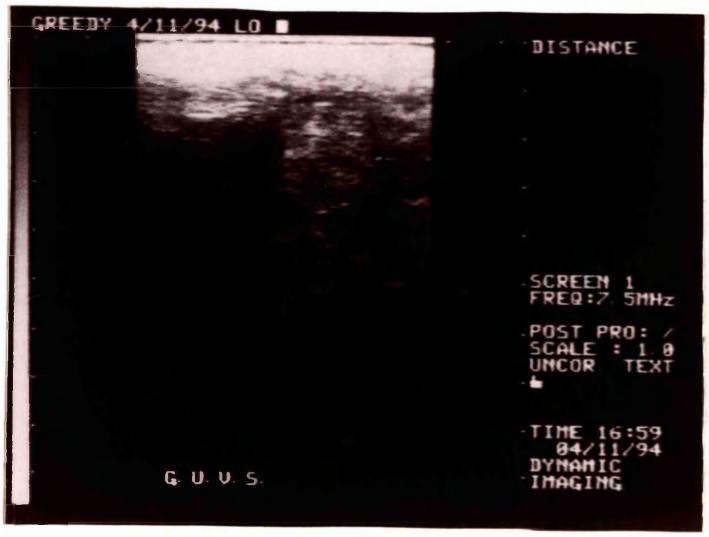


Photo 1.19a - Jenny a. CL in right ovary (top right of photo). Hyperechoic and reduced in size as undergoing lysis. (Markers are artefactual).

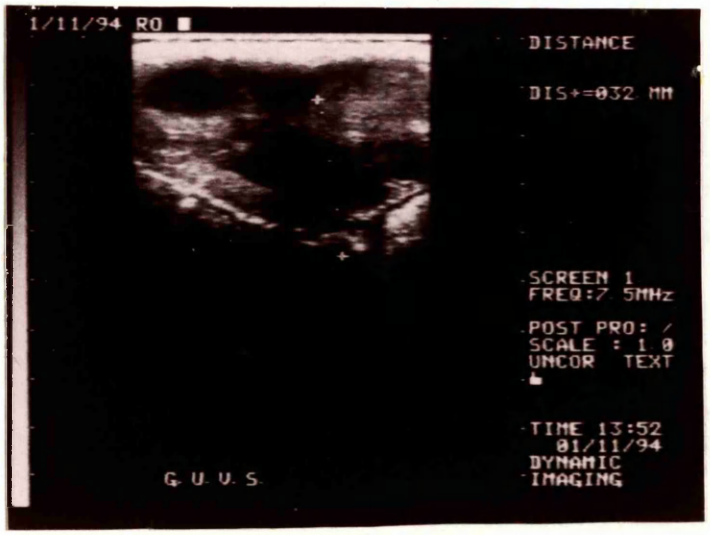


Photo 1.19b - Jenny a. Mature CL in left ovary associated with dioestrous ovulation. Present at same time as CL in 1.19a and produced prolonged dioestrus. (Markers are artefactual).

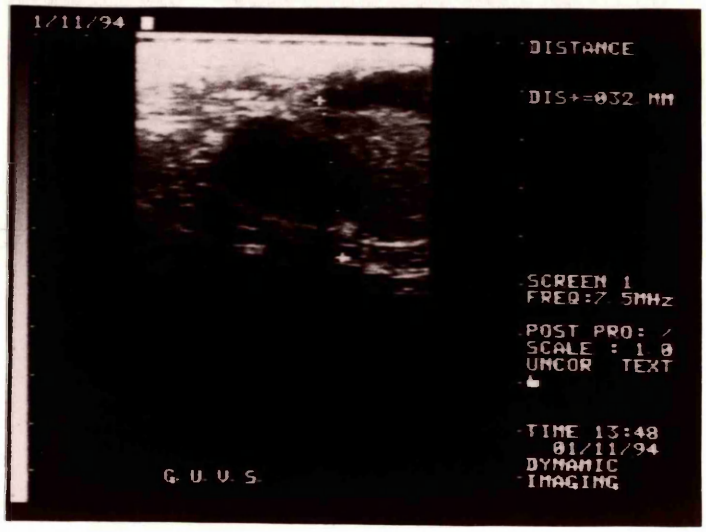


Photo 1.20a - Jenny b. Recently formed, centrally hypoechoic CL in left ovary.

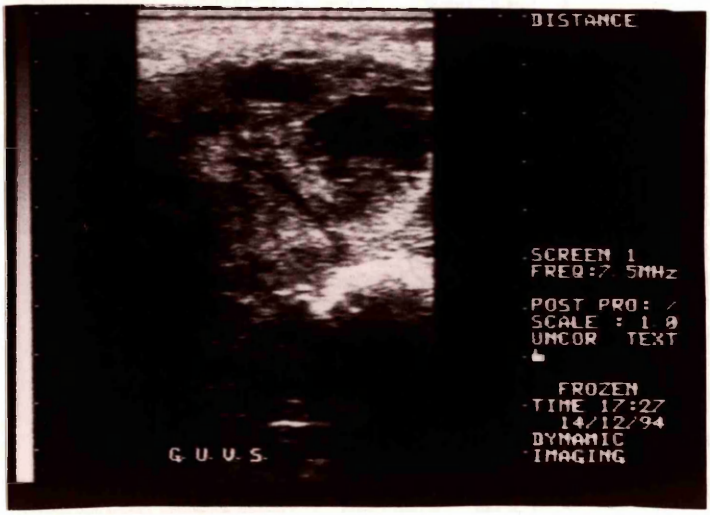


Photo 1.20b - Jenny b. Markers indicate same CL as shown in 1.20a 22 days later. Central clot organised so uniformly dense.

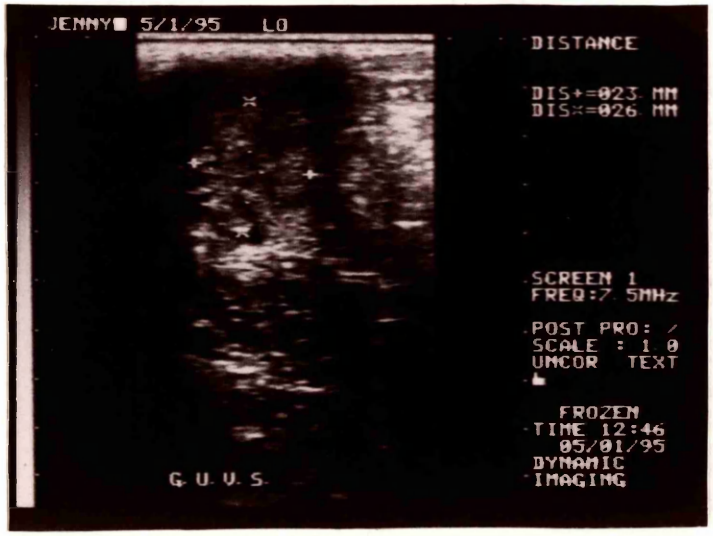


Photo 1.20c - Jenny b. Same CL as shown in 1.20b. Becoming hyperechoic as regresses in association with declining progesterone concentrations

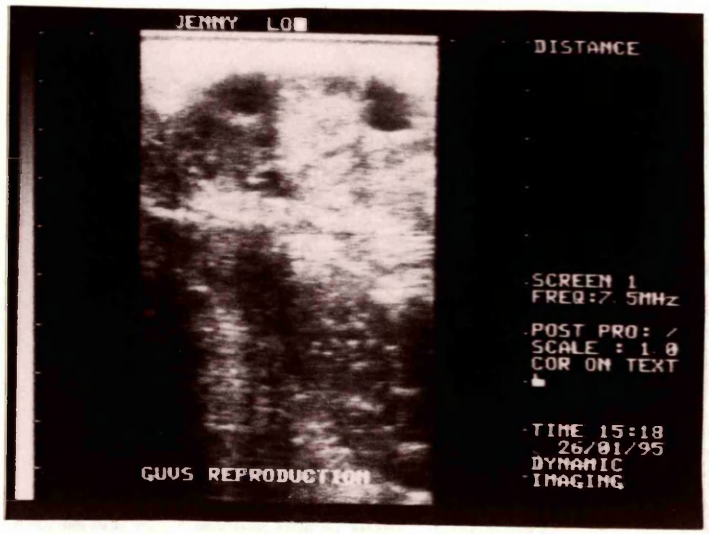


Photo 1.21 - Lady g. Newly formed, hyperechoic CL in left ovary associated with ovulation during a silent oestrus.

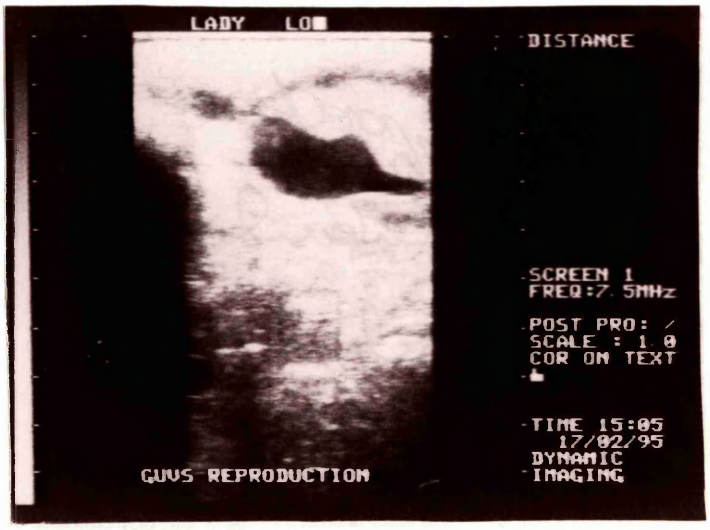


Photo 1.22a - Lady h. CL present in left ovary during dioestrus preceding a silent oestrus.

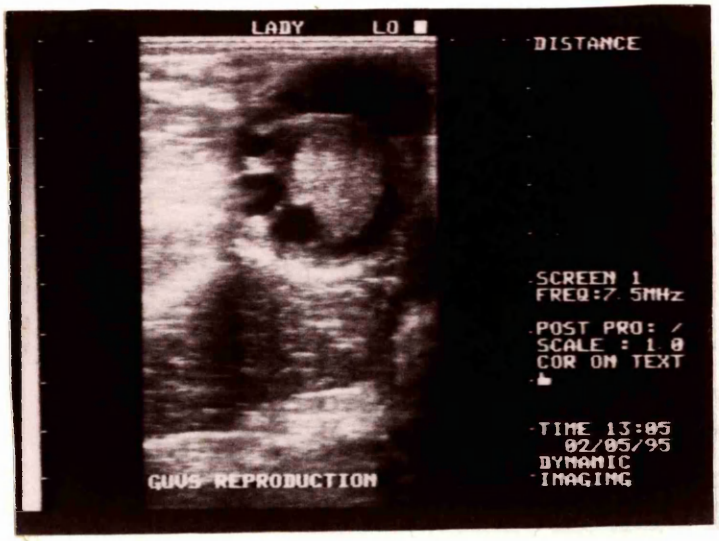


Photo 1.22b - Lady h. CL formed from ovulation in left ovary in association with a silent oestrus

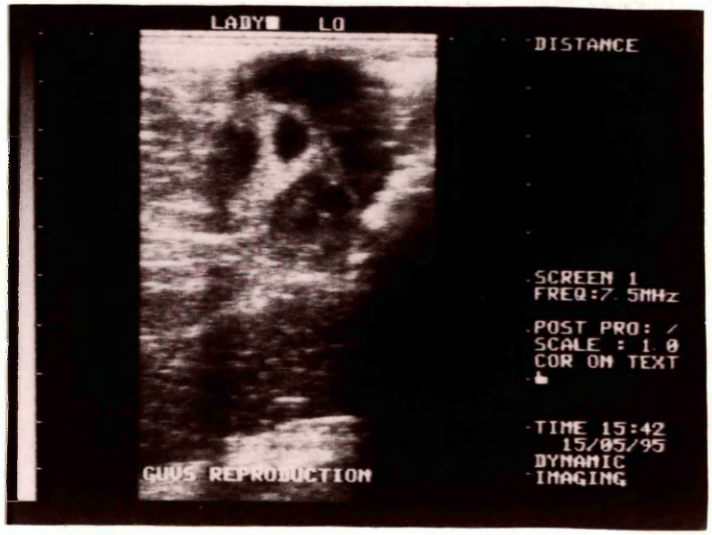
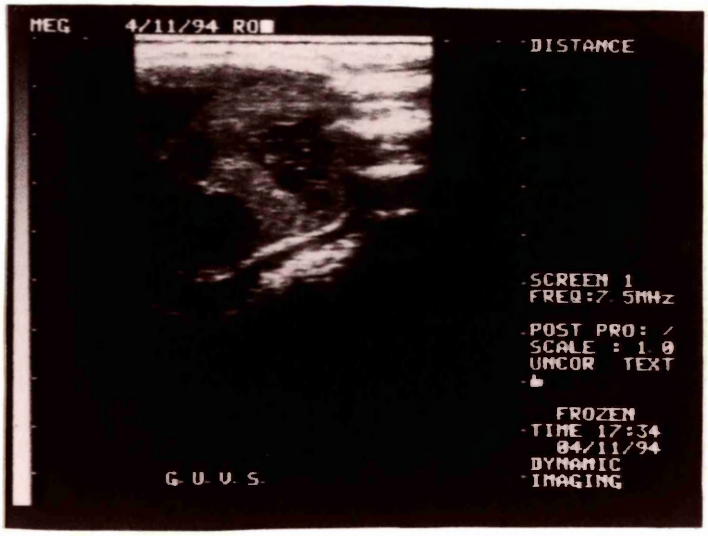


Photo 1.23a & b - Meg a. Double ovulation from different ovaries i.e. bilateral



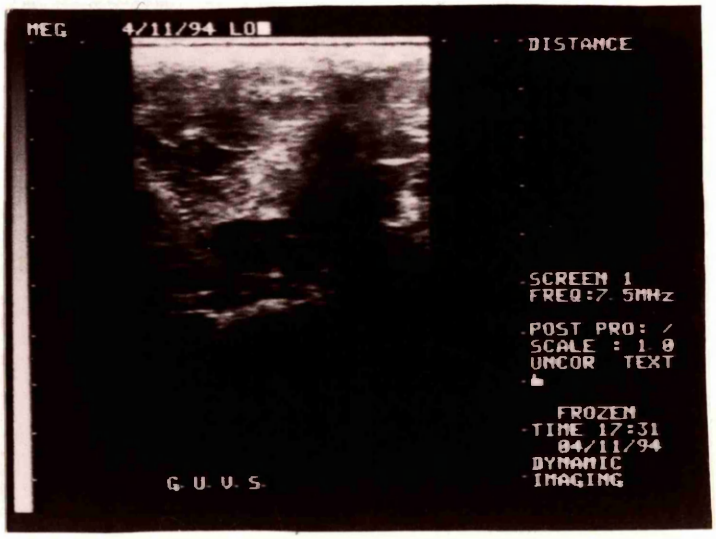


Photo 1.24 - Meg c. Anovulatory follicle (indicated by markers) associated with the last oestrus of the year before became atretic and regressed.

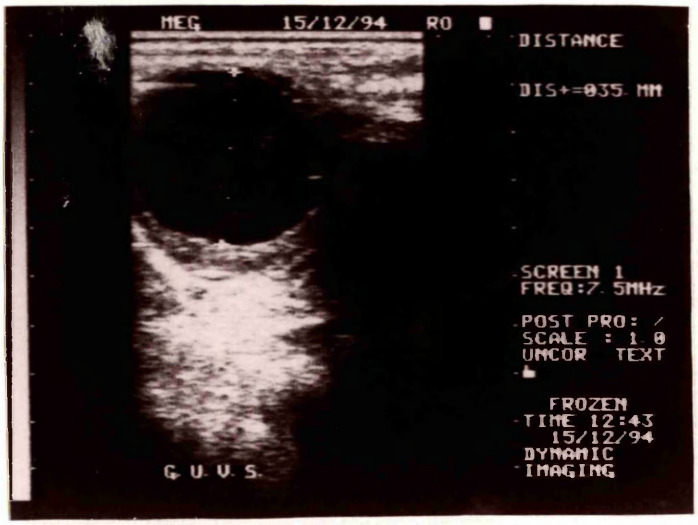
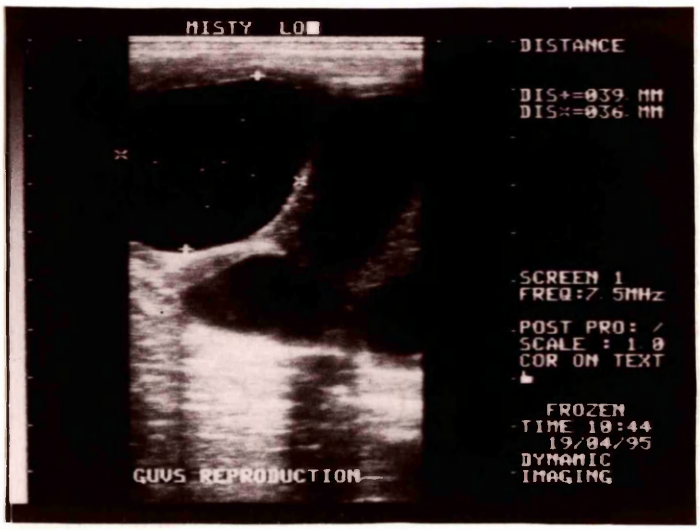


Photo 1.25a & b - Misty e. Three large follicles in left ovary. One is indicated by markers.



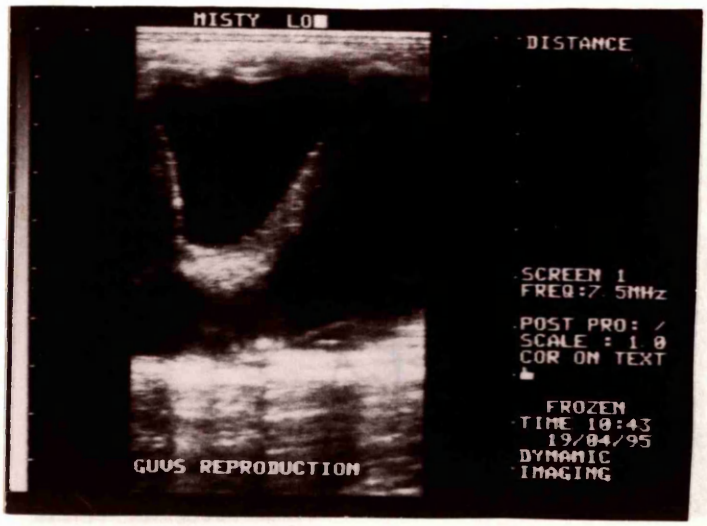


Photo 1.25c - Misty e. Two CLs formed from ovulation of follicles in 1.25a & b (indicated by markers) and one follicle regressed.

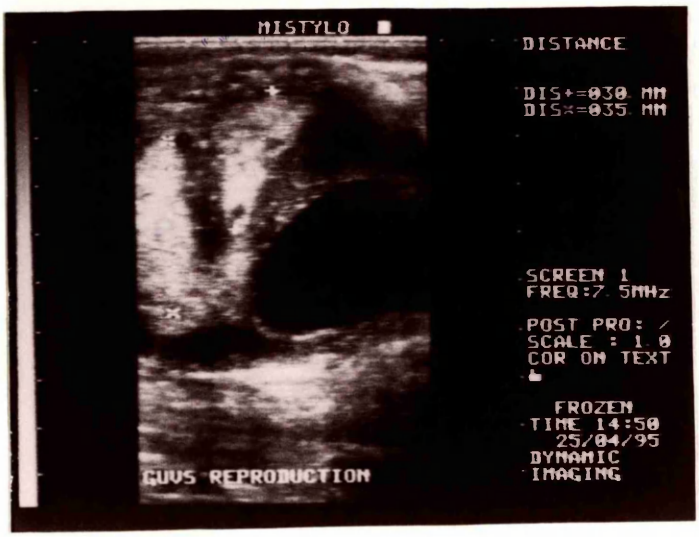
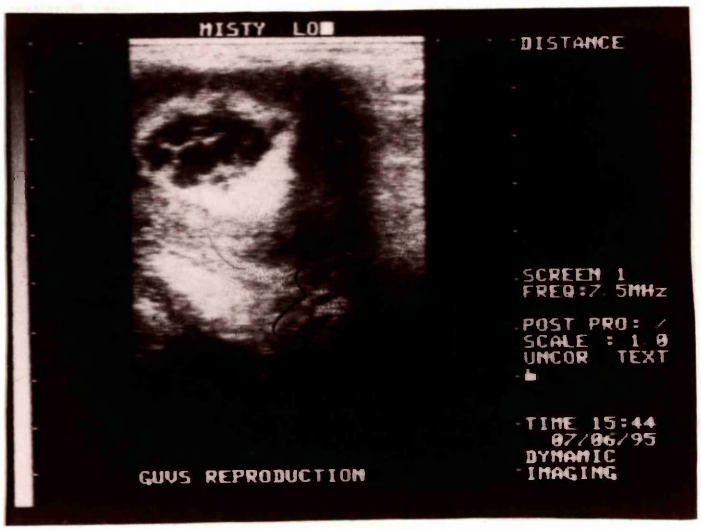


Photo 1.26a & b - Misty g. Two centrally hypoechoic CLs in left ovary due to double ovulation i.e. unilateral



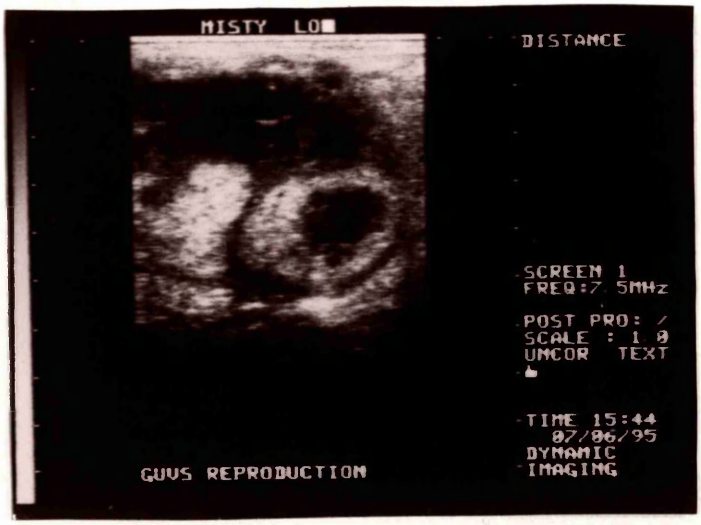


Photo 1.27a - Suzy c. Uniformly dense CL in left ovary.

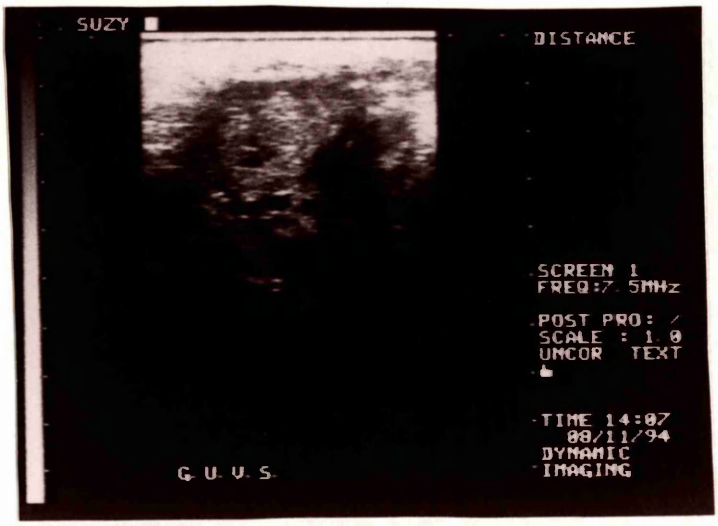


Photo 1.27b - Suzy c. Second CL formed from dioestrous ovulation in left ovary (indicated by markers) while original CL becoming smaller and more hyperechoic as regressing

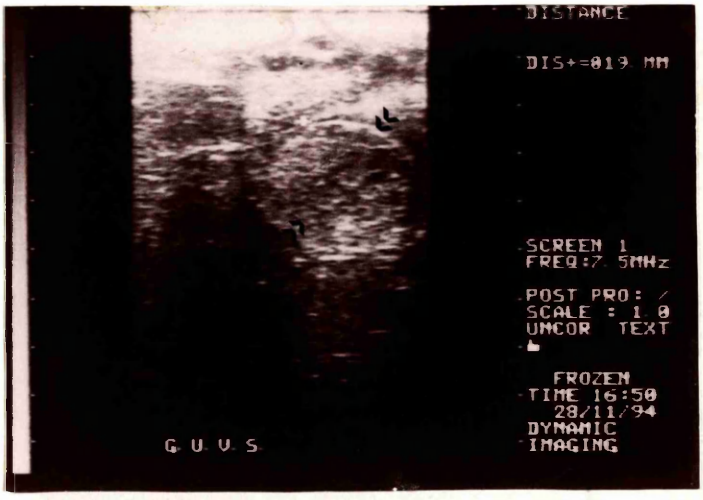


Photo 1.28a - Suzy d. Remnants of previous two CLs in left ovary (indicated by markers). Both non-functional

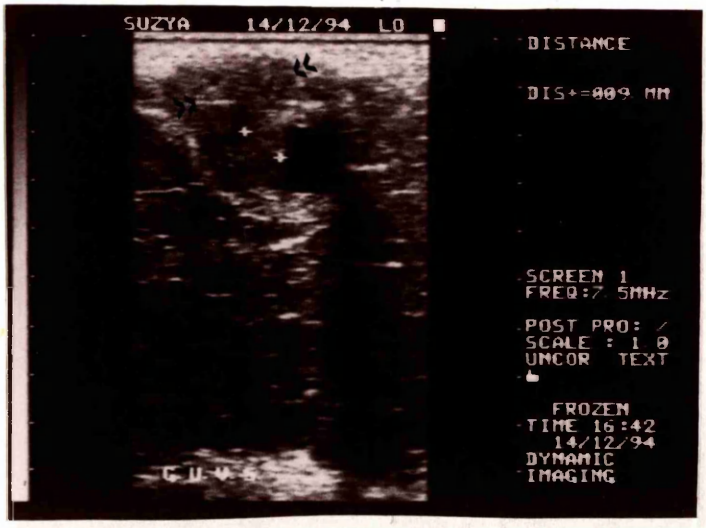


Photo 1.28b - Suzy d. Either spontaneously luteinised follicle in right ovary or new CL following silent oestrus associated with progesterone production

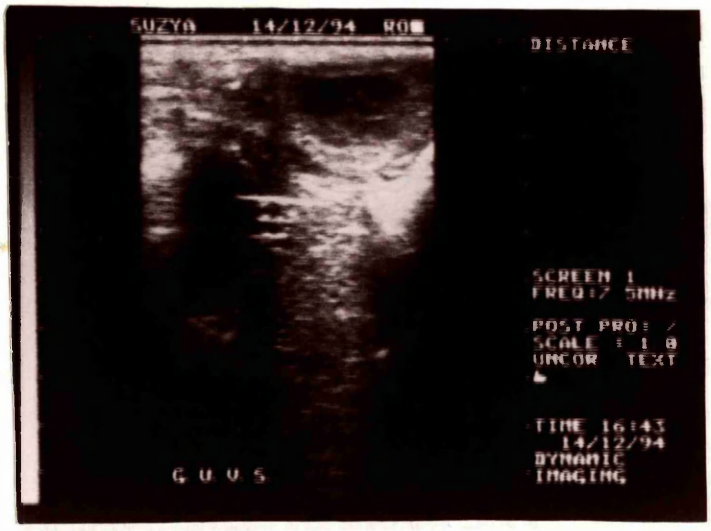


Photo 1.28c - Suzy d. Remnants of luteinised follicle in right ovary (indicated by markers). No longer functional

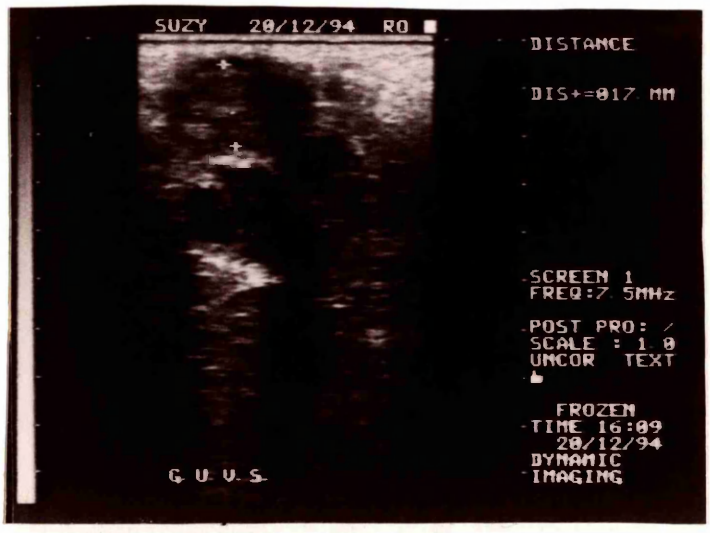


Photo 1.29 - Suzy e. Luteal like tissue (indicated by markers) associated with small, short lived rise in plasma progesterone concentrations during anoestrus

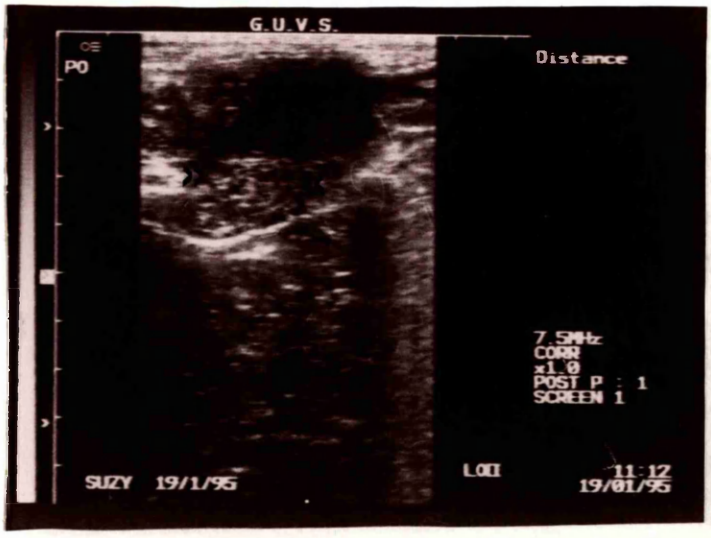


Photo 1.30 - Fluid present in Lady's cranial vagina and the body of her uterus. The ventral hypoechoic area is the bladder

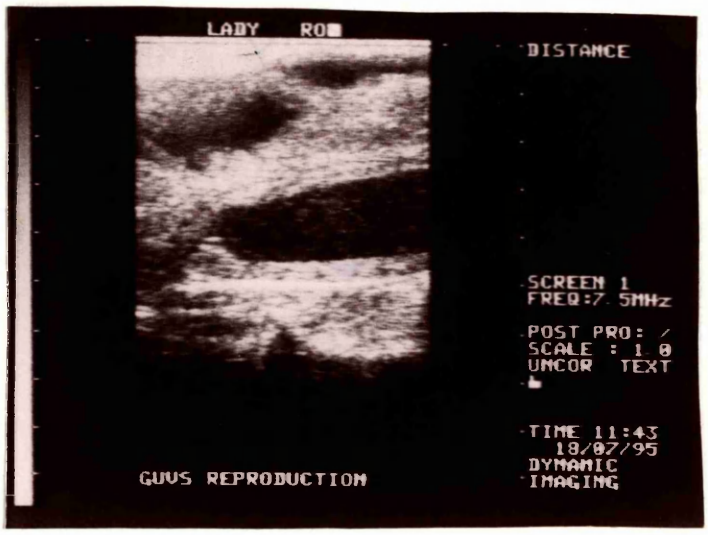


Photo 1.31 - Endometrial cysts in Lady's uterus

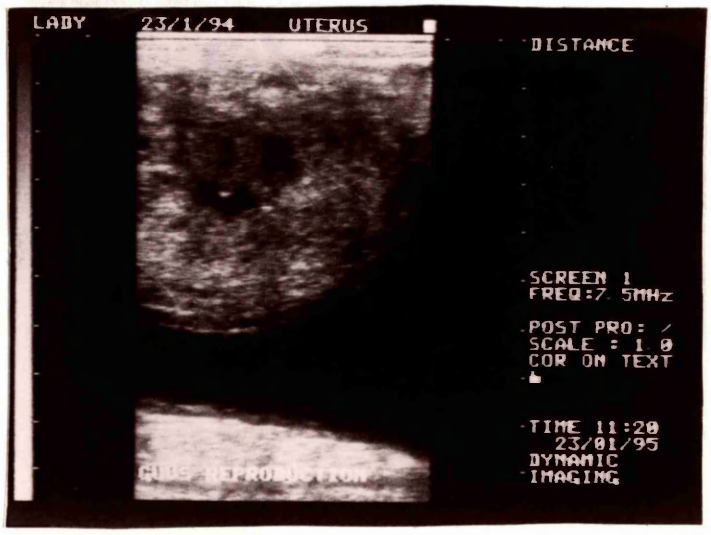


Photo 1.32 - Area of dense stroma which resembled luteal tissue in an anoestrous mare. Not associated with progesterone production.

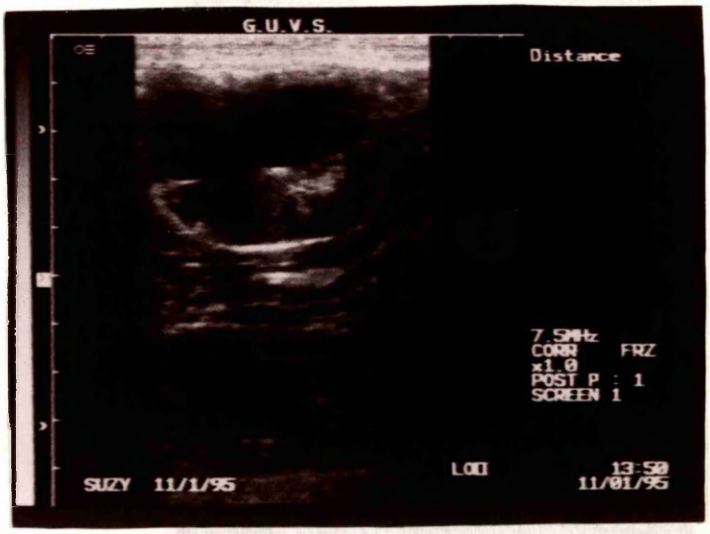
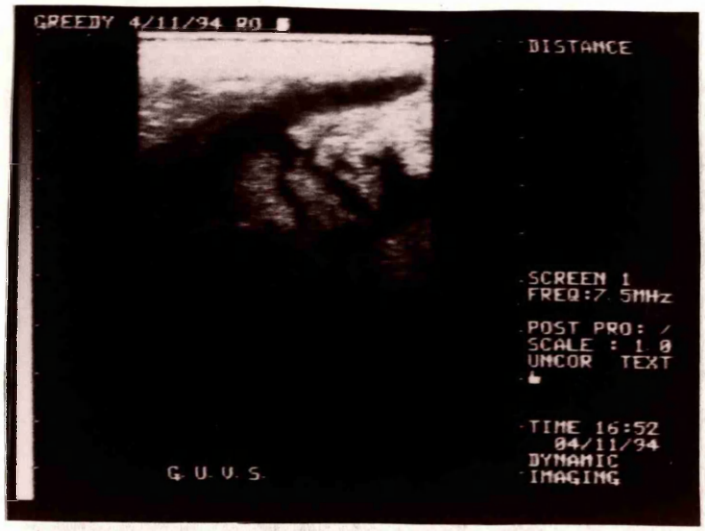


Photo 1.33a & b - Appearance of the granulosa cell tumour in Greedy's right ovary on ultrasound. Note dense tissue interspersed with fluid filled areas.



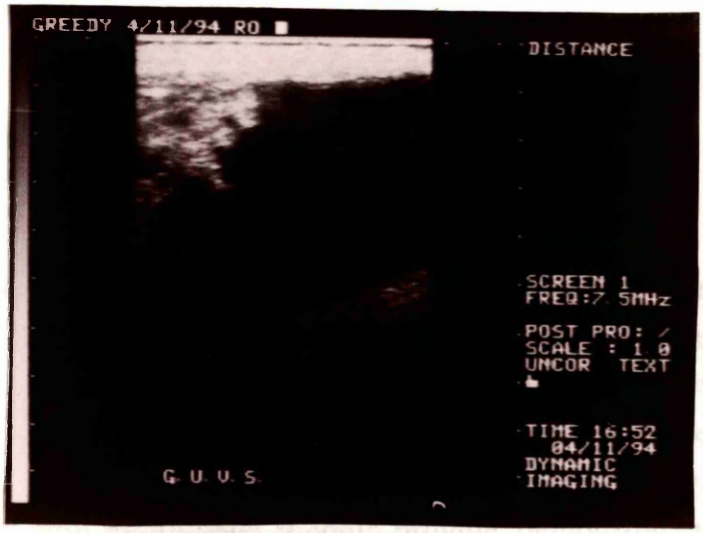
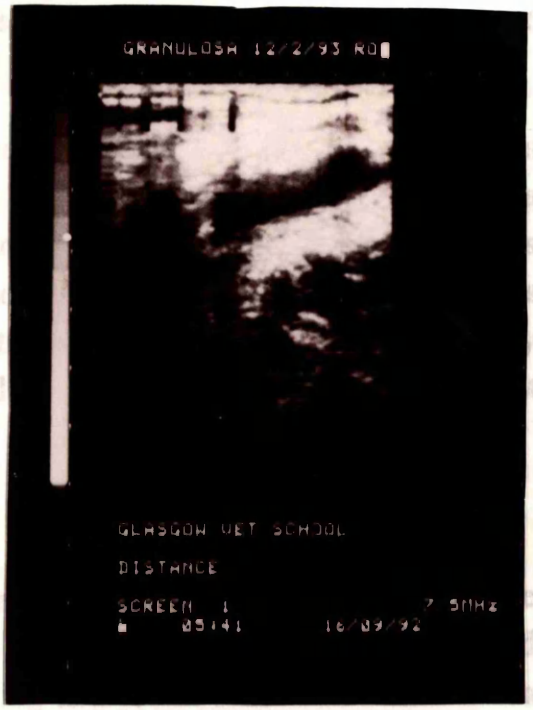


Photo 1.33c - Previous appearance of tumour. Note more honeycombed structure



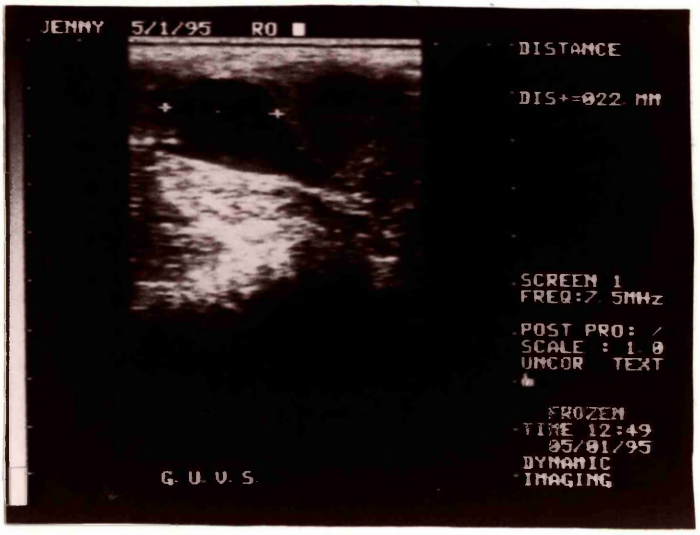


Photo 1.34 -

Peri-ovarian cyst

associated with

Jenny's right ovary

1.4 Discussion

The mares used in this study were not selected for any reason other than their availability and so represented a relatively random cross section of the general mare population. It can be seen from the results that the cyclic behaviour throughout the study varied greatly between mares and in this regard each mare should be considered as an individual rather than as a group. Each procedure used in this study had limitations in the amount of useful information it could provide, demonstrating that the combination of results from a range of techniques was the most accurate way to determine the cyclic state of a mare at a given time.

The information obtained from observing oestrous behaviour during teasing was limited with regard to determining the cyclic state of the mare. Also, teasing was a subjective assessment of the mare's behaviour which varied with the observers. This presented a problem in the present study where different groups of undergraduates performed the teasing each week. The attempt to standardise the results by introducing a scoring system worked well and produced interpretable results in most instances.

Coat-A-Count Progesterone Assay

The Coat-A-Count progesterone assay used in this study proved to be simple to use and produced results which corresponded to the mare's cyclic state as determined by the use of other procedures such as teasing and rectal ultrasound examination

which was in agreement with the findings of Hughes et al. (1972). The results obtained during luteal and non luteal stages of the oestrous cycle were in agreement with previously reported values (Ginther, 1992) as were the progesterone profiles displayed by the mares.

Consequently, the Coat-A-Count radioimmunoassay was considered to be a reliable and accurate method of determining whether ovulation had occurred and if luteal tissue was functional. However, it could not differentiate between the various causes of prolonged dioestruses which also corresponded with the findings of Ginther (1992).

Progesterone concentrations varied between the mares in the present study, as was seen by the different scales on the charts. There also appeared to be differences between dioestrous concentrations within the same mare on occasions. Progesterone secretion has been reported as being episodic (Evans, 1991), so the frequency of sampling or the variation in high progesterone values between Coat-A-Count runs could have accounted for this.

LH Assay

There appeared to be a wide variation in basal LH concentrations between the mares in this study, which was probably due to the degree of inter-assay variation and so was not considered to be of any significance. Peak LH concentrations occurred towards or just after the end of oestrus and the values were greater than the highest standard of the assay so it was only possible to determine that they were >25ng/ml. However, these values corresponded with the findings of Evans and Irvine (1975) who also reported ovulatory peaks of this magnitude. If the samples in the present study were reassayed, it would be beneficial to dilute them in zero standard to enable their actual value to be determined.

The instances where ovulation occurred in the absence of a detected LH increase were probably because the peak was missed due to the infrequency of the sampling.

Ultrasound Examination

The failure to accurately predict the timing of ovulation using the criterion described in materials and methods corresponded with the findings of Pierson and Ginther (1985). The indication that ovulation would occur in the following few days would be beneficial when natural covering was being used but in conjunction with the use of artificial insemination, a greater degree of precision is often required.

Other ultrasound properties such as follicular wall width, follicular wall echogenicity and follicular fluid echogenicity have also been evaluated for this purpose (Ginther, 1986). Although follicular wall width has been shown to increase during the preovulatory period, none of these three parameters have proved to be reliable indicators of impending ovulation. Ginther (1986) also commented on the quality of the equipment used for such assessments and it may be that the equipment used in the present study affected the accuracy with which these parameters were assessed and that in the future, more accurate predictions using these parameters will become possible with the availability of better equipment.

Ultrasound was useful to detect the occurrence of ovulation or follicles which did not ovulate.

Average Cycle Lengths

The average length of oestrus in the present study corresponded with the 6.5 days reported by Ginther (1979). However, the range was wider in this study than the reported 4.5 - 8.9 days (Ginther, 1992). This observation may be due in part to the present study being conducted from October to June, encompassing only part of the normal breeding season. The length of oestrus has been shown to be longest during the winter and shorten as the breeding season commences due to a faster rate of follicular development and maturation prior to ovulation (Hughes et al., 1972). In particular, prolonged oestruses can be associated with the first and last oestruses of the breeding season (McKinnon and Voss, 1993). This seasonal variation in length of oestrus was also observed in the present study and could account for the wider variation in lengths which occurred.

Ginther (1992) reported that oestrous length tends to be repeatable with the same mares having short or long periods (Ginther, 1992). However, this did not correspond with the findings of the present study and again provides a reason for the variations observed.

The average dioestrous length in this study was longer than the average of 14.9 days reported by Ginther (1979). Also the range was wider than the reported 12.1-16.3 days which again highlights the amount of variation present in the general mare population.

Variations in Oestrous Behaviour

There are many circumstances which have been reported to affect the expression of oestrous behaviour in mares. For example, the protective instincts of a post partum mare towards her foal during teasing or the stress associated with separation from it

for teasing, can both prevent her showing signs of oestrus. This is particularly relevant in mares such as Thoroughbreds where mating is carried out as soon after parturition as possible and therefore the foal at foot is very young. Other potentially stressful situations such as new environment, inclement weather, management changes, teasing near feeding time and the use of unfamiliar stallions have also been reported to affect some mares as well as poor body condition, chronic pain, debilitating disease and inadequate teasing time (Ginther, 1979).

None of the post partum mares in the present study seemed affected by the presence or absence of their foal at teasing and of the other factors mentioned above, only the management changes associated with housing in October and the introduction of the new stallion seemed to affect any of the mares.

It was interesting that Corrie and Poppy reacted in such different ways to the change in circumstances at housing, despite it being an annual occurrence. Given Corrie's nervous nature, the stress caused by being stabled in a different box and the introduction of a new stallion probably caused the suppression of oestrous behaviour. However, Poppy acted precociously, presumably due to exposure to a stallion after a long period without contact. Filly's dislike of the new stallion was shared only by Corrie initially with the rest of the mares accepting him immediately and Misty displayed no alteration in behaviour throughout the study despite being in probably the most stressful situations. These observations highlight the amount of variation between individual mares in the way they respond to changing circumstances.

Poppy's expression of 'mouth clapping' was unusual and there has only been one previous report of it occurring in a mare, although it is a sign commonly displayed by oestrous jennys and zebras (Ginther, 1992). It is also observed in foals as a submissive gesture and may have been displayed as such by Poppy given her low place in the dominance hierarchy and its absence during individual teasing. Jenny would not come forward to be teased due to the presence of dominant mares and these both highlight the problems with group teasing mares.

Silent oestruses presented a particular problem since these were not detected even with regular teasing and so information from the other procedures had to be used to confirm their occurrence. Repeated examinations were necessary to differentiate them from other circumstances which would cause an apparently prolonged period without oestrous behaviour such as prolongation of the CL and dioestrous ovulations, especially when the new CL formed at the silent oestrus was in the same

ovary as the original one. This was in agreement with the observations of Hughes et al. (1972) who also noted that confusion could arise in this manner.

Silent oestrus has been associated with lower than normal oestrogen concentrations, the total amount produced appearing to be more important than the peak values in producing oestrous behaviour (Nelson et al., 1985). The poor follicular development in Lady during her silent oestruses may therefore have contributed to the lack of behavioural signs. Also, where there was a reduced interval between luteolysis and ovulation, such as that caused by premature luteolysis, it has been suggested that the oestrogen peak occurs before progesterone concentrations drop below 1 ng/ml. The behavioural centres are therefore not as receptive to its action, as well as there being a shorter period of time for it to have effect (Nelson et al., 1985).

Nelson et al. (1985) also suggested that in other species a period of progesterone priming followed by oestrogen is required to produce oestrous behaviour. This has never been proved in the horse but if it was the case, it may have accounted for Lady's silent oestrus which followed the presence of a non-functional CL.

The negative signs associated with dioestrus are not only associated with an absence of oestrogen but are produced by the influence of progesterone on the behavioural centres (McKinnon and Voss, 1993). The effects of progesterone are in fact dominant to those of oestrogens, as demonstrated by dioestrous ovulations not being associated with oestrous behaviour (Vandeplassche et al., 1979). Without stimulation from one or other of these hormones, mare show indifferent signs at teasing and this is usually associated with anoestrus, when hormone concentrations are basal (McKinnon and Voss, 1993).

This type of behaviour would also occur in a mare in which luteolysis had occurred and progesterone concentrations had therefore fallen but for some reason, such as those previously suggested, the behavioural centres were not responding to the effects of oestrogen thus producing a silent oestrus. Why Lady displayed such actively negative behaviour during periods when it was demonstrated that her progesterone concentrations were basal was not known.

The presence of the LH peaks in association with two of Lady's silent oestruses demonstrated that the oestrogen was capable of positively feeding back to induce LH release despite not being capable of producing oestrous behaviour. This suggested there may be some defect in Lady's behavioural centres that do not allow her to respond to oestrogen stimulation.

Conditions which cause endometrial irritation, such as endometritis, have been shown to stimulate early release of prostaglandin and so premature luteolysis (Neely et al., 1979). Accumulations of fluid in the uterus have been shown to represent the presence of an inflammatory process (Adams et al., 1987) and the detection of polymorphonuclear leucocytes in smears is consistent with the presence of endometritis. Endometrial cysts are particularly common in older mares (Adams et al., 1987) but are not thought to interfere with uterine function and a link between them and infertility has yet to be established.

Lady's tendency to pool urine in her cranial vagina in combination with an incompetent cervix may have lead to pooling of urine in the uterus itself. This could have explained the fluid accumulations observed in the uterus on ultrasound examination but the failure to detect polymorphonuclear leucocytes on smears. The resulting endometrial irritation may have cause premature luteolysis and therefore shortened dioestrous intervals. This may in turn have affected her expression of oestrous behaviour due to the reduced interval between luteolysis and ovulation as previously mentioned, which is in agreement with the findings of Nelson et al. (1985).

Pooling of urine in the cranial vagina or urovagina is associated with poor perineal conformation (McKinnon and Voss, 1993). This can be caused in a number of ways but the most common in older, multiparous mares is the occurrence of splanchnoptosis, where the abdominal and pelvic organs become relaxed and fall forward into the abdomen, pulling the reproductive tract and rectum cranially (Pascoe, 1979). This causes alteration in the positioning of the urethral opening with relation to the vulvovaginal fold and causes urine to flow back into the vagina instead of out through the vulva. This problem is compounded by poor body condition and age associated muscle atrophy but since Lady's body condition and perineal conformation were both good, this was unlikely to have been the cause of the problem. Pascoe (1979) suggested that poor fertility in these mares was either to other faults or poor management.

Since most of the other mares in the present study successfully conceived at least once throughout the study, it seemed unlikely that poor management was to blame for her failure to become pregnant. It seemed more likely that during her previous abortion of a late term foetus, as well as her cervix being damaged, either her urethra itself or her vulvovaginal fold was also affected since these are responsible for the passage of urine from the urethral opening out through the vulva during urination. This may also have been the cause of the small amounts of urine which she expelled while displaying negative behavioural signs during silent oestruses.

McKinnon and Voss (1993) also reported the occurrence of urovagina due to foaling injuries to the urethra or vulvovaginal fold.

Variations Associated with Dioestrus

There are many causes of prolonged dioestrous intervals. Those associated with pregnancy will be discussed in Chapter Four. The average length of prolonged dioestrus in mares has been reported as 63 ± 15.6 days with a range of 35 to 95 days (Stabenfeldt et al., 1974). Only one of the intervals in the present study lasted as long as 64 days while the rest were between 34 and 39 days.

The cases where only one CL was present throughout the entire period, suggested that there must have been a defect in the luteolytic mechanism allowing maintenance of the primary CL. This could theoretically have taken place at the level of the prostaglandin (PGF2a) production, transport or action on target (McKinnon and Voss, 1993).

Prolongation of the CL has been found in mares with chronic endometrial damage such as pyometra (McKinnon and Voss, 1993), where the ability of the endometrium to produce and secrete prostaglandin is impaired. There was no clinical or ultrasound evidence of endometrial abnormalities in any of the four mares who displayed maintenance of the CL. However, both Jenny and Laura underwent early embryonic death at other stages of the study. This will be discussed in Chapter Four but could have indicated the presence of an endometrial abnormality which may have affected the induction of luteolysis as well as causing early embryonic death.

Newly formed CLs are not susceptible to the luteolytic effects of PGF2a for 1-4 days (Douglas and Ginther, 1975) but all the mares had mature CLs at the time luteolysis should have occurred so this was obviously not the case. Theoretically since the PGF2a must travel through the circulation to reach the ovary, any factor which affected its metabolism or that was antagonistic to it could have reduced its effect but there was no evidence to support this suggestion.

Since there were no obvious causes for these prolonged dioestrous intervals, they were considered to be idiopathic and spontaneous. Ginther and Pierson (1989) cited a report which observed that this was due to a biochemical or cellular predisposition occurring in up to 25% of oestrous cycles in association with normal uteri. However, Ginther and Pierson (1989) themselves did not find any evidence to support this and questioned the occurrence of spontaneously prolonged CLs.

In Jenny's case, the dioestrous interval ended when the CL regressed naturally. However, the progesterone profiles seen in Greedy and Laura suggested that the factors normally controlling CL regression were re-established and the CL was lysed, causing a sudden decline in progesterone concentrations and allowing the mare to return to oestrus. This was in agreement with the findings of Stabenfelt et al.(1974).

Stabenfelt et al. (1974) reported marked follicular development during prolonged luteal phases and also the occurrence of further ovulations. In the present study, follicular development was noted in all four of these mares although no more ovulations were detected.

Another cause of prolonged dioestrous intervals in mares is the occurrence of dioestrous ovulations, which refers to the formation of CLs after the end of an oestrous period (Ginther et al., 1982). Vandeplassche et al. (1979) reported that mature follicles can develop at all stages of the oestrous cycle but most regress due to the low concentrations of LH present at this time. Only about 10% ovulate in association with a much lower LH rise than that associated with oestrus, although oestrogen concentrations are the same no matter what the stage of cycle is. There were no signs of sexual receptivity or relaxation of the cervix associated with these ovulations (Hughes et al., 1972), although they have been proven to be fertile with the use of A.I. (Hughes and Stabenfeldt, 1977).

No increase in LH was detected in association with the dioestrous ovulations in the present study. This could reflect the sensitivity of the assay used since concentrations are reported not to be as high as those associated with ovulation during oestrus. Alternatively, a different isoform of LH may be responsible for these ovulations in the same way as Alexander and Irvine (1982) reported the occurrence of two isoforms of LH during oestrus. This could reflect the specificity of the assay used suggesting that although it cross reacted with eCG, it did not recognise this different isoform of LH.

The three instances of prolonged intervals due to dioestrous ovulations all related to ovulations which occurred around day 11 of dioestrus. Douglas and Ginther (1975) reported that newly formed CLs are not susceptible to the luteolytic effects of PGF2a for 1-4 days which meant that they were not lysed at the same time as the original one. However, in Gemma's case it appeared that second CL was partially susceptible to the luteolytic effects of the prostaglandin, as progesterone concentrations during the second half of the interval are much reduced and reflect a poorly functioning CL. The resulting prolonged dioestrous interval was of 37-39 days duration, which was short compared to the average reported by Stabenfelt et al.

(1974). This may have been due to the partial lysis of the secondary CL reducing its lifespan.

The two instances of dioestrous ovulations which did not result in prolonged luteal phases were associated with ovulations early in dioestrus. The second CL underwent complete lysis along with the original one at the usual time giving a normal dioestrous interval.

A more straightforward cause of prolonged dioestrous intervals in the mare could be a missed oestrus. This is not as common in horses as cattle due to the long oestrous period and the widespread use of regular teasing on breeding establishments. It can be a problem for single mare owners with no access to a stallion or with a mare which either does not show well or is only in oestrus for a short period (Ginther, 1992). This occurred with Meg due to the holidays and the progesterone results demonstrated the return to basal concentrations associated with oestrus in the absence of teasing.

Seasonal Variations

Adams and Bosu (1988) reported that 0-10 % of ponies and 20-50 % of horses continue to ovulate all year round without entering a period of anoestrus. Only one of the four pony mares in the present study continued to cycle so the high result of 25% is probably due to small group size. It may have been the favourable environmental conditions under which Lady was kept throughout the winter compared to the other ponies which prevented her entering anoestrus.

All of the horse mares in this study were housed under the same conditions and two entered anoestrus which suggested that environmental conditions were not the only factors affecting reproductive function at that time. 71% of the horse mares continued to cycle which is greater than the previous report and may have been due to the additional extra light which the unit received during the night due to its situation in the city.

Photoperiod has been reported as the main factor controlling seasonal variations in the mare, although other influences such as environmental temperature and body condition also played a role (McKinnon and Voss, 1993). The good body condition of all the mares except Misty may therefore have also contributed to the higher percentage continuing to cycle than expected. Colquhoun (1984) suggested that not only was body condition important but also the level of nutrition and that this was especially relevant in larger mares where feeding may have been enough for maintenance but no extra was available to support reproductive function. Meg and

Gemma, who entered anoestrus, were both large mares but Greedy was the largest mare in the study and continued to cycle on the same diet as the other two. This does not support the findings of Colquhoun (1984).

Colquhoun (1984) also suggested that mares who expended a lot of energy on vices such as weaving may use their feed up quicker and so be more likely to become anoestrus. However in this study, Laura continued to cycle despite weaving almost continually.

Snyder et al. (1979) reported a decline in circulating LH concentrations at the end of the breeding season due to a reduction in its production and this eventually caused ovulation failure and the entry into anoestrus. FSH is slower to decline and can cause the production of follicles and oestrous behaviour into and in same cases throughout the winter. However, the lack of LH means ovulation cannot occur. This type of behaviour was displayed by three of the five mares in the present study where sufficient FSH was present to produce follicles competent enough to cause oestrous behaviour in association with LH concentrations too depleted to allow ovulation. However, Gemma's lack of follicular development at this time suggested that her FSH concentrations were reduced along with her LH and she went straight into anoestrus after regression of the previous CL. Hughes et al. (1972) also found a similar variety of ways in which mares entered winter anoestrus including the presence of a persistent CL as displayed by Suzy in the present study.

Suzy's behaviour after this time was unusual and did not correspond with any previous reports. The structures in her ovary which were observed during two periods as she entered winter anoestrus were both functional, as shown by the raised progesterone concentrations and therefore must have contained luteal tissue, although neither of them produced progesterone for as long as a normal CL. Their appearance on ultrasound suggested that spontaneous luteinisation of follicles could have occurred since the characteristic shape and size associated with ovulation and CL formation were absent.

Ovulation failure in the mare is not common. One report recorded only two cases of ovulation failure in ponies out of 100 cycles during the ovulatory season (Ginther, 1974) and in Thoroughbreds a rate of 3.1% has been found (Hughes et al., 1972). This low incidence is thought to be due to the large follicular size and the long LH increase that occurs in the mare (Colquhoun, 1984).

Haemorrhagic or autumn follicles have been reported to occur in mares towards the end of the ovulatory season due to ovulation failure, presumably due to a lack of LH, although they have also been reported following ovulation (Ginther, 1979).

They can develop up to 10-15 cm in diameter due to an influx of blood and persist for around 60 days while a fibrinous clot forms, before regressing. They are non-functional and therefore do not affect the mares reproductive cycle (Hughes and Stabenfeldt, 1972). Ginther and Pierson (1989) reported three instances of prolonged interoestrous intervals which were supposedly caused by these structures. However, they did not confirm they were functional by measuring plasma progesterone concentrations so it seems likely that these intervals were due to some other cause.

It seemed unlikely that the structures present in Suzy were haemorrhagic follicles since they were clearly functional. An alternative explanation could be the occurrence of two ovulations accompanied by silent oestruses. Lack of behavioural signs of oestrus could have been due to declining FSH concentrations not being sufficient for maturation of the dominant follicle. Oestrogen production may therefore have been reduced in a similar manner to the early follicles in the spring transitional period which are steroidogenically incompetent due to a lack of androgen precursors (Adams and Bosu, 1988). Alternatively, Suzy's behavioural centres may not have been responsive to the effects of oestrogens at this time.

The short lifespan of these structures may reflect a decline in LH concentrations at this time since LH has been shown to contribute to the early development of the CL. Also, the pituitary is thought to produce a substance which performs a luteotrophic role throughout the lifespan of the CL (Pineda et al., 1972) which may also be declining due to the time of year.

It therefore seemed most likely that Suzy's behaviour at that time was due to silent oestruses and the formation of functional CLs while the structure identified prior to the start of the study was probably a haemorrhagic follicle.

The instances where dense stroma in the ovary were mistaken for luteal tissue highlights the problems which can arise with inexperienced operators but taken in conjunction with all the other available information such as teasing scores and progesterone results, the significance of these findings can be evaluated.

Oestrous behaviour in anoestrous mares can occur without a perceptible increase in ovarian activity (Hughes et al., 1972) or detectable increases in plasma oestrogen concentrations and it has been suggested that the behavioural centres are more sensitive to oestrogen stimulation during this time of basal progesterone concentrations. This allows signs of oestrus to be displayed despite oestrogen concentrations being too low to measure with conventional assays. Alternatively, other substances may be produced by the adrenals which also affect the behavioural

centres (Colquhoun, 1984). In the present study, variations were observed in the mares behaviour throughout the anoestrous period with the mares in technically the most unfavourable environment showing most ovarian activity. The reason for this was not known and suggests there are many factors involved in how mares respond to this inhibitory period.

The first ovulation of the year is considered to be April 7th ± 9.1 days in horses (McKinnon and Voss, 1993) and around the start of May in ponies which corresponded with the findings of the present study. However, Gemma was an exception since she did not produce her first ovulation after anoestrus until May 2nd, which is relatively late in the breeding season. This may have been due to her correspondingly late entry into anoestrus in January.

The pony mares all displayed periods of oestrous behaviour longer than seven days before undergoing their first ovulation of the year which corresponded with the findings of Sharp (1980b) who reported some mares showing prolonged oestruses of 40 or 50 days at this time of the year.

However, Meg did not display a prolonged oestrus which suggested variations between mares with regard to how rapidly their gonadotrophin concentrations recovered after anoestrus. Again, additional light may have hastened her entry into the breeding season.

Multiple Ovulations

The cause of multiple ovulations in the mare is not known but is thought to occur at the level of the follicle, possibly due to the development of LH receptors on more than one follicle (Urwin and Allen, 1983). It may also be encouraged by the long period of increased LH in the mare (Geschwind et al., 1975). Follicles associated with multiple ovulations are structurally identical to those that ovulate singly but tend to attain a smaller diameter before ovulation (35 - 40 mm compared to 44 mm) (Ginther and Pierson, 1989). The incidence of double ovulations varies between studies from 2% (Ginther, 1974) to 22% (Ginther et al., 1982) and triple ovulations are reported in 1.4% of cases (Ginther and Pierson, 1989). The findings of the present study correspond with this range.

A distinct breed predisposition has been reported which may be the cause of the range of incidences found between these reports, with Thoroughbreds showing a 19% incidence compared to 9% in Quarterhorses (Ginther et al., 1982) and almost 0% in ponies (Arthur and Allen, 1972). It also seems to be repeatable in individual mares (Arthur, 1968) as well as related ones (Ginther et al., 1982).

Of the mares displaying double ovulations in the present study, one was Thoroughbred and another part Thoroughbred which corresponds with the higher incidence of double ovulations reported in Thoroughbreds by Ginther et al. (1982). However, Arthur and Allen (1972) reported an incidence of multiple ovulations in pony mares approaching 0% and since the third mare was a Highland pony cross Thoroughbred she might therefore have been expected to have had a low incidence of multiple ovulations. That this was not the case suggests that the Thoroughbred influence must be dominant to that of the Highland pony. This same mare also produced a double ovulation at her first ovulation of the year following a period of anoestrus, which is surprising considering that Ginther et al (1982) reported a 42-67% reduction in the rate of multiple ovulations during other similar inhibitory periods where ovarian function is not maximal, such as during lactation. However, none of the other part bred Thoroughbred mares in the study displayed any double ovulations.

In this study, unilateral and bilateral ovulations occurred with the same frequency which corresponded to the report by Squires et al. (1987). Ovulations can be described as synchronous when they occur within 24 hours of each other and asynchronous when they occur more than 24 hours apart but still within the same oestrous period. Squires et al (1987) reported that whether they were unilateral or bilateral did not affect the interval between ovulations which corresponds with the findings of the present study.

It has been suggested that there may be a connection between mares which have a tendency to multiple ovulations and the occurrence of dioestrous ovulations. In this study, there appeared to be two distinct groups with Misty, Meg and Poppy being responsible for the double ovulations and Gemma, Jenny and Suzy for the dioestrous ovulations. In addition, because Suzy is a pony, she might be expected to have a low incidence of double ovulations, suggesting the mechanisms responsible for dioestrous ovulations are different from those associated with multiple primary ovulations.

It has been suggested that ovulation occurs from the left ovary of maiden mares with a greater incidence than from the right (62% v 38%) but that in mares which have had a foal there is no difference. This is presumed to be due to the hypothesised inequality in the blood supply to the maiden ovaries being corrected by the generally improved supply to both ovaries during pregnancy (Ginther, 1983a). The findings of the present study do not support this but do highlight the variation between individual mares.

Prostaglandin Administration

Prostaglandin administration was used on a number of occasions throughout this study to bring mares into oestrus for A. I. or to provide an oestrous mare for semen collection as part of another project.

Douglas and Ginther (1975) reported that the CL was not sensitive to the effects of prostaglandin until it was five days old which could explain the prolonged time between administration and oestrus observed in one instance with Suzy. It appeared that there was individual variation in the way the mares responded to prostaglandin administration early in lifespan of the CL but that the response became more uniform if given later in dioestrus.

Nelson et al (1985) reported that silent oestruses have been associated with prostaglandin administration in some cases as it shortens the interval between luteolysis and ovulation. However, since Lady was the only mare to demonstrate silent oestruses in association with treatment and she displayed a number of such oestruses throughout the course of the study, it seemed likely that she was predisposed to such behaviour rather than it being induced by the prostaglandin.

Ovarian Abnormalities

Ovarian tumours are relatively rare in the mare and the most common of these are granulosa cell tumours (McKinnon and Voss, 1993). They derive from granulosa and theca cells, gradually increasing in size until they destroy the ovary and commonly produce oestrogens and testosterone (Stabenfeldt et al., 1979) as well as other substances such as inhibin (McKinnon and Voss, 1993). These can produce behavioural changes such as nymphomania, virilism or anoestrus as well as atrophy of the contralateral ovary causing the mare to stop cycling, which helps differentiate this tumour from other causes of ovarian enlargement (Hinrichs and Hunt, 1990). Transrectal ultrasound is also useful in identifying this tumour but is not diagnostic due its variable appearance. Previous reports have described the surgical excision of the granulosa cell tumour on diagnosis and the subsequent return to normal cyclicity of the mare. This is due to the recovery of the remaining ovary following removal of the inhibitory substances produced by the tumour (McKinnon and Voss, 1993).

Throughout the course of the present study, Greedy not only appeared to cycle normally but also conceived and maintained a pregnancy until terminated at day 41. This confirms that her granulosa cell tumour had spontaneously become non-functional following her previous incidence of nymphomania, which may be reflected in the change in appearance of the tumour. There are no reports describing

the course of events following the diagnosis of such a tumour in a mare without its excision and it seems likely that unless a mare was being used as a brood mare or was displaying disruptive behavioural signs, such a tumour could easily remain undetected. This suggested that a proportion of mares in the general population have granulosa cell tumours which remain as incidental findings.

The most commonly recognised type of peri-ovarian cysts in the mare are hydatids of Morgagni which are cystic remnants of mesonephric tubules and can reach sizes of 2cm. This corresponds with the one observed in the present study in Jenny. They are reported to be of no clinical importance, although care must be taken not to confuse them with follicles (McKinnon and Voss, 1993). In this case, careful palpation confirmed the cyst was outwith the boundaries of the ovary.

Chapter 2 - Comparison of 2 Progesterone Assay Kits.

2.1 Introduction

As discussed in Chapter One, there are many cyclic aberrations that occur in the general mare population and various information is required to determine a mare's cyclic state and stage. Plasma progesterone concentrations are useful as they indicate the presence or absence of luteal tissue and can be used in a number of situations amongst which are differentiating between anoestrous and cycling mares during the non-breeding season, determining if the first ovulation of the year has occurred and identification of oestrous mares during the breeding season which do not display obvious oestrous behaviour.

In a veterinary practice situation, it is often uneconomical and impractical to run assays especially if the mare population is small and to send samples to commercial laboratories is time consuming, especially later on in the breeding season when time is short. However, there are progesterone kits available which are designed for convenient use in veterinary practice that can be used economically for single samples and give rapid results.

The Target Equine Progesterone Kit (Biometallics, Princeton, NJ, USA) is one such kit and results obtained from mares in various cyclic stages using it were compared to those obtained using the Coat-A-Count Radioimmunoassay described in Chapter One, to allow the accuracy and reliability of the Target kit to be calculated. This investigation was prompted by the confusion that arose early in the study when progesterone concentrations indicated by the Target kit did not correlate with the stage of the mares cycle as determined by the other methods discussed in Chapter One.

2.2 Materials and Methods

2.2.1 Plasma Samples

69 of the plasma samples collected for use in Chapter One were selected to represent a range of different stages throughout the oestrous cycle. Collection and storage procedures were the same as described in Chapter One.

2.2.2 Radioimmunoassay

The progesterone concentrations obtained in Chapter One for each of these samples using the Coat-A-Count Progesterone Radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA) were used in this Chapter also. The accuracy and quality control data for this assay was discussed in Chapter One.

2.2.3 ELIZA

The Target Progesterone ELIZA (Biometallics, Princeton, NJ, USA) was used in accordance with the manufacturer's instructions, as described in Appendix A. It was run in individual tubs and gave a semi-quantitative result. A colour change indicated low (<1 ng/ml = dark blue), intermediate (1-5 ng/ml = pale blue) or high (>5 ng/ml = white) progesterone concentrations.

IPACT (Immuno Precision Colour Technology) was built into the tubs during manufacture so standards were not required and samples were run singly. It was therefore not possible to calculate the intra-assay variation and financial constraints prevented the number of assays required to assess inter-assay variation being performed.

The control samples prepared as described in Chapter One were also run as unknown samples.

Some of the Target assays were performed by veterinary undergraduates.

2.3 Results

As discussed in Chapter One, the Coat-A-Count inter-assay coefficient of variation was acceptable for the low and intermediate control samples Therefore the results from all the runs were combined and taken to be correct in order to allow comparison with the Target kit. The inter-assay coefficient of variation regarding the high control was poorer but since these concentrations are well into the 'high' range as defined by the Target kit, it was felt that they would not affect the outcome of the comparison.

Figure 2.1 shows the results obtained when the ICN standards were run on the Target and Coat-A-Count kits compared with the manufacturer's values.

Figure 2.1

MANUFACTURERS VALUES	TARGET n = 2	C-A-C (± st dev)
LOW 1.29 (0.93 - 1.65)	LOW	1.2 ± 0.1
MED 6.18 (5.08 - 7.28)	INT	4.34 ± 0.28
HIGH 24.2 (20.1 - 28.3)	HIGH	18.28 ± 3.7

The low and high samples were in agreement but since the manufacturers stated that the Target kit would read any sample above 5ng/ml as high, it gave a false intermediate result for the medium control. The medium control was run again and the same result was obtained. This could have suggested that the kit was producing results that were lower than they should be. However, the Coat-A-Count also read the medium standard as lower than the manufacturer's values and the mean that it gave of 4.45 ng/ml was within the range of concentrations that would have correctly given an intermediate Target result.

Because these kits were designed to be run individually it was not possible to calculate the intra-assay coefficient of variation for the Target kit and financial reasons prevented the number of assays necessary to calculate the inter-assay coefficient of variation being performed. There was also no information accompanying the kit regarding its sensitivity or specificity.

Figure 2.2 shows the results obtained for the unknown samples when the Target kit was interpreted according to the manufacturer's instructions with a low result indicating a sample with a progesterone concentration <1ng/ml, an intermediate result a sample from 1-5 ng/ml and a high result a sample >5ng/ml. These were then compared to the results obtained when the same samples were run on the Coat-A-Count and the percentage of results that agreed calculated.

The Target kit only produced results that agreed with those obtained using the Coat-A-Count in 61% of cases. The low samples agreed satisfactorily, but only about half of the intermediate and high results obtained on the Target kit agreed with those of the Coat-A-Count. In other words, if a high result was obtained on a Target kit, there was only a 50% chance that the mare actually had a progesterone

concentration >5ng/ml and likewise if an intermediate result was obtained only 47% were due to mares with progesterone concentrations of 1-5ng/ml.

The results from the samples which disagreed are shown in Appendix C. Because nine of these were due to high results obtained for samples at the upper end of the 1-5ng/ml or intermediate range, it was decided to alter the interpretation of the Target to try and improve its accuracy. Figure 2.3 shows the results obtained when the intermediate range was changed to 1-4ng/ml and the high to >4ng/ml with the low remaining as <1ng/ml, before comparison with the Coat-A-Count results.

The agreement between the high samples improved markedly so that 78% of the high results obtained on the Target kit were due to samples with progesterone concentrations >4ng/ml and consequently the overall rate of agreement improved to 74%, which was much more acceptable. It suggested however, that the Target kit had a tendency to read high and in fact out of the 27 samples which were in disagreement (See Appendix C), 23 or 85% were due to the Target reading being one category too high. This did not agree with the medium standard producing an intermediate result, especially since the Coat-A-Count gave an average value of 4.45 ng/ml, which under this new interpretation should have achieved a high result anyway. The intermediate readings were still very inaccurate with under 50% of them being due to samples in the 1-4ng/ml range and the rest being due to values <1ng/ml.

Since from a clinical point of view it was usually only necessary to determine if there was luteal tissue present or not, the interpretation of the Target kit was once more altered. Again, a low result indicated a sample with a progesterone concentration < 1 ng/ml but the intermediate and high results were combined into a 'luteal' category, denoting a sample with a progesterone concentration >1ng/ml. Again, these were compared to the Coat-A-Count results and Figure 2.4 shows the outcome. The agreement between the two kits when interpreted this way was 85.5% which was very much improved and meant that 85.5% of the 'luteal' results obtained on the Target kit were from mares with progesterone concentrations >1ng/ml. The seven samples or 15% that gave intermediate results but had progesterone concentrations <1ng/ml were again the main source of inaccuracy.

The situation was then reversed to determine what percentage of mares with progesterone concentrations in a given range would obtain correct results when run on the Target kit. The Coat-A-Count results were put into categories of <1ng/ml, 1-4ng/ml and >4ng/ml to correspond with the interpretation of the Target kit and then compared to the Target results. The outcome is shown in Figure 2.5. The overall result was the same as in Figure 2.3 but this represented the likelihood of a sample

producing a result that would be correctly interpreted. Again, the agreement between high and low concentrations were acceptable but mares with progesterone concentrations between 1 and 4ng/ml had a less than 50 % chance of producing an intermediate result on the Target kit.

Again, from a clinical point of view it was usually only necessary to determine if a mare was luteal or not, so the Coat-A-Count results were grouped as <1ng/ml and >1ng/ml. These were then compared to the Target results to determine the percentage of mares with progesterone concentrations >1ng/ml that would produce 'luteal' results i.e. intermediate or high on the Target kit and these are shown in Figure 2.6. This demonstrates that although 93% of mares with progesterone concentrations >1ng/ml gave luteal i.e. intermediate or high results on the Target, 27% of mares with concentrations <1ng/ml also did so.

One sample was collected from Corrie in the ninth month of pregnancy and produced a white high result on the Target kit but was not run on the Coat-A-Count.

Figure 2.7 shows the results obtained when some samples which were not in agreement initially were reassayed. Four samples were now in agreement (1, 2, 4 & 5) after being reassayed on the Target kit but the other four continued to disagree (3, 6, 7 & 8) which suggested an equal chance of a reassayed sample correcting itself or continuing to disagree although more would have had to be carried out to confirm this. Of the three samples with progesterone concentrations <1ng/ml which gave intermediate results initially, two read correctly on being reassayed (1 & 2).

The remaining samples (6, 7 & 8) unlike those mentioned previously, gave Target results that were lower than their Coat-A-Count ones and all three produced the same result on being reassayed. With sample 6, there was definitely a blue colouring present although it was very faint and therefore by the manufacturer's guidelines was considered intermediate despite the Coat-A-Count results indicating progesterone concentrations significantly higher than the manufacturers stated threshold (9.1 v 5 ng/ml). Sample 7 was the only sample to produce a Target result more than one category away from its Coat-A-Count result when high readings were taken as being >5ng/ml, but sample 8 also did so when the interpretation was altered to include concentrations >4ng/ml in the high range.

2.4 Discussion

The manufacturers recommend the Target kit for use in 'determining where the mare is in her cycle, whether there is a functional CL and thus whether to use

Figure 2.2 Target v Coat - A - Count when Target is interpreted according to the manufacturers instructions

TARGET	CAC		TOTAL % AGREES
	AGREES	DISAGREES	
LOW (< 1 ng/ml)	19	3	86%
INT (1 - 5 ng/ml)	7	8	47%
HIGH (> 5ng/ml)	16	16	50%
TOTAL	42	27	61%

Figure 2.3 Target v Coat - A - Count when a high result is interpreted as > 4 ng/ml and an intermediate as 1 - 4 ng/ml

TARGET	CAC		TOTAL % AGREE
	AGREES	DISAGREE	
LOW (1 ng/ml)	19	3	86%
INT (1 - 4 ng/ml)	7	8	47%
HIGH (> 4ng/ml)	25	7	78%
TOTAL	51	18	74%

Figure 2.4 Target v Coat - A - Count when a luteal category of high and intermediate results is interpreted as > 1 ng/ml

TARGET	CAC		TOTAL % AGREE
	AGREE	DISAGREE	
LOW (< 1 ng/ml)	19	3	86%
LUTEAL (> 1 ng/ml)	40	7	85%
TOTAL	59	10	85.50%

Figure 2.5 Coat - A - Count v Target when > 4 ng/ml is interpreted as high and 1 - 4 ng/ml as intermediate

COAT - A - COUNT	TARGET		TOTAL % AGREE
	AGREE	DISAGREE	
> 1 mg/ml (LOW)	19	7	73%
1 - 4 ng/ml (INT)	7	8	46%
> 4 ng/ml (HIGH)	25	3	89%
TOTAL	51	18	74%

Figure 2.6 Coat - A - Count v Target when a result > 1 ng/ml is interpreted as luteal

COAT - A - COUNT	TARGET		TOTAL % AGREE
	AGREE	DISAGREE	
< 1 ng/ml (LOW)	19	7	73%
> 1 ng/ml (LUTEAL)	40	3	93%
TOTAL	59	10	85.50%

Figure 2.7 Results from samples in disagreement after being repeated

SAMPLE	C - A - C ORIGINAL	C - A - C REPEAT	TARGET ORIGINAL	TARGET REPEAT
1	0.11		INT	LOW
2	0.16	0.19	INT	LOW
3	0.8	0.7	INT	INT
4	1.6	1.4	HIGH	INT
5	3.9	3.7	HIGH	INT
6	9.5	8.7	INT	INT
7	6.48	6.9	LOW	LOW
8	4.1	4.7	LOW	LOW

prostaglandin, monitoring progesterone concentrations during pregnancy and monitoring progesterone for embryo transfer.'

The Target kit was only 61% accurate, as demonstrated in Figure 2.2, which was poor from a clinical viewpoint and meant this assay was of limited value when performed as the manufacturers intended. The tendency of the kit to produce results which were higher than the actual plasma concentrations could have lead to potential problems if the results wee to be used in the circumstances suggested above.

If it was performed to confirm mares were luteal before prostaglandin was administered, it may have lead to treatment being given after luteolysis had already begun or to repeated administration once the mare had entered oestrus, if the manufacturers guidelines were followed in this respect. This could have affected the subsequent timing of artificial insemination, natural mating or embryo transfer. It could also have given the false impression that an anoestrous mare had undergone her first ovulation of the year or that an oestrous mare was either still undergoing luteolysis or had already ovulated. Again, this may have affected the timing of artificial insemination or mating especially in mares which did not show signs of oestrus well such as Lady or mares like Meg who had short oestrous periods and ovulated before large follicles were detected. It could also have affected the degree to which mares were thought to be synchronised in association with embryo transfer procedures.

For the kit to be of use in a clinical situation, the interpretation of its results was modified. Using a high white result to indicate a sample with a progesterone concentration >4ng/ml rather than 5ng/ml not only improved the accuracy of the assay to an overall 74%, but was also of more clinical significance since high progesterone concentrations in the mare are generally regarded as being over 4 ng/ml (Ginther, 1992) rather than 5ng/ml.

The main inaccuracy when using the Target kit was associated with the intermediate results. Only 46% of the plasma samples with progesterone concentrations of 1-4ng/ml actually produced intermediate results on the Target kit which was unsatisfactory from a clinical viewpoint. When an intermediate result was obtained, the manufacturers recommended that a repeat sample was taken two days later to determine if the result was due to concentrations rising or falling. However, if the original intermediate Target result was not due to a sample with a progesterone concentration of 1-4 ng/ml, as was the case with 53% of the intermediate results

obtained, then the outcome of the second sample could confuse rather than resolve the situation.

By eliminating this intermediate range and using the Target kit to produce qualitative i.e. high or low results, rather than the semi-quantitative ones the manufacturers intended, the accuracy of the kit improved significantly. Since in the mare it was usually only necessary to determine whether luteal tissue was present or not, this interpretation was justified and also represented the most reliable way to use the kit to produce clinically useful results.

The Target kit is also recommended by the manufacturers for the monitoring of progesterone concentrations throughout pregnancy and they state that 'low concentrations of progesterone at any stage of pregnancy are a major cause of early embryo loss or late-term abortion'. Although there is much debate as to the minimum progesterone concentrations required to maintain pregnancy in the mare and therefore the need for progesterone supplementation, a very faint blue or white result indicating at least 4 ng/ml on the Target kit is recommended by the manufacturers as the minimum safe level.

In the present study, progesterone concentrations as low as 1.39 and 1.54 ng/ml gave high white results so the accuracy of its use in such a quantitative manner was debatable. Also, since mares with progesterone concentrations as low as 2.5 ng/ml have not aborted (Shideler et al., 1982) and that progesterone secretion is reported to be episodic (Evans, 1991), it would seem unwise to diagnose progesterone insufficiency on the basis of an isolated blood sample.

During the last half of gestation, progesterone production by the ovaries ceases but progestagens are produced by the placenta and so the measurement of circulating concentrations can give very variable results depending on the cross reactivity of the assay used (Ginther, 1992). This could lead to low results being obtained on any assay despite a normal pregnancy. The sample from Corrie when she was in her ninth month of gestation produced a high white result on the Target kit, which suggested that this kit did crossreact with the placental progestagens, although more samples would need to be run from varying stages of pregnancy to confirm this. The sample was not run on the Coat-A-Count, so it was not known whether this assay would also have given a value >4 ng/ml. Since the manufacturer's state a high specificity of the Coat-A-Count for progesterone alone, it was likely a low result would have been given despite the pregnancy.

There are many reasons why an assay may give results which do not truly reflect the hormone concentrations present in a plasma sample. The Target kit was designed as

a rapid and convenient assay for use in veterinary practice and there were therefore very few steps involved and little room for error in technique. However, there were some areas where inaccuracies could have occurred such as in the timing of certain steps, the age of the reagents, the length of time the kit was allowed to warm up and the prevailing room temperature.

In the present study, the Target kit often produced results higher than the actual progesterone concentration of the sample. However, before this could be considered significant, the inter-assay coefficient of variation would have to be calculated for this assay. This calculation gives an indication of the precision or reproducibility of the assay by measuring the variation between repeated determinations of the same sample (Chard, 1995). It would help to determine whether an aberrant result was due to assay variation or was in fact real. As previously mentioned however, lack of funds prevented the number of assays being performed to allow this parameter to be calculated.

Since these higher than expected results obtained on the Target kit involved the difference between the development of a pale or dark blue colouring, it may be that the colour was slow to develop due to low room temperature or that the results were read too early by inexperienced operators. This was especially likely to explain the four samples where a correct result was obtained when the Target assay was repeated. However, by the very nature of the kit, it was designed to be a 'one-off' test which does not require standards, duplicate samples or controls and therefore there was nothing with which to compare the result to confirm its accuracy.

Three of the plasma samples in the present study produced results on the Target assay which were lower than their actual progesterone concentrations, unlike the rest of the incorrect results which were all higher. These cannot be explained in the same way as the previous discrepancies since the appropriate colour change for their concentrations would have been paler than those obtained. All of these samples were from the same mare which suggested that there was some factor present in her plasma sample affecting the ability of the progesterone to bind to the antibodies in the tubs.

Immunoassays for progesterone have been shown not to suffer from species specificity but can display problems referred to as a 'matrix effects' or non-specific non-specificity, where some factor interferes with an assay which is not clearly identifiable by its physiochemical similarity to the ligand (Chard, 1995). Examples of this are incompatibility between the type of fluid the sample is within and the assay such as the use of equine plasma in a kit designed for use with human plasma or substances present in the plasma such as lipids which interfere with the binder-

ligand reaction of the assay. The samples from the other mares all produced acceptable results which excludes a problem with the use of equine plasma with this kit but it was only Jenny's plasma which produced this effect suggesting it could be some substance within it that was responsible. Further investigations would have to be carried out to confirm this though.

Another possibility could have been incorrect handling of the plasma samples since prolonged storage at room temperature has been shown to cause a reduction in progesterone concentrations. However, the results obtained on the Coat-A-Count for these samples were consistently higher than those of the Target kit on a number of occasions which rules this out. It may be possible to identify individual mares for which this assay is not suitable but more work would need to be done to confirm this observation.

Unfortunately, it was not possible financially to perform the number of repeated assays necessary to calculate the inter-assay variation of the Target kit and therefore these observations are speculative.

The Target kit did prove to be very quick and easy to use, required a minimum of expertise and equipment and was useful in helping determine the plasma progesterone concentrations in the mare. However, when used as the manufacturers intended its accuracy was poor and it was the confusion that this caused in the interpretation of the cyclic behaviour of the mares in this study that prompted the investigation. Its main downfall was a tendency to read high and this was alleviated by combining high and intermediate progesterone concentrations into a 'luteal' category and using it as a qualitative assay rather than in the semi-quantitative way the manufacturers suggest. However, for the accurate determination of the mares' cyclic state and stage, it should be used in conjunction with information obtained from other techniques such as ultrasound scanning, rectal palpation and teasing records.

When quantitative results are required, such as those concerned with the maintenance and supplementation of pregnancy, progesterone concentrations should be confirmed by another more accurate assay.

Chapter 3 - Artificial Insemination and Pregnancy Detection.

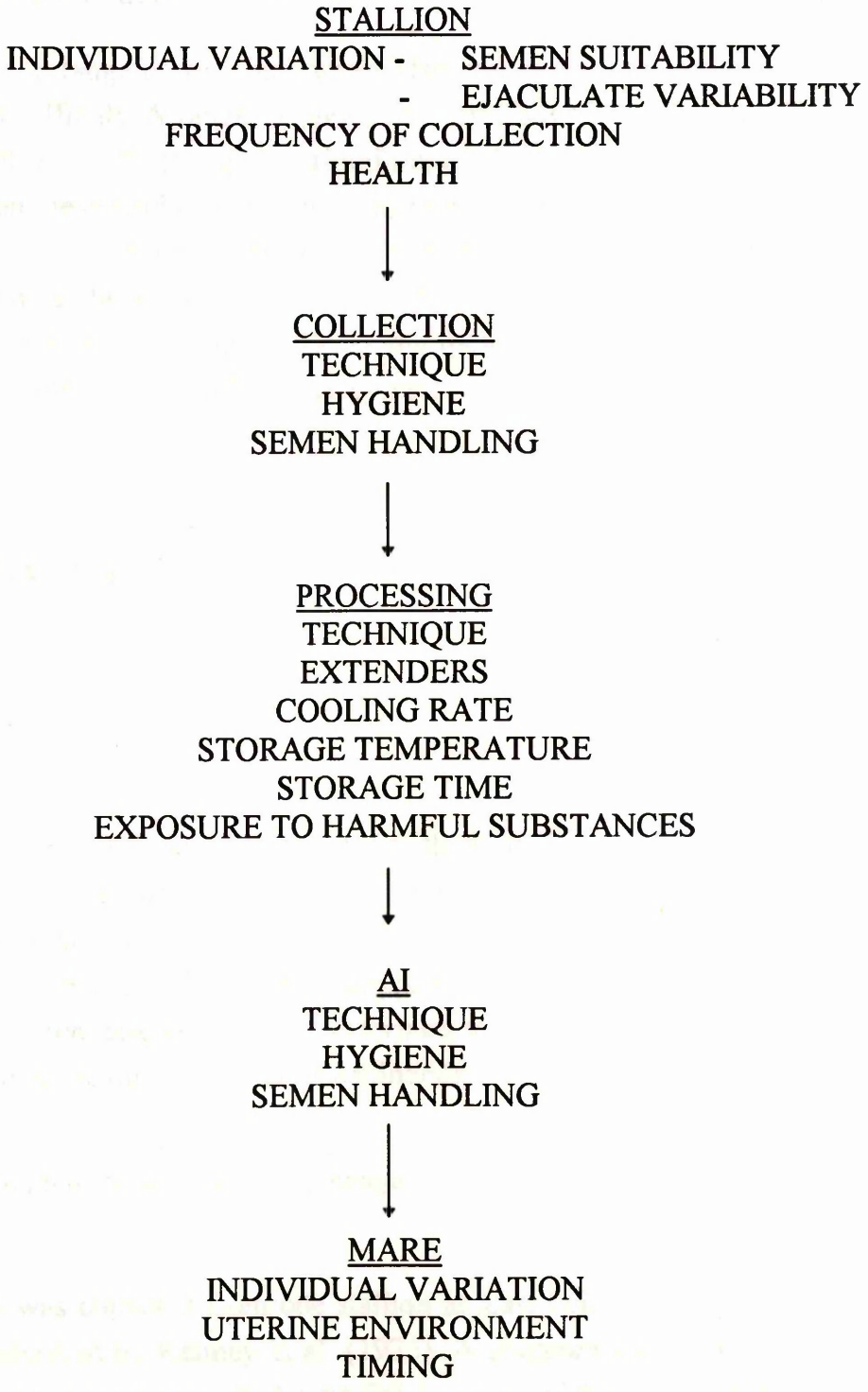
3.1 Introduction

The use of artificial insemination in horses was reported as early as 1322 in Arabic texts although its scientific investigation only commenced at the end of the 19th Century. The technique is now widely used but is still not universally accepted by breed societies in Europe and North America despite the success of its application in large scale breeding programmes in China. These programmes, involving thousands of mares, claim overall pregnancy rates comparable to, if not better than those attained with natural service (Bowen, 1969).

During natural covering, the stallion ejaculates against the patent oestrous cervix depositing semen directly into the uterus, as described by Kenney et al (1975). This can be demonstrated by ultrasound examination immediately after covering (Ginther and Pierson, 1984b). Consequently, at insemination, the semen is also deposited through the cervix into the uterus. The sperm travel to the uterine tubes by a combination of smooth muscle contraction, ciliary action, fluid currents and flagellar activity under the influence of oestrogen (Ginther, 1992). Maximum numbers of sperm are present in the uterine tube four hours after insemination following which numbers decline (Bader, 1982) as they are eliminated from the tract, presumably through the cervix (Bader and Krause, 1980). Once in the mare's tract, exposure to secretions causes the sperm to undergo capacitation which makes the occurrence of the acrosome reaction possible and allows the sperm to penetrate the ovum (Bedford, 1983). Once one sperm has penetrated, the outer layers of the ovum undergo a change that prevents the entry of any other sperm (Metz and Monray, 1967). Fertilisation takes place in the ampulla of the uterine tube.

Many factors are involved in determining the outcome of an insemination (See Figure 3.1) and these must all be taken into account if a programme is to be successful. Many different procedures for the processing and handling of semen are employed around the world, but there is not one which has been proven in large scale breeding programmes to be better than any other. Because breed societies have put different constraints on the use of AI within their breed, the development of a

Figure 3.1 SOME FACTORS AFFECTING THE OUTCOME OF A.I.



universal method has been hampered. There is also a great variation between stallions, and even on occasion between ejaculates, in the suitability of sperm for processing (Pickett and Amann, 1987). Since most stallions are selected for breeding on attributes such as athletic ability and physical appearance rather than on the quality of their sperm and its suitability for processing, this further compounds the problem of developing a standard procedure that will suit all programmes.

This wide range of variable factors also makes comparing the findings of different studies difficult. A range of pregnancy rates have been reported from 0% (Voss et al., 1981) to 91% (Douglas - Hamilton et al., 1984) but there are many differences between these studies which need to be taken into consideration before conclusions can be drawn. Attempts to predict the fertility of semen by measuring a range of criterion has been attempted and threshold concentrations for many parameters have been suggested (Jasko et al., 1992), but these have proven very variable and the only accurate indicator is still pregnancy rate.

3.2 Materials and Methods

Introduction

Mares were artificially inseminated with chilled, extended semen of varying age and quality at different times in relation to ovulation. The outcome of each insemination was correlated with the variable factors associated with it in order to determine the success rate obtainable with an approach to an A.I. programme as flexible as this. Basic semen assessment was performed to evaluate the use of such procedures in predicting the outcome of an insemination.

3.2.1 Semen Collection and Storage

Semen was collected from one stallion at least twice a week in a manner similar to that described by Kenney et al. (1975). A phantom mare was used with or without an oestrous mare present. An artificial vagina of the Cambridge type was used and the semen collected into a plastic container through a milk filter to remove the gel

fraction. The semen was diluted with Kenney's extender (Kenney et al., 1975) to a final concentration of 100 million sperm per ml. It was then either :

a/ placed in an Equitainer (Hamilton-Thorn Research, Danvers, MA, USA) in accordance with the recommended procedure for its use and stored there until used (Douglas-Hamilton et al., 1984)

or

b/ cooled in a cryofreezer at a rate of -0.4°C / min to 4°C and then stored at that temperature in a waterbath in a fridge.

3.2.2 Semen Assessment

This was conducted as close to the time of insemination as possible.

a/ Percentage Progressively Motile Sperm (% PMS)

A drop of semen was placed on a glass slide at room temperature and then warmed on a heated stage to 37°C. After five minutes it was examined under a x40 microscope objective and the % live and progressively motile were assessed subjectively.

b/ Percentage Live (% Live)
A drop of semen was added to five drops of nigrosin-eosin stain, mixed and allowed to stand for five minutes. A smear was made and 100 sperm were counted using a x100 objective, recording the number that were alive (unstained) and dead (pink stained) at staining.

3.2.3 Artificial Insemination

The mares described in Chapter One were used for this section of the study. A procedure similar to that described by Kenney et al. (1975) was used. The mare was restrained in stocks with her tail placed in a clean rectal glove and held to the side. Her perineum was washed thoroughly using a dilute Savlon solution (Pitman-Moore, Cheshire, UK) and dried. A clean, gloved, lubricated hand guarded the end of a sterile, rigid, plastic pipette as it was introduced into the vagina. A finger was passed through the cervix and used to guide the end of the pipette through into the uterine lumen. The semen was placed in a 60 ml syringe and either flushed down

the pipette or run in under gravity. A small volume of extender or air was used to flush the pipette prior to removal.

Inseminations were performed at varying intervals before ovulation and only one insemination was carried out during each oestrous period with the exception of one mare who was inseminated twice. Inseminations were carried out during eight oestruses which were induced using prostaglandin, either 5mg Dinoprost (Lutalyse (Upjohn, Crawley, UK) or 250mcg Cloprostenol (Estrumate (Coopers, Crewe, UK) administered intramuscularly. Records were kept of the procedure associated with each insemination especially since most were performed by veterinary undergraduates.

3.2.4 Time of Ovulation

Ovulation was assumed to have occurred 24-48 hours before the end of oestrous behaviour unless ultrasound evidence indicated otherwise.

3.2.5 Pregnancy Detection

Mares were scanned 11-14 days post ovulation using the same procedure and ultrasound scanner as previously described.

The uterus was systematically examined for the presence of an embryonic vesicle by scanning cranially from the cervix along the body of the uterus then up the left horn to the left ovary, back down the left horn and up the right horn to the right ovary, back down the right horn and caudally along the body of the uterus to the cervix, in a manner similar to that described by McGladdery and Rossdale (1992).

If no vesicle was found, this procedure was repeated and non-pregnancy confirmed by rescanning 2-4 days later.

3.3 Results

Because this chapter was not the main area of the present study, there was no attempt made to maximise its efficiency through the use of ultrasound examination, more than was necessary for other parts of the study, or manipulation of the timing of ovulation by hCG administration. As well as being available for this study, the

Figure 3.2 Information Associated With Each Artificial Insemination.

No.	Mare	Age of Semen	Days before Ovulation	Total no. days	Volume Inseminated	Semen Assessment		Pregnant	Comments
						% LIVE	% PMS		
1	LAURA	1d	4d	5d				Y	EED
2	POPPY	1d	3-5d	4-6d				Y	
3	CORRIE	1d	2d	3d	45ml	76%	30%	Y	
4	POPPY	2d	1d	3d	50ml	37%	25%	Y	
5	JENNY	2d	1d	3d	50ml	40%	5%	Y	EED
6	GREEDY	1d	0d	1d	45ml	65%	25%	Y	
7	JENNY	1d	1d	2d				Y	EED
8	MISTY	3d	1d	4d	20ml	78%	5%	Y	
9	FILLY	3d	3d	6d	30ml	78%	5%	Y	
10	JENNY	1d	0 - 3d	1 - 4d	60ml			Y	
11	MEG	1d	1 - 2d	2 - 3d	60ml	79%	50%	Y	
12	POPPY	0d	1 - 4d	1 - 4d	50ml	60%	60%	Y	
13	SUZY	0d	2d	2d	30ml	60%	60%	Y	
14a	MISTY	1d	3 & 5d	4 & 6d	60ml				Double Insemination
14b	MISTY	1d	1 & 3d	2 & 4d	60ml			Y	Double Insemination
15	MEG	2d	2d	4d				N	
16	LAURA	1d	1d	2d	50ml	70%	70%	N	
17	MEG	0d	1d	1d	45ml	65%	25%	N	
18	LAURA	1d	4d	5d				N	
19	POPPY	2 d	3d	5d	60ml	62%	10%	N	
20	GEMMA	1d	1 -3d	2 - 4d	50ml	69%	50%	N	
21	LAURA	1d	1 - 3d	2 - 4d	40ml	69%	50%	N	
22	LADY	2d	1 - 2d	3 - 4 d	60ml			N	
23	GEMMA	2d	1d	3d	60ml	25%	10%	N	

Figure 3.3 Mares Excluded From the Calculations.

	Mare	Age of Semen	Days before Ovulation	Total no. days	Volume Inseminated	Semen Assessment		Pregnant	Comments
						LIVE	PMS		
24	GEMMA	1d	5d	6d	50ml	80%	15%	N	Poor technique
25	SUZY	3d	0d	3d	25ml	78%	5%	N	Poor technique
26	SUZY	2d	0d	2d	60ml	60%	15%	N	Poor technique
27	GREEDY	2d	7d	9d	50ml	65%	10%	N	Too early
28	LAURA	3d	> 24 hrs post ovn	3d	45ml	85%	75%	N	Too late - ovd
29	MEG	2d	</= 24 hrs post ovn	2d	40ml	60%	15%	N	Too late
30	LADY	2d	0 - 1d	2 - 4d	120 ml	0%	0%	N	Poor chilling technique

Figure 3.4 Correlation Between Interval From Insemination Until Ovulation, Age of Semen & Pregnancy Rates.

Days Before Ovulation	Number of Inseminations	Number of Pregnancies	Age of Semen		Number Pregnant
			0 - 1 day	> 1day	
4 - 5 days	3	2 (66%)	3	0	2 0
0 - 3 days	19	11 (58%)	11	8	7 4
TOTAL	22	13(59%)	14	8	13(59%)

Age of Semen	
0 - 1 day	64% pregnant (9/14)
> 1 day	50% pregnant (4/8)

Figure 3.5 Correlation Between % Live, % Progressively Motile Sperm and Pregnancy Rates Using 0 - 1 Day Old Semen.

% Live Sperm	Number of Inseminations	Number of Pregnancies	% PMS		Number Pregnant
			< 50%	> 50%	
< 60%	0	0			
60 - 69%	6	3 (50%)	2	4	1 2
70 - 80 %	3	2 (66%)	1	2	1 1
TOTAL	9	5(55%)	3	6	5(55%)

% PMS	
0 - 49	66% Pregnant (2/3)
50 - 100	50% Pregnant (3/6)

Figure 3.6 Correlation Between % Live, % Progressively Motile Sperm and Pregnancy Rates Using 1 - 6 Day Old Semen.

% Live Sperm	Number of Inseminations	Number of Pregnancies	% PMS		Number Pregnant
			< 50%	> 50%	
30 - 39%	2	1(50%)	2	0	1
40 - 49%	1	1(100%)	1	0	1
50 - 59%	0	0	0	0	
60 - 69%	1	0(0%)	1	0	0
70 - 79 %	2	2(100%)	2	0	2
TOTAL	6	4(66%)	6	0	4(66%)

Figure 3.7 Outcome of Inseminations Peformed at Oestruses Induced Using Prostaglandins

Mare	Pregnant	Not Pregnant	Total
Gemma		1	1
Lady		1	1
Misty	1		1
Poppy	2	1	3
Suzy	1	1	2
Total	4(50%)	4(50%)	8

mares were also used for teaching, so many of the procedures were carried out by veterinary undergraduates under supervision. Towards the end of the study, prostaglandin was used as much to bring mares into oestrus to provide a mare for the collection of semen for another project, as to allow prediction of ovulation.

Pregnancy Rates

The outcome of each insemination and any related information is shown in Figure 3.2. The inseminations which produced pregnancies are presented first, followed by the unsuccessful ones. Day 0 refers to semen inseminated on the same day as it was collected. Day 0 also refers to insemination less than 24 hours before ovulation. When the exact day of ovulation was not known, due to the infrequency of examinations, a range of possible intervals is given.

In total, 30 artificial inseminations were performed with one mare receiving two inseminations during the same oestrous period (Misty 14a & b) and the rest being single. 14 pregnancies were achieved giving a success rate of 45% (14 out of 31 inseminations) or 47% (14 out of 30 oestruses).

Figure 3.3 shows the seven inseminations which were excluded from the calculations for various reasons. The resulting total was then 61% (14 of 23).

Three of these cases were related to poor insemination technique. With Gemma (24) the pipette was caught in a cervical fold and therefore not right through into the uterus, causing the semen to run back out of the cervix. The other two cases involved Suzy (25 & 26). Although the pipette was well through the cervix on both occasions and the semen infused slowly, it still ran back into the cranial vagina following removal of the pipette. This suggested that the semen was not entering the uterine body but pooling immediately cranial to the cervix and following the path of least resistance back out. The reason for this was not known but may have been due to close apposition of the endometrial folds forming a seal against ascending material or may have been due to uterine tone or contractions as she also very effectively expelled the fluid introduced during flushing (See Chapter Four). However, the problem was overcome at her next insemination (13) by using the finger in the cervix to lift it so the internal os of the cervix was above the level of the uterine body. This seemed to separate the endometrial folds and create a path for the semen to run down into the body as it was slowly infused. The fact that she became pregnant at this insemination confirms that the technique had been at fault in the past, especially since two other mares Misty (8) and Filly (9), inseminated with the same semen previously had conceived.

Another three cases were related to poor timing of insemination in relation to ovulation. Laura (27) was inseminated blind on day six of oestrus and had entered dioestrus by the next day. Since most mares ovulate 24-48 hours before the end of oestrus (Hughes et al., 1972), she could have been inseminated between 4 and 48 hours after ovulation. Meg (28) was inseminated around 24 hours post ovulation with three day old semen. This was due to her ovulating despite the dominant follicle being less than 3cm in diameter at the ultrasound examination 24 hours prior to insemination and therefore its ovulation not being anticipated. Because the exact time of insemination compared to ovulation was not known they were not included in the current total calculation, although it did give a post ovulation insemination pregnancy rate of 0% for this study.

Greedy (29) was inseminated seven days before ovulation using two day old semen and this insemination was excluded due to the total interval between collection and insemination of the semen being considered excessive.

The last case, Lady (30), was excluded as the semen with which she was inseminated contained no living sperm due to a poor chilling technique.

Timing of Insemination

The longest total interval between collection of the semen and ovulation that resulted in pregnancy was six days (Filly 9). The oldest samples that achieved conception was three days (Misty 8 & Filly 9) and the longest intervals between insemination and ovulation were 3-5 days (Poppy 2) or four days (Laura 1).

Figure 3.4 shows the correlation between interval from insemination until ovulation, age of semen and pregnancy rates. There appeared to be no significant difference in pregnancy rates achieved when insemination was performed 0-3 days or 4-5 days before ovulation or when semen more than or less than 48 hours old was used ($p<0.05$). However, no inseminations were performed more than three days before ovulation using semen that was greater than one day old, so beyond this time young and old samples could not be compared. Also, more inseminations would need to be carried out to confirm these results this due to the small group size in the statistical analyses.

Semen Assessment

Figure 3.5 gives the correlation between % live, % progressively motile sperm and pregnancy rates using 0-1 day old semen and Figure 3.6 gives the same parameters when using 2-6 day old semen. This information was not available for all the samples in the study so not all of the inseminations could be included in these two

figures. The determination of % PMS was found to be a highly subjective visual assessment with variable results while the % Live was found to be much more accurate.

The 0-1 day old semen samples, as shown in Figure 3.5, all had % Live values between 60 and 80% while the samples 2-6 days old, as shown in Figure 3.6, gave a wide range of results from 30 to 79%. The group of younger samples gave a range of values for % PMS while the older group were all below 50%. This shows that the parameters assessed in this study demonstrate the ageing of the semen, with % PMS falling most rapidly to below 50% after 48 hours and the % Live decreasing more slowly. However, there appeared to be no correlation between the % PMS and % Live results of a sample and the pregnancy rate achieved ($p<0.05$). Samples with 37% live sperm produced pregnancy while those as high as 70% failed. Misty (8) had a PMS of 5% and became pregnant yet Laura (16) with a PMS of 70% did not

Mare Fertility

Nine of the eleven mares became pregnant at least once throughout the course of the study. The three pony mares had very good fertility rates with Misty and Filly conceiving to all their inseminations and Suzy becoming pregnant as soon as her insemination technique was modified. Poppy conceived at three out of four inseminations and Jenny three out of three although she subsequently lost two of the conceptuses. Laura became pregnant at her first AI and underwent early embryonic death after which despite another three inseminations she failed to conceive again. Greedy conceived at one of two insemination despite the presence of a granulosa cell tumour in her right ovary and Corrie became pregnant at a single one. Meg conceived to one out of three inseminations. On all three occasions, the dominant follicle was less than 3cm in diameter 24 hours prior to ovulation and the appearance of the follicle present at the time of the successful insemination is shown in Photo 3.1.

The two exceptions were Lady and Gemma. As discussed in Chapter One, Lady is an aged mare with a poor uterine environment. One of her inseminations was associated with dead semen and can be discounted but the other was not associated with any obvious reason for failure and so is likely to be due to the state of her uterus. Gemma was, as far as is known, a maiden mare although in her early teens. She was inseminated three times during the study and although one was associated with poor insemination technique, she failed to conceive to either of the other two. No abnormalities were detected throughout the course of the study and no reason was found as to why she did not conceive, although more inseminations would have to be performed before this could be considered significant. If however, further

investigations found both these mares to be infertile, this would obviously affect the results obtained by the insemination programme. Exclusion from the calculations would increase the success rate to 70% (14 of 20).

Prostaglandin Induced Oestruses

Figure 3.7 shows the outcome of inseminations performed at oestruses induced using prostaglandin, giving an overall success rate of 50%. Two of the mares who failed to conceive were the same ones that failed to become pregnant throughout the entire period of the study and the other two failures occurred in mares who conceived at subsequent induced oestruses. This suggests that the use of prostaglandin to bring mares into oestrus does not adversely affect their subsequent fertility.

Pregnancy Detection

In most of the mares, the embryonic vesicle was easily identifiable after approximately 12 days using the ultrasound scanner. Embryonic death occurred after initial detection of the conceptus in three of the pregnancies, giving an incidence of 21.5%. Two cases occurred in Jenny (5 and 7) and the other in Laura (1), all of which are discussed in Chapter Four.

In two pregnancies, the conceptus was missed due to inexperienced operators and the mares falsely diagnosed non-pregnant. Corrie was an older mare with numerous endometrial cysts in her uterus and the vesicle was mistaken for a cyst during early pregnancy detection (See photo 3.2).

Another occurrence with older, multiparous mares is that their uterus becomes more pendulous, causing the dip associated with the body horn junctions to become very pronounced. When scanning was performed by inexperienced operators, they found it difficult to follow the entire length of this type of uterus without losing contact at some point, especially at the most ventral parts. This caused the transducer to pass from the horn to the body in one horizontal move, failing to follow the ventral dip associated with the junction. Since it is in this area that the embryo becomes lodged, this explains how it was missed during subsequent examinations until it was detected around day 40.

To further confuse the issue, Corrie displayed two days of oestrous behaviour 17 and 18 days after the preceding oestrus, therefore appearing to return to oestrus. Since she had displayed a similar two day oestrus at the start of the study followed

by a completely silent oestrus (See Chapter One), this short period was not thought unusual.

In Greedy's case, it was again inexperienced operators who missed the conceptus. Because of the weight of the tumour in her right ovary, her whole tract was pulled ventrally and towards the midline. The right horn travelled laterally and then dipped sharply ventrally to connect to the tumour which made following the tract with the transducer very difficult. The caudo-ventral areas of the tumour which were beneath the horns of the uterus consisted of fluid filled areas surrounded by dense stroma and a fluid filled conceptus could easily have been mistaken for part of the tumour (See Photo 3.3 and photos 1.33a & b).

Photo 3.1 - Meg h. Largest follicle present at time of successful AI (Dots down left of photo are at 1cm intervals)

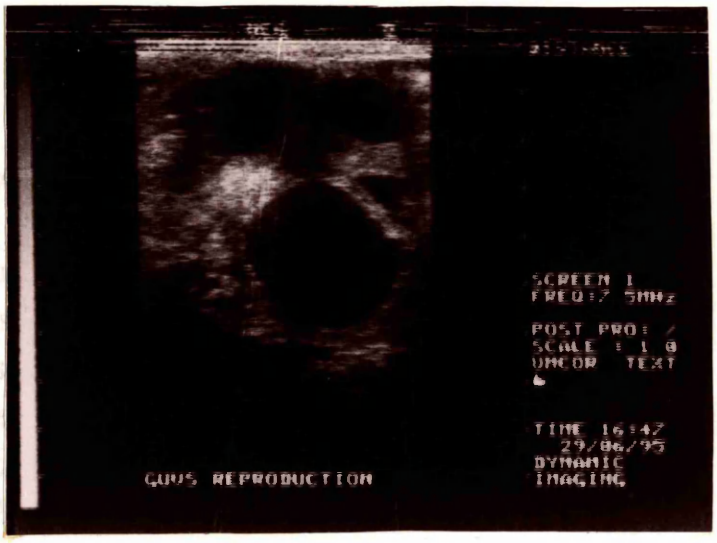


Photo 3.2 - Endometrial cysts and an embryonic vesicle in Corrie's uterus

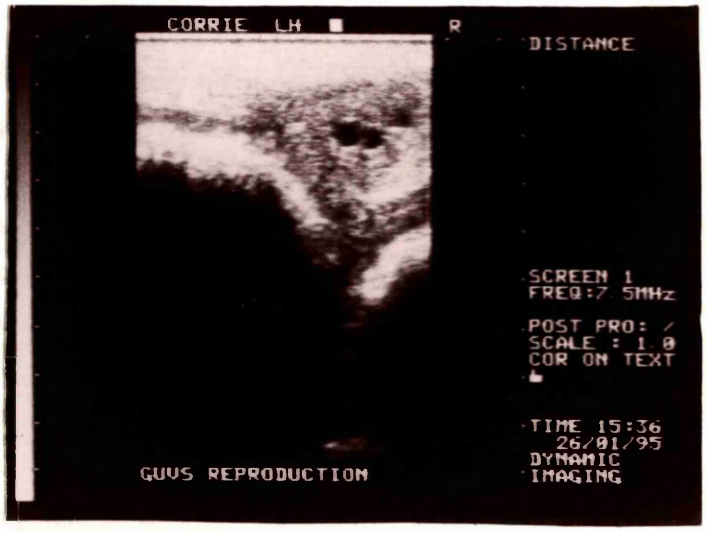
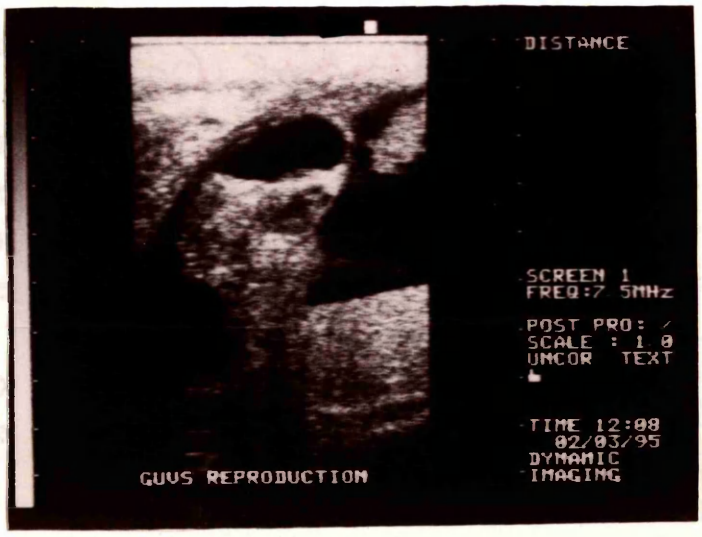


Photo 3.3 - Fluid filled area in Greedy's tumour for which a conceptus could have been mistaken



3.4 Discussion

Many studies have been carried out regarding artificial insemination in horses (Pickett and Amann, 1987) but because no universal method has been adopted, comparing these studies is confusing and drawing conclusions difficult. Variations include the use of different states of semen (raw, extended, extended and chilled or frozen), cooling rates, extenders, semen storage times, insemination volumes and concentrations, semen assessment techniques and number of inseminations per oestrous period. It was therefore not possible to directly compare the results obtained in the present study with those of other studies involving semen assessment and A.I. in the horse.

Pregnancy Rates

The initial pregnancy rate of 47% obtained in this study was disappointing until closer examination of the records revealed some of the problems encountered with individual inseminations. With an approach to an insemination programme as flexible as this, it was not surprising that on occasions, the circumstances surrounding an insemination would lead to its inevitable failure to produce a pregnancy. However, careful record keeping can identify these and allow their exclusion thus producing results which more accurately reflect the success of the procedure. Seven such cases arose in the present study and their exclusion produced a considerably improved total of 61% (14 of 23). This result is relatively high considering the lack of cycle manipulation and interference and also the wide range of operator experience. It therefore indicates that acceptable results can be achieved without the need for intensive management of all the mares.

The three instances of poor technique leading to failure of the insemination highlights the need to treat mares as individuals to get the best results and also that although the technique itself is relatively straightforward to perform, there is still the potential for errors especially where inexperienced operators are concerned. Many of the inseminations in the present study were carried out during the non-breeding season and although all the mares did ovulate during the oestruses at which an insemination was performed, this could be a potential problem when inseminating mares early in the breeding season. Again, careful record keeping would identify these mares and allow them to be eliminated from the calculations.

Timing of Insemination

In the present study, there appears to be no significant difference between the pregnancy rates obtained using chilled, extended semen less than or greater than one day old. There is little with which to compare this observation since most other studies investigating extended, chilled semen have stored it for less than 24 hours before insemination (Douglas-Hamilton et al., 1984). It suggests however, that under the regimen used in the present study, sperm survival and fertility is the same in a chilled, extended state outwith the mare as it is within, although more work would need to be done to confirm this.

Extending and chilling semen has been shown to improve its longevity (Pickett and Amann, 1987 ; Varner et al., 1988) although much variation has been found between stallions and also between ejaculates from the same stallion, which suggests individual variation plays a role (Pickett and Amann, 1987). Semen from only one stallion was used in this study so it may be that his semen was particularly suited to this method of preservation and storage

Surprisingly, the results of the present study also suggested there was no statistical difference in the pregnancy rates obtained when insemination was carried out 0-3 or 4-5 days before ovulation, although again more inseminations would need to be performed to confirm this. Most previous studies using chilled, extended semen have involved inseminations every other day throughout oestrus with a variety of different extenders (Pickett and Amann, 1987) or the interval from insemination to ovulation was not recorded (Douglas-Hamilton et al., 1984) and so direct comparisons between the pregnancy rates achieved in different studies was not possible. Woods et al. (1990) looked at the effect of the interval between insemination and ovulation on pregnancy rates using fresh, extended semen and reported greater pregnancy rates with inseminations performed within the three days before ovulation than prior to three days (76% versus 46%) which was not reflected by the findings of the present study. However, no inseminations were performed more than three days before ovulation using semen greater than 48 hours old, so beyond this time young and old samples could not be compared and neither could the present results be compared to those of Woods et al.(1990).

After ovulation, Woods et al. (1990) report that the ovum is usually viable for approximately 12 hours before becoming degenerate. They achieved a conception rate of only 6% when insemination was performed 24-30 hours after ovulation, which dropped to 0% after 30 hours and was much lower than that obtained in the 1-12 hours following ovulation. This was thought to be due to the sperm reaching a non-viable ovum and a similar situation occurred in the present study despite the

difference in semen handling. None of the inseminations performed after ovulation in the present study produced pregnancies.

The average lifespan of chilled, extended semen in the mare is considered to be around 72 hours (Ball et al., 1984) so it was decided to omit Greedy's insemination seven days before ovulation from the calculations on the grounds that the semen would no longer be capable of fertilisation after this length of time. However, there has been one report of a pregnancy being achieved after an insemination seven days before ovulation (Woods et al., 1990) but again Greedy received two day old chilled extended semen and so this was not directly comparable with the previous report in which fresh extended semen was used.

These observations highlight the difficulty involved in comparing results from different studies and drawing conclusions due to the number of variable factors involved.

Semen Assessment

No correlation was found between the %PMS and %Live of a sample and whether the mare became pregnant or not. This suggests that the use of this type of semen assessment was limited when trying to predict the outcome of an insemination. This was in agreement with the findings of Douglas-Hamilton et al. (1984) who observed that variations in motility occurring between samples from different stallions under the same regimen was not related to their subsequent fertility and Voss et al. (1981) who reported stallions with good semen motility but poor fertility.

The determination of %PMS was found to be a highly subjective assessment which was in agreement with the findings of Voss et al. (1981). This parameter can be affected by many factors among which were the length of time the sample was warmed, the temperature it reached, the length of time it spent on the slide and the interval between insemination and assessment, although an attempt was made to standardise these as explained in the Materials and Methods. Pickett and Amann (1987) reported that the number of motile sperm was increased by 20% after incubating an aliquot of semen at 37°C for ten minutes before preparing the slide. Also, since only one person carried out the semen assessment in the present study, there was the possibility of operator bias.

%PMS and %Live did reflect the ageing of the semen with %PMS being the first to decline. However, since some of the samples still had a high % Live after three days, this suggested that there were many factors associated with the longevity of sperm in stored samples. Varner et al. (1988) reported an average % PMS of 63%

after 48 hours decreasing to 46% by 72 hours when extended semen was stored at 4°C. This was higher than the corresponding average recorded in the present study. However, differences in assessment techniques and stallion variation could have accounted for this.

The only instance where semen assessment was useful in predicting the outcome of an insemination was in the case of Lady where there were no live sperm present in the sample. This highlighted the need to perform basic semen assessment at insemination to check there were viable sperm present although the results were subjective and did not give a real indication of the fertility of the sample. The best indicator of fertility therefore remained the resulting pregnancy rate.

Mare Fertility

The mares in the present study represent a relatively random cross section of the general mare population, being selected for their availability and not for any reproductive reason and therefore reflect the types of mares likely to be included in a breeding programme. Most of the mares conceived at least once throughout the study indicating that artificial insemination is a technique that can be used successfully in the majority of mares although the variations in conception rates between mares indicates a degree of individual mare variation in the outcome.

Again, careful record keeping helped identify mares who's reproductive behaviour was not as anticipated and allowed their management to be altered to achieve the best results. In Meg's case, the anticipation that ovulation would occur after a relatively short period of oestrous behaviour despite the follicle being less than 3cm diameter allowed a successful insemination to be performed towards the end of the study.

However, a percentage of mares in the general population will not conceive. This may be for unknown reasons as in the case of Gemma or due to identifiable problems such as a poor uterine environment in Lady's case. Adams et al. (1987) found both lower pregnancy rates at day 11 and increased rates of early embryonic death by day 20 in mares similar to Lady. Obviously a large number of subfertile or infertile mares in a group would affect the subsequent pregnancy rates of the programme as seen by the improvement to 70% in the present study when Gemma and Lady were excluded. This again highlighted the need for good record keeping to allow problem mares to be identified early in the programme and allow appropriate action or treatment to be instituted.

The use of prostaglandin to bring mares into oestrus did not appear to adversely affect their subsequent fertility, which was in agreement with McKinnon and Voss (1993). Its use was therefore unlikely to have affected the results of the present study.

This study demonstrated that there were many factors involved in the outcome of an insemination including stallion variations, semen collection and processing, timing of insemination and mare variations and it was very difficult to control and assess them all. Pregnancy rates could probably have been improved by intensive management such as the use of prostaglandin to bring mares into oestrus at known times, daily ultrasound examinations and the use of hCG to allow the prediction of ovulation. This was especially the case with mares such as Lady who did not show oestrous signs well or Meg who tended to ovulate before a particularly large follicle was present. However, using chilled, extended semen acceptable results could be obtained without this type of management being necessary.

Pregnancy Detection

Rectal ultrasound examination is the earliest method of pregnancy detection currently available and can be carried out as soon as the conceptus becomes visible at day 9-11 although most operators prefer to wait until after the vesicle has become fixed as will be discussed in Chapter Four (McGladdery and Rossdale, 1992). The accuracy of this method has been reported as >95% for detection of pregnancy and >84% for non-pregnancy (Hinrichs, 1990).

The occurrence of early embryonic death must be taken into account as any losses which occur before the conceptus becomes visible on ultrasound will not be detected. It was therefore important to differentiate between conception rates which were reported as between 79 and 92% and pregnancy rates which are considerably lower (Ball et al., 1984). Ball et al.(1984) also reported the incidence of early embryonic death in this period as 9% in young fertile mares and 62 - 73% for aged subfertile mares (Ball et al., 1984). Embryonic defects, abnormal uterine tubal transport and abnormal uterine environment were among the suggested potential causes although more work in this area is needed.

The three cases of embryonic death in the present study occurred after the pregnancy was detected and therefore will not affect the pregnancy rate achieved. It could be suggested that the long interval of five days between collection of the semen and ovulation was responsible for the early embryonic death that occurred in Laura (1) due to degeneration of the sperm at fertilisation. However, Filly (9) also had a total interval of 6 days but maintained her pregnancy until flushed at day 13.

This was in agreement with the findings of Woods et al. (1990) who reported a higher incidence of early embryonic death in mares inseminated after ovulation but not in the group inseminated more than three days before ovulation. Due to the relatively simple nature of the sperm compared to the ovum, it seems unlikely that if the sperm was degenerate enough to interfere with cell division and cause early embryonic death, that it would be capable of fertilisation in the first place.

These three cases will be discussed more fully in Chapter Four but no reasons associated with the insemination programme could be identified as possible causes of the deaths of the embryos.

Endometrial cysts are often found in older mares and are not thought to contribute to infertility (Adams et al., 1987). Although they did not interfere with conception and maintenance of the pregnancy in this case, they did interfere with its detection. Cysts and embryonic vesicles are both fluid filled sacs and therefore appear the same on ultrasound (Ginther and Pierson, 1984b). Both even display the hyperechoic specular reflection dorsally that is in fact an artefact although it has in the past been mistaken for the embryo. The only way to differentiate between the two structures is over consecutive examinations looking for the increase in diameter and change in position which will indicate a vesicle. Alternatively, at a prior scanning, the size and position of the cysts can be mapped allowing differentiation at a later date although in this case, even that did not work.

Corrie's display of oestrous behaviour 17-18 days after conception was not unusual either. However, again it can easily lead to a mis-diagnosis of non-pregnancy in a mare, especially if it occurs around the time when the mare would be expected to return to oestrus if the insemination had been unsuccessful. This phenomenon has been reported to occur in 10 - 20% of normal pregnant mares, usually around 14-26 days after conception (Arthur and Allen, 1972), and has been anecdotally connected with the presence of a female foetus (Ginther, 1992). Although this has not yet been proven, it seems to hold true for some pregnant mares we worked with which were not involved in this study. Whether this theory is found to be correct in the case of Corrie will have to wait until she foals.

In Greedy's case, inexperienced operators were again to blame for the conceptus going undetected until such an advanced stage of pregnancy. However, the abnormal positioning of the tract due to the presence of the granulosa cell tumour did make a systematic examination of the whole uterus difficult. The close apposition of abnormal ovarian tissue and the uterine horn containing the conceptus could easily have lead to the various structures being wrongly identified.

Both of these cases highlighted the need to ensure the whole uterus was systematically scanned when detecting a pregnancy. This was easiest to perform if a set procedure was followed such as the one described in Materials and Methods to ensure the entire uterus is examined and to allow experience to be gained with time. If no pregnancy was detected in a mare who's reproductive tract was abnormal in some way, the examination should be repeated at a later date when the conceptus is more easily detected to confirm the result. The lower pregnancy detection rate achieved in this study reflected the findings of Squires et al. (1983) who observed that the operator required skill in ultrasound examination as well as rectal palpation in order to avoid false negatives.

Chapter 4 - Early Pregnancy and Embryo Retrieval

4.1 Introduction

4.1.1 Early Pregnancy

In the mare, only fertilised ova eventually reach the uterus with unfertilised ones being retained in the middle third or ampulla - isthmus junction of the uterine tube (Van Niekerk and Gerneke, 1966). Various theories have been suggested for this phenomenon but the most likely is the production of prostaglandin E2 by the conceptus, causing preferential transport through the uterine tube and allowing it to by-pass degenerating unfertilised ova from previous cycles (Weber et al., 1991a ; Weber et al., 1991b).

The conceptus enters the uterus at about day five or six post ovulation (Flood et al., 1982) as a morula or early blastocyst. A thin, uniform layer begins to develop on the inner surface of the zona pellucida within the first 24 hours in the uterus. By day eight, the zona pellucida has peeled off to leave the conceptus surrounded by a homogenous capsule 1 um thick, which progressively thickens until day eleven (Flood et al., 1982). Since its formation is a continuous process, this implies that initially the trophoblast is associated with the deposition of capsular material but that the endometrium also plays a role (Betteridge, 1989). It is thought to provide protection for the conceptus as it allows the passage of macromolecules from the uterine milk produced by the endometrial glands (Ginther, 1992) but excludes bacteria and viruses (Flood et al., 1982). Its strength and elasticity may be essential for the extensive mobility of the conceptus, as demonstrated by Ginther (1985b), allowing it to withstand the intra-uterine forces to which it is subjected. However, conceptuses of other species with similar coverings do not display such mobility (Betteridge, 1989), so this may not be its sole function.

The capsule is present until at least day 21 (Betteridge et al., 1982) but is gone by day 26 of gestation (Denker et al., 1987). By this time, the amnion is complete and so the embryo proper is never exposed to the uterine lumen. The mechanism of its loss is not yet known (Flood et al., 1982).

Figure 4.1 summarises the development of the conceptus in the uterus and Figure 4.2 shows its appearance on ultrasound examination. As previously mentioned, it first becomes visible on ultrasound from 9 - 11 days (McGladdery and Rossdale, 1992). While the vesicle is spherical and mobile, a bright white spot is seen at the dorsal pole (See Figure 4.2). This represents a specular reflection or artefact caused by exaggerated reflection of the ultrasound beam due to the outer surface of the vesicle being at right angles to the beam (Ginther, 1986) and should not be confused with the embryo proper as has been done in the past (Squires et al., 1983). The embryonic vesicle at this stage is still a blastocyst, with a single layer of ectoderm forming the trophoblast. After day 11, a layer of endoderm is complete forming a bilaminar yolk sac (Ginther, 1992).

The conceptus is very mobile, travelling extensively throughout the entire uterus from the tips of both horns to the cervix (Leith and Ginther, 1984). This mobility is partly caused by contractions of the myometrium as demonstrated by the fact that administration of Clenbuterol, a B2 sympathomimetic blocker of smooth muscle contractions causes a reduction in vesicle mobility (Leith and Ginther, 1985). Maximum mobility is seen from day 11 - 14 (Leith and Ginther, 1984) and is associated with maximum motility of the uterus from day 12 - 16 (Bessent et al., 1988). However, it appears that the vesicle also contributes to its mobility as simulated 12 - 13 day vesicles made from the finger tips of surgical rubber gloves filled with water and inserted into the uterus, moved at the slower rate associated with 9 - 10 day vesicles (Ginther, 1985a). This period of maximum mobility is temporally related to the time at which the luteolytic mechanism is blocked (Ginther, 1985a) and is thought to be due to the need for the conceptus to 'neutralise' the whole of the endometrium to prevent prostaglandin release.

The conceptus is thought to start initiating maternal recognition as early as day 11 but the mechanism by which it does this is not yet known (Ginther, 1992) although it seems most likely that it causes a reduction in prostaglandin production by the endometrium (Ginther, 1992 ; Berglund et al., 1982). Conceptus membranes incubated with endometrium caused a reduction in prostaglandin production (Sharp and McDowell, 1985) and it has also been found that PGE2 produced by the conceptus binds to the endometrium during this period and so may also play a role (Vanderwall et al., 1993).

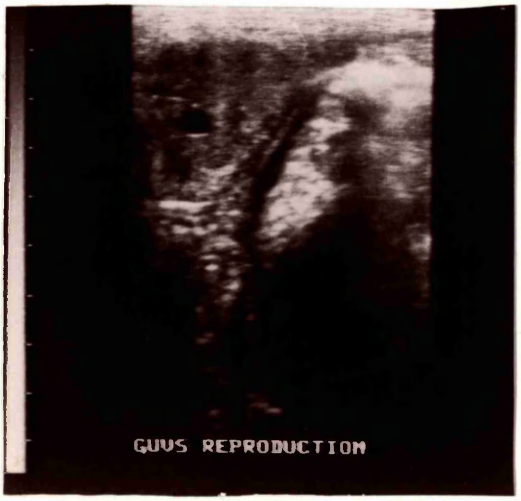
At day 15 - 16 , the vesicle becomes fixed at one of the horn body junctions (Leith and Ginther, 1984 ; Enders and Liu, 1991), this area being associated with a major branch of the uterine artery. There is a marked curvature in the tract at this point and the vesicle becomes trapped due to its increasing diameter and the progressive

Figure 4.1 Development of the Equine Conceptus

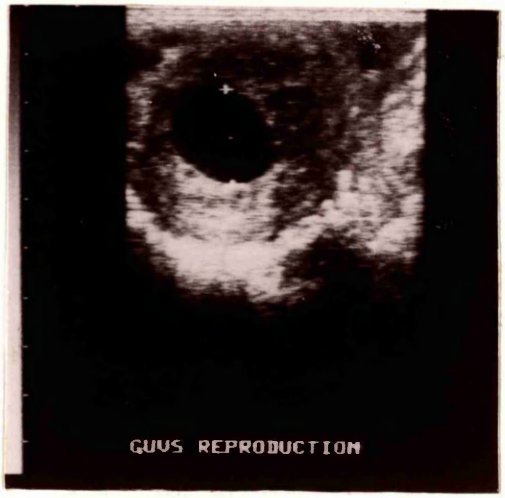
Age (days)	Diameter on ultrasound (mm)	Actual Diameter (mm)	Shape (% of conceptuses)				Stage
			Spherical	Oblong	Triangular	Irregular	
9		2					Blastocyst
11	7.3	4.5	100	0	0	0	Yolk sac formed
12	10.7		100	0	0	0	
13	13.7	16	100	0	0	0	
14	17.3	25	86	7	0	7	Mesoderm grows out from embryonic disc
15	21.3		82	12	0	6	
16	23.4	52	83	11	0	6	Mesoderm envelopes half of yolksac.Amnion devs.
							Embryo 14 pairs of somites
17	25.4		34	39	11	16	
18	24.8	60	18	9	30	43	Sinus terminalis at leading edge of mesoderm. Allantois starts to dev.
							Embryo 16 pairs of somites.
19	25.8		0	15	45	40	
20	26.5	66 x 58	0	9	61	30	Capsule lost
21	25.9	65	0	0	59	41	Amnion envelopes embryo.
23							Small bilaminar area remains at abembryonic pole
							All major internal organs & limb buds formed.
24	27.2		0	0	29	71	
25		68					Allantochorion froms as allantois & chorion fuse at embryonic pole.
							Chorionic girdle starts to dev.
27	30.6		0	0	35	65	
30	36.4	75	0	0	19	81	Allantochorion 1/2 surface area.
							Embryo moving dorsally as yolk sac regresses.
33	44.5		0	0	9	91	
36	51.4	90	0	0	0	100	Allantochorion 3/4 surface area
							Chorionic girdle invades endometrium.
39	59.4	110	0	0	0	100	Chorionic girdle separated.
							Embryo recognisable as horse.
42	68.8	1.3	0	0	0	100	
45	77.9	1.3	0	0	0	100	Rudimentary villi start to dev over surface of complete allantochorion
48	88.7	1.6	0	0	0	100	

Refs Ginther, 1983 ; Van Niekerk and Allen, 1975 ; Allen, 1975a

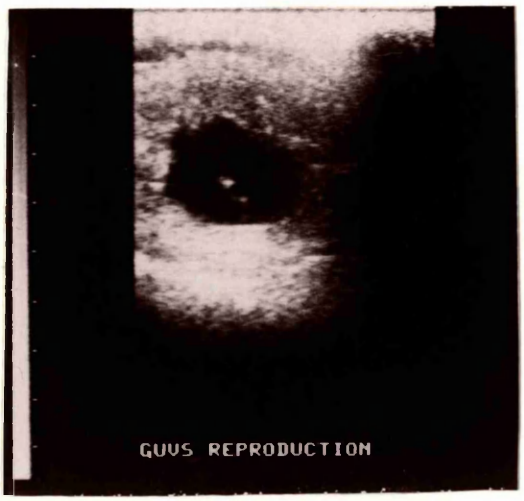
Figure 4.2 Appearance of the Developing Equine Conceptus on Ultrasound Examination



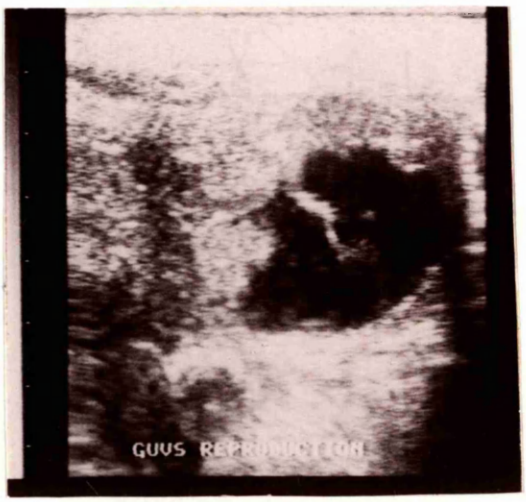
Day 11 - 12



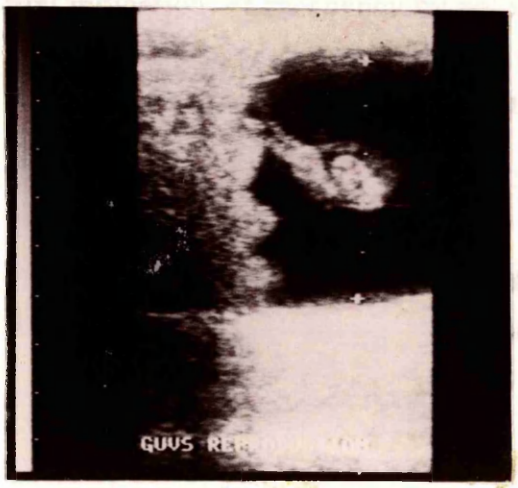
Day 14 - 15



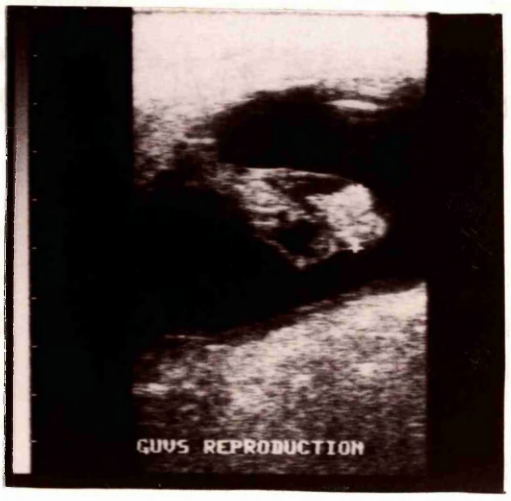
Day 26



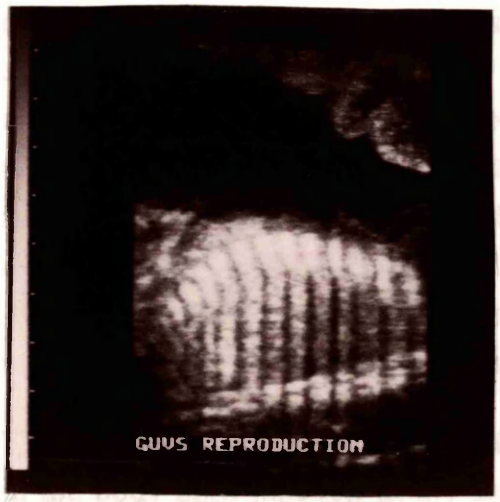
Day 28



Day 38



Day 44



Day 98

increase in uterine tone. This is associated with progesterone stimulation which increases luminal resistance to mobility of the vesicle (Ginther, 1985a).

There is a marked increase in uterine tone associated with pregnancy and it is first discernible from a non - pregnant uterus after day 14 (Ginther, 1992). This gives the first indication of pregnancy by palpation although it is not consistent for all mares. The tone increases in the non-gravid parts of the uterus to reach a maximum around 25 days of gestation (Hayes and Ginther, 1986) and is present until around day 50 (Kooistra and Ginther, 1976).

There is some disagreement as to whether this increase in tone is due to the combined effects of progesterone and oestrogen stimulation or to progesterone alone. It was found in one study that prolonged progesterone treatment produced tone comparable to that of dioestrus only. However, the addition of a low dose of oestrogen produced the same degree of tone associated with pregnancy (Hayes and Ginther, 1986) and caused fixation to occur earlier (Bessent et al., 1988). Since it did not affect the rate of movement of the vesicle, this was presumably due to the increase in tone. That this oestrogen comes from the conceptus and not the ovary is supported by the finding that oestradiol is produced by the conceptus membranes from 8 - 20 d of gestation (Zavy et al., 1979).

This tone is thought to be the cause of the plateau in vesicular growth and the loss of its spherical shape seen between days 16 and 21 (Ginther, 1985a). The vesicle loses the tension in its yolk sac, allowing it to conform to the irregular uterine lining (Ginther, 1979). This is demonstrated by removed intact conceptuses being spherical when free standing at day 14 and at day 18 - 26 regaining a smooth outline although tending to flatten into an oval shape (Ginther, 1979). It has been suggested that the loss of the capsule begins at this time, commencing at the abembryonic pole and that this may be linked to the deformation of the conceptus due to the differential response of thick and thin walled areas to the uterine tone (Betteridge, 1989). The embryonic vesicle expands at a rate of around 3 - 4 mm / day as seen on ultrasound before the growth plateau and 2 - 3 mm / day after (Ginther, 1983b).

The enlargement due to the conceptus is palpable as a bulge of increasing diameter in the body horn junction of the uterus (Enders and Liu, 1991), first detectable by day 16 and consistently by day 23 (Ginther, 1992).

Myometrial contractions continue beyond fixation of the vesicle (Bessent et al., 1988) and are thought to play a role in orientation of the vesicle. This involves rotation of the vesicle so the embryo proper is at the ventral pole of the vesicle and opposite the mesometrial attachment of the uterus (Ginther, 1985a). This

presumably occurs between the cessation of mobility at day 15 and the time when the embryo proper becomes visible on ultrasound at day 19 / 20 as it is already in a ventral position at this time (Ginther, 1985a). The endometrial folds interlock on either side of the vesicle and the dorsal wall of the uterus becomes disproportionately thick trapping it (Enders and Liu, 1991) and allowing the myometrial contractions to massage the thicker embryonic pole into a ventral position (Ginther, 1985a).

From day 14, mesoderm invades between the endoderm and ectoderm forming an increasing area of three layered membranes, the trilaminar omphalopleure. The amnion forms by day 20 and at day 21, the allantois starts to form (Ginther, 1992). This gradually increases in size, taking over as the main source of physiological exchange for the embryo and causing regression of the yolk sac. The embryo is consequently lifted away from the ventral embryonic pole and the embryo is seen to move dorsally on ultrasound examination (Ginther, 1983b).

At the junction of the allantois and yolk sac around day 25, a single layer of trophoblast cells undergoes marked proliferation and hypertrophy to form a series of tightly packed, branching, villous projections known as the chorionic girdle. By day 36, this whole girdle separates from the foetal membranes and actively invades the endometrium (Van Niekerk and Allen, 1975). The cells phagocytose the endometrial epithelium and migrate into the uterine glands and stroma where they differentiate into large, sessile, endometrial cup cells. These cells produce equine Chorionic Gonadotrophin (eCG) (Moor et al., 1975) which is released into the general circulation.

Round these foreign cells, maternal lymphocytes, plasma cells and eosinophils aggregate, their numbers increasing over the lifespan of the cups. It has been suggested that eCG provides an immunoprotective barrier around these cells protecting them from attack by maternal cells. At day 50, maternal cells are clustered round the endometrial cups and by day 70 they start to degenerate. By day 90, the maternal cells have walled off the cups and invade, phagocytosing the foreign cells. This causes eCG concentrations to fall (Allen, W. R., 1975).

At day 33 only about a quarter of the vesicle is still yolk sac and the allantois has taken over as the dominant placenta. By day 40 the embryo is positioned dorsally in the vesicle and the allantochorion is complete with only a vestige of the yolk sac remaining (Van Niekerk and Allen, 1975). The allantochorion then comes together to form the umbilical cord and as this elongates, the foetus will drop back down towards the ventral wall again (Ginther, 1992). The remnant of the yolk sac remains

as part of the umbilical cord, playing an important role in hormone production and the absorption of macromolecules (McKinnon and Voss, 1993).

4.1.2 Endocrinology and ovarian changes during pregnancy

The hormone changes associated with early pregnancy in the mare are summarised in Figure 4.3.

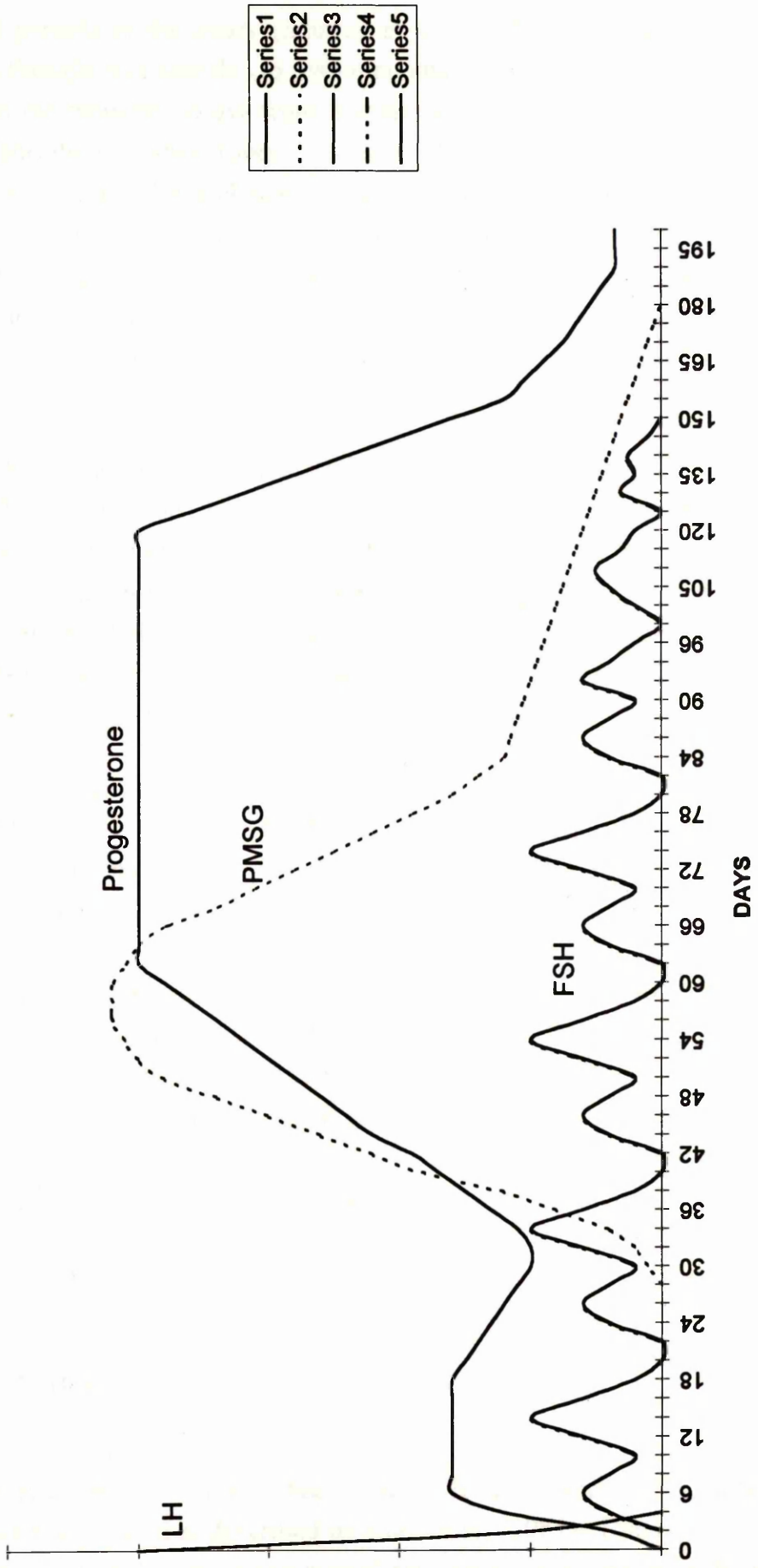
Hormone concentrations are similar for the first 14 days after ovulation whether the mare is pregnant or not (Allen and Hadley, 1974). If a conceptus is present at day 13, there is an absence of the PGF2a peak due to the occurrence maternal recognition (Kindahl et al., 1982) and the primary CL is maintained. Consequently, progesterone concentrations remain raised.

Because FSH is not inhibited by high progesterone concentrations, it continues to be produced and is responsible for the follicular development associated with early pregnancy (Urwin and Allen, 1982). There is disagreement as to whether this occurs in distinct waves or at random (Urwin and Allen, 1982 ; Allen, W. E., 1975) and it may be that there are individual mare variations. Follicular development reaches its greatest around day 50 - 60 of gestation (Squires and Ginther, 1975).

LH is suppressed by the high concentrations of progesterone and so concentrations remain low throughout pregnancy (Vanderwall et al., 1993). This is first noticed as what would have been the next wave of preovulatory follicles develop around day 15 of pregnancy but fail to ovulate due to the lack of LH (Allen, W. E., 1975).

Progesterone is the only ovarian factor required for the maintenance of pregnancy. This has been shown by mares which were ovarioectomised during the ovarian dependent part of gestation maintaining their pregnancies if administered exogenous progesterone (Holtan et al., 1979). Progesterone concentrations begin to drop slowly over 8 - 28 days (Holtan et al., 1975) as the primary CL regresses (Bergfelt et al., 1989) but start to rise again as eCG production commences around day 32 - 37 (Nett et al., 1975 ; Kindahl et al., 1982). Initially this is due to eCG causing a resurgence of the primary CL, as demonstrated by the rise in progesterone occurring before any accessory CLs have been formed and an associated increase in the weight of the primary CL (Bergfelt et al., 1989). eCG then causes the formation of secondary and accessory CLs which are first detected around day 38 - 40 (Bergfelt et al., 1989) and increase in number in association with a decrease in the number of large

Figure 4.3 Hormone Changes During Pregnancy in the Mare



follicles present in the ovary (Squires et al., 1974). Approximately 30% of these CLs are thought to occur due to ovulation and so are termed secondary CLs. This is based on the recovery of ova from the uterine tubes and there being a tract between the CL and the ovulation fossa (Squires et al., 1974). There appears to be a greater incidence of ovulation and corpus haemorrhagicum formation early in gestation, with 72% of ovulations occurring between days 54 and 72 (Allen, W. E., 1975). After this, spontaneous luteinisation of follicles is more common, producing accessory CLs (Squires et al., 1974). eCG is not responsible for follicular development, which is caused by FSH (Squires and Ginther, 1975), but takes over the role of LH (Urwin and Allen, 1982).

The peak concentrations of progesterone coincide with maximum eCG production of 14 - 22 mg / l at day 50 - 80. eCG concentrations decline slowly after day 80 to reach basal concentrations at around day 150 (Kindahl et al., 1982) in association with the destruction of the endometrial cups (Marrable and Flood, 1975). Regression of all the CLs occurs together, starting around day 140 - 160 and is complete by day 210 - 220 (Squires and Ginther, 1975) caused by the dropping eCG concentrations.

The primary CL is therefore the only source of progesterone before day 40 (Squires and Ginther, 1975) as shown by the ovaries being necessary for the maintenance of pregnancy in all mares until day 45 of pregnancy and until between day 50 and 70 for 50% of mares (Holtan et al., 1979). The primary, secondary and accessory CLs combine as the progesterone source until day 160 - 220 (Squires and Ginther, 1975) but by day 140, the pregnancy is independent of the ovaries as shown by 0% mares aborting after ovarioectomy at this time (Holtan et al., 1979). The minimum progesterone concentrations required to maintain a pregnancy are not clear but Shideler et al. (1982) suggest an average of 4 ng/ml. However, concentrations as low as 2.5 ng/ml have been reported on occasions without loss of the conceptus. This suggests that progesterone deficiency should not be diagnosed on the basis of a single low sample. In the same study, of the mares with concentrations consistently below 2 ng/ml, 83% aborted.

4.1.3 Embryo Retrieval

Embryo retrieval for transfer has been performed since 1979, usually using a procedure similar to that described by Imel et al. (1981). This involves flushing the mares uterus via a flexible, transcervical catheter to obtain embryos between day six and nine which are suitable for introduction into a recipient mare (McKinnon and

Voss, 1993). These embryos are between 0.2 and 2 mm in diameter (Ginther, 1992) and so are easily collected with the returning fluid. However, once the conceptus becomes older, its diameter makes it more difficult to recover intact by this method. Previously, the retrieval of embryos at later stages of gestation for research purposes has been carried out at slaughter or by surgical means (Flood et al., 1982 ; Betteridge et al., 1982). However, recovery of intact 10 - 16 day conceptuses via the transcervical route has been successfully achieved using rigid catheters of appropriate diameter by Sirois and Betteridge (1988).

Embryo recovery rates at 6 - 9 days are reported as being 55 - 80% (including twin pregnancies), which is less than corresponding pregnancy rates at day 14 (Hinrichs, 1990). This suggests that not all embryos are recovered during flushing and various alterations in the flushing procedure have been advocated in an attempt to improve the retrieval rate such as manipulation of the uterus per rectum, use of different flush volumes and frequencies and allowing a longer period of contact between the medium and the uterine lumen (Hinrichs, 1990). In day six pregnancies, some embryos may still be in the uterine tubes at flushing (Flood et al., 1982) and so are not retrieved.

Recovery programmes are subject to all the factors previously mentioned that affect conception rates. In addition, there is no easy way to tell if a mare is pregnant or not prior to flushing at this early stage, so interpretation of results is difficult. However, once the embryo reaches day 9 - 10, it becomes visible on ultrasound examination (Leith and Ginther, 1984) and so attempts to recover embryos after this stage can be restricted to mares which are known to be pregnant. 100% recovery rate was reported from days 10 - 16 with 86% of the vesicles remaining intact (Sirois and Betteridge, 1988).

Potential problems associated with the retrieval of older embryos are the diameter of the conceptus, the increased tone of the uterus and the lodging of the conceptus in the body horn junction (Ender and Liu, 1991). There appears to be no published data concerning embryo retrieval via the transcervical route after day 16 except for one case (Meadows et al., 1995) where videoendoscopy was used to locate the embryo and allow aspiration of the foetal fluids. The conceptus was then flushed from the uterus in a manner similar to that used for younger embryos, using a wide diameter catheter.

4.2 Materials and Methods

Introduction

The mares previously described were also used for this chapter of the study. In those found to be pregnant after insemination in Chapter Three, the pregnancy was either terminated or an attempt was made to retrieve the embryo. This was done via a transcervical approach at varying stages of development from day 12-28 for inclusion in a study of the early embryological development of the equine eye.

4.2.1 Pregnancy Detection

Mares inseminated in Chapter Three underwent ultrasound examination at day 11-14 post ovulation to detect the presence of an embryonic vesicle and then at regular intervals to monitor its progress.

Daily teasing, ultrasound examinations and plasma hormone assays were carried on throughout the course of the whole study, as described in Chapter One.

4.2.2 Pregnancy Termination

In three mares 5mg Dinoprost (Lutalyse (Upjohn, Crawley, UK) or 250mcg Cloprostenol (Estrumate (Coopers, Crewe, UK)) was administered intramuscularly either on one occasion, on consecutive days or at an interval of three days in an attempt to terminate the pregnancy.

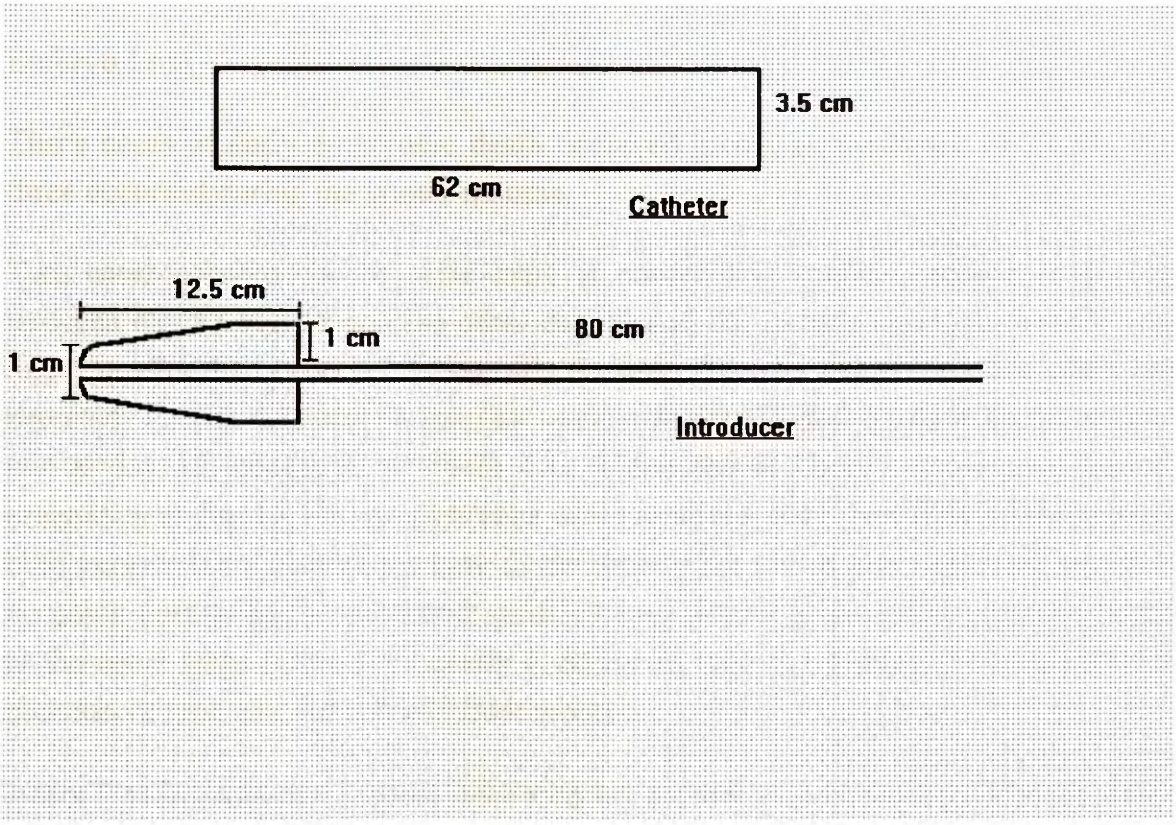
In another two cases, one litre of warmed, sterile 0.9% saline (Baxters Healthcare Ltd., Glasgow, Scotland) was introduced into the uterus. This was performed to evaluate the effect of the flushing medium and technique on the embryo.

4.2.3 Embryo Retrieval

The mare was prepared in the same manner as for artificial insemination. A gloved hand was introduced into the vagina and an increasing number of fingers used to gently dilate the cervix. A lubricated sterile aluminium catheter and introducer, as

shown in Figure 4.4, was then introduced through the cervix. One litre of warmed, sterile 0.9% saline (Baxters Healthcare Ltd., Glasgow, Scotland) was run in under gravity through the central introducer until the bag was empty or the mare showed signs of discomfort. The introducer was then removed and replaced in its sterile covering while a plastic tube was connected to the end of the remaining section of the catheter to collect the returning fluid. It was run into a plastic 2.5 litre cylinder and examined visually for the presence of an embryo. If present, the embryo was trapped in an embryo filter by running the fluid back through it and placed in fixative (2% glutaraldehyde in cacodylate buffer).

Figure 4.4 Embryo Retrieval Equipment



If no embryo was present initially, the uterus was massaged per rectum to ensure most of the fluid had returned. If still not present, the introducer was replaced in the catheter, taking care to keep it sterile and the procedure repeated.

If the mare did not return to oestrus within three days of flushing, 5mg Dinoprost (Lutalyse (Upjohn, Crawley, UK) or 250mcg Cloprostenol (Estrumate (Coopers, Crewe, UK)) was administered intramuscularly.

4.3 Results

The results of daily teasing, ultrasound examinations and plasma hormone assays throughout the course of the study are presented in Figures 1.7 - 1.17 in Chapter One.

14 mares were found to be pregnant but because the conceptus was not consistently visible on ultrasound before around day 11, any losses before this time were not detected and the mares considered to be non-pregnant.

In all the pregnancies that occurred, the embryo became fixed in the right horn of the uterus. There was no information available for the three lactating mares regarding which horn the previous conceptus had lodged in.

Embryo Loss

Three cases of early embryonic death occurred giving an incidence of 21.5%. All three occurred during the winter months.

Two cases occurred in the same mare at consecutive cycles (Jenny c & d), one between day 13 and 18 and the other day 19 and 20. In both cases, the vesicle was much smaller than expected, being only 5 mm in diameter and still mobile when last detected. Photos 4.1a shows the first vesicle at day 13 and photo 4.1b the second one at day 19, compared to Photo 4.1c which shows Jenny's subsequent normal pregnancy at day 14. Her progesterone concentrations were over 6 ng/ml throughout both periods but a distinct increase in the tone of her uterus was not noted on either occasion despite her progesterone profile being similar to the other pregnant mares at the same stage (Corrie c, Greedy e, Misty h) who did display the characteristic increase in tone and did not lose their embryo.

Jenny was brought into oestrus following her second case of early embryonic death with a single luteolytic dose of prostaglandin on day 20 (Jenny d) in the same way the prolonged dioestruses in Chapter One were ended (Lady i & Poppy d).

Jenny conceived later in the study (Jenny f) and maintained the pregnancy until flushing at day 18. In this case, the embryo was 24 mm in diameter which can be considered normal for an embryo of that age, and had become fixed in the horn body junction at the correct time.

In Laura's case (Laura b) , the embryo was 26 x 22 mm, fixed in the body horn junction and appeared normal at the time of last examination, before being lost

Figure 4.5 Outcome of Pregnancies

Reference	Outcome	Returned	No.days	Total Interval	Comments
Laura a	EED d 19 - 26	Sp	49 - 56	75	
Jenny c	EED d 12 - 18	Sp	43 - 49	61	
Jenny d	EED d18 - 20	PG	2	22	
Poppy b	Pregnancy Terminated d18 - 20	PG	2	20 - 22	Conceived at next oestrus
Poppy c	Fluid Introduced d21	Sp	3	24	Recovery not attempted
Greedy e	Pregnancy Terminated d39 - 41	PG	2	89	Pseudopregnant till PG d87
Corrie c	Attempted Pregnancy Termination d39 & 44	PG			Termination Unsuccessful
Misty f	Flushed d12	Sp	4	11	No Embryo Retrieved
Filly e	Flushed d13	Sp	2	15	Embryo Retrieved
Jenny f	Flushed d18	Sp	6	24	Embryo Retrieved
Misty h	Flushed d24 - 25	PG	*	*	Embryo Retrieved
Meg h	Flushed d23 - 25	PG	*	*	No Embryo Retrieved
Poppy f	Flushed d23	PG	*	*	No Embryo Retrieved
Suzy i	Flushed d20	PG	*	*	Embryo Retrieved

PG = Prostaglandin Administration

* = Length of Interval Not Known

Sp = Spontaneous Return to Oestrus

EED = Early Embryonic Death

between days 19 and 24 (See Photo 4.2). Unlike Jenny, there was a palpable increase in uterine tone in the horn where the conceptus had been, which remained for a period after the loss. Laura's progesterone concentrations were lower for most of this period compared to those of the other pregnant mares at the same stage (Corrie c, Misty h, Greedy e), although they did remain just over 4 ng/ml. However, the increase in uterine tone suggested that these concentrations were sufficient to produce physiological changes. She did not conceive again despite being inseminated another four times throughout the course of the study, which may or may not be related to this loss (Laura c).

In the two instances where the embryo was lost before day 35 and prostaglandin was not administered (Jenny c & Laura b), progesterone concentrations remained elevated for the natural lifespan of the primary CL, which was 61 and 75 days respectively.

Twin Pregnancies

There were three mares responsible for the eight double ovulations detected throughout the study but artificial insemination was only carried out twice in Misty and once in Poppy during such oestruses. Meg was not inseminated in association with a double ovulation.

The three cases when insemination was performed resulted in two sets of twins and a singleton detected on day 12-14. This gave a 14.2% incidence of twin pregnancies for the present study (2 of 14 pregnancies).

The first set of twins occurred in Poppy (f) and the vesicles were of different diameters (20 and 24 mm) on day 17 as shown in Photo 4.3a. This suggested that they were of different ages and had therefore arisen from an asynchronous double ovulation. Photo 4.3b shows only one follicle present in the ovary at insemination and Photo 4.3c demonstrates the 2 CLs present early in dioestrus. The lower one is more hyperechoic suggesting it is the younger of the two. The embryos both became fixed in the right body horn junction of the uterus and the smaller one had been resorbed spontaneously by day 22. The remaining one is shown in Photo 4.3d

In Misty (h), the twin vesicles were of equal diameter (10 mm at day 12-14) and were associated with CLs which had a similar appearance on ultrasound indicating they were of similar age and had therefore arisen from a synchronous double ovulation (See Photo 4.4a and b). Photo 4.4c shows another view of the CL in 4.16b. In this photo, it looks like there are two CLs present which demonstrates that it can sometimes be difficult to determine the number of CLs that are present in an

ovary at any one time. One vesicle became fixed at day 14 while the other was still mobile within the body of the uterus. It is unknown whether fixation of the second vesicle occurred or not since it had been resorbed by the next examination at day 18-20 (See Photo 4.4d).

If pregnancy detection in the present study had not been carried out until after day 20, these mares would have appeared to have normal single pregnancies.

In the third instance of insemination at a double ovulation (Misty f), only one embryonic vesicle was detected on day 12. It was impossible to know whether only one conception occurred or whether twins were present but one was lost before the vesicles became identifiable on ultrasound.

Pregnancy Termination

Attempts were made to terminate three of the pregnancies using prostaglandins at different stages of gestation. Two of the attempts were successful but one mare maintained her pregnancy and subsequently foaled normally six months after the end of the study.

Poppy was administered a single luteolytic dose of prostaglandin on day 18-20 of gestation (Poppy b). She came into oestrus three days later and ovulated 11 days after treatment. There were no lasting effects on Poppy's fertility caused by this treatment and she conceived again at the induced oestrus (Poppy c).

Corrie and Greedy were treated with prostaglandin on days 39 and 41 of gestation respectively, due to the conceptus being missed in earlier ultrasound scans as discussed in Chapter Three.

Corrie's first prostaglandin injection on day 39 (See photo 4.5) caused a reduction in progesterone concentrations from 8 to 4.6 ng/ml. A second injection given on day 44 further reduced concentrations to 2.5 ng/ml, suggesting that partial lysis of the CL had occurred. Despite these drastically reduced progesterone concentrations, the conceptus survived.

Corrie's progesterone concentrations began to rise again due to eCG support stimulating the development of more luteal tissue and the pregnancy continued with eCG concentrations remaining elevated until the end of the study.

Greedy was first given prostaglandin on day 41 (See photo 4.6a) and this caused a reduction in progesterone concentrations from 9.17 to 1.8 ng/ml. As recommended

on the data sheet, it was repeated to ensure abortion but since concentrations declined by only a further 0.63 ng to 1.17 ng/ml after the second injection, it seems likely that one injection would have been sufficient in this case. As previously mentioned, Greedy has only one functional ovary so it seems possible that the total mass of luteal tissue was less than Corrie's at a similar stage and so a greater proportion of it was lysed after the first injection.

Following the progesterone decline, the foetus was present for a further 10-16 days but no heartbeat was detectable. There was a slight decrease in the size of the vesicle with the membranes appearing disorganised and the foetus floating freely. Photos 4.6b - d show the appearance of the foetus from day 44 and 50. Between day 52 and 59, Greedy's conceptus was completely resorbed leaving no other sign of its presence than a slight increase in diameter of the right horn compared to the left. At day 82 (Photo 4.6e) some fluid was seen on ultrasound in the uterine lumen but the cervix remained closed throughout so resorption must have been responsible for elimination of the foetal fluids. Since the embryonic tissues are no longer visible on ultrasound once the fluid is removed, the fate of the rest of the conceptus was not known.

Progesterone concentrations rose again due to the continued production of eCG (as seen by the LH results, Greedy k) from the endometrial cups despite the death of the foetus. Concentrations never rose above 2.4 ng/ml which may reflect the relatively small amount of ovarian tissue that was present for stimulation to form luteal tissue and thus progesterone. Due to the infrequency of sampling and the fluctuations in results, it is unclear whether Greedy's eCG concentrations remained high or began to decline after the pregnancy was terminated, although her progesterone concentrations decreased steadily suggesting lack of eCG support and regression of the luteal tissue.

Greedy responded to the single prostaglandin injection at day 87 by coming into oestrus two days later (Greedy g). The prostaglandin must have lysed what luteal tissue remained as evidenced by the progesterone concentrations falling to basal values. The eCG profile (Greedy l) although unclear, suggests that the endometrial cups were also regressing at that time as eCG production was dropping although there appeared to be a surge in response to the low progesterone after prostaglandin treatment (Greedy m). It is not known whether concentrations remained elevated throughout this induced oestrus of more than 34 days duration but if so, it was not able to suppress her behaviour. There was little follicular development associated with this period and Photo 4.7 shows the largest follicle that was present, so the

cause of this behaviour was unknown as was whether it finally ended in ovulation as this was after the end of the study.

The appearance of Greedy's granulosa cell tumour appeared to change in conjunction with the prostaglandin treatment (See photo 4.8). This showed an increase in density with a reduction in fluid filled areas.

Misty was flushed 12 days after ovulation (Misty f) using a 1.75 cm diameter rigid pipette but no attempt was made to retrieve the embryo. Photo 4.9a shows the vesicle before flushing and in photo 4.9b, it can be seen floating freely in the infused fluid. She returned to oestrus spontaneously four days post flushing and ovulated 4 days later giving an interovulatory interval of 20 days. Misty's embryo was not removed during the flushing but her return to oestrus confirms that luteolysis occurred.

Poppy was flushed on day 21 (Poppy c) using a 1 cm diameter, insemination pipette and 1 litre of 0.9% warmed saline but again no attempt was made to recover the embryo. Photos 4.10a & b show the vesicle before and during flushing. The uterus was scanned and gently balloted but not palpated or massaged. She returned to oestrus three days later.

Embryo Retrieval

A 3.5 cm diameter catheter was used and four embryos were successfully retrieved out of six attempts, giving a recovery rate of 66%.

With Filly (f) and Jenny (f), the vesicle was retrieved intact at day 14-15 and 17 respectively and both consisted of a bilaminar yolk sac within an outer capsule. On retrieval of Filly's embryo, the trophoblastic membranes had collapsed within the intact capsule. Photo 4.11a and b show the conceptus before and after collection. Traumatic damage to the conceptus after collection resulted in damage to the yolk sac and loss of the capsule. The embryonic disc is visible but the primitive streak is not clear due to the rupture of the tissue in that area. It is reported to be approximately 1 cm in length at this stage (Van Niekerk and Allen, 1975).

Jenny's embryo was retrieved intact 2-3 days later than Filly's and the trophoblastic membrane had not collapsed away from the capsule. The capsule of Jenny's embryo was intact at collection but split when the conceptus was removed from the fixative for photographing and can be seen clearly in Photo 4.12a as two halves of a rigid,

transparent shell. There is no sign of any attachment between the trophoblast and the capsule. The embryo is visible by this stage and Photo 4.12b shows it was at the 14 somite stage. The neural groove is well developed and ends in a primitive gut, which corresponds with the findings of Van Niekerk and Allen

Misty (h) was the most advanced retrieval attempt at day 25 (See photo 4.13a). The first flush resulted in the fluid forming a localised accumulation round the conceptus at the body horn junction of the uterus. The second flush successfully dislodged the conceptus which then took up position beside and behind the opening of the catheter. Retrieval was only completed by altering the angle of the catheter downwards and to the right to allow the embryo to enter it.

The membranes were ruptured on retrieval due to the diameter of the conceptus being greater than that of the catheter lumen and so the conceptus was collapsed. The embryo was clearly visible to the naked eye with a prominent neural fold. All the major internal organs were formed, including the eye, and limb buds were beginning to sprout, which again corresponds to the observations of Van Niekerk and Allen (1975). There was no capsule present at this stage but the developing allantois and chorionic girdle were visible (See Photo 4.13b).

By 3 days post flushing Misty had not returned to oestrus although on ultrasound she had marked follicular development (See photo 4.13c). She also had some free fluid in her uterus and so it was decided to treat her with prostaglandin as were all the mares flushed subsequently. Because she was flushed at a much more advanced stage than the other mares, it would have been interesting to find out if the procedure had stimulated prostaglandin release or if the uterus was incapable of inducing luteolysis at this stage. However, Misty's falling progesterone concentrations at this time (Misty h), suggest that she probably would have returned to oestrus spontaneously if left.

The attempts to flush Meg and Poppy (Meg i & Poppy f) were both unsuccessful. Photo 4.14 shows Meg's conceptus before flushing. Both were at the 21-23 day stage and the fluid accumulated round the conceptus as described before but repeated flushes failed to dislodge it. Meg is an older, multiparous mare and her uterus was consequently quite pendulous. It was impossible to lift the ventral areas of the body horn junctions dorsally enough to retrieve the fluid. In Poppy's case, uterine tone was very marked making uterine massage difficult and preventing both the fluid and the conceptus leaving the dilated area.

However, Suzy's embryo was successfully retrieved at day 22 (Suzy i). Her uterus also displayed a marked increase in tone and was difficult to manipulate but it also

seemed very contractile. The entire 1 litre of saline was expelled with some force immediately on removal of the introducer and the embryo with it. On post flushing ultrasound examination there was no fluid visible at all, unlike the other cases where a small amount of residual fluid remained in the dilation the conceptus had occupied. Again the embryo was clearly visible with a prominent neural fold and most of the organ systems were formed, including the eye. The membranes still consisted of mainly bilaminar yolk sac (See Photo 4.15).

Pregnancy Monitoring

The hormone profiles produced by Corrie and Greedy up until the time of prostaglandin administration were similar to those widely reported during pregnancy (Holtan et al., 1975 ; Nett et al., 1975). This demonstrates that conception during the winter has little effect on the course of the pregnancy. It also indicates that Greedy was able to produce a hormone profile indistinguishable from other pregnant mares despite the presence of a granulosa cell tumour in her right ovary. Photo 4.16a shows marked follicular development in Greedy's right ovary and photo 4.16b shows an anovulatory follicle undergoing spontaneous luteinisation both due to eCG stimulation.

Vanderwall et al. (1993) demonstrated that LH concentrations in the mare remain low throughout pregnancy. However, according to the LH assay used in the present study, a distinct increase in concentrations were seen in both mares whose pregnancies progressed beyond 35 days. Since it is at this time that eCG concentrations are reported to rise in the pregnant mare, it was assumed that the apparently elevated LH concentrations were in fact due to cross reaction between eCG and the LH assay. This coincided with the findings of Nett et al. (1975) who also reported the occurrence of a crossreaction between eCG and an LH assay due to the similarity in structure between the two hormones.

The wide fluctuations in eCG concentrations between subsequent samples seen in the present study suggests an episodic secretion of eCG and since these samples were days rather than hours apart, they may represent long term variations in secretion. However, the samples would have to be reassayed to confirm that the fluctuations observed were real and not due to assay variation.

A particular difficulty was observed in detecting pregnancy at a certain stage of gestation. When scanning both Corrie throughout her pregnancy and other pregnant mares not involved in this study, a period of time around day 60 of gestation was

identified where the foetus was difficult to visualise on ultrasound. The uterus appeared as a domed fluid filled area sometimes with scintillating particles suggesting the presence of debris and the foetus was frequently resting on the ventral aspect where the 7.5 MHz transducer could not penetrate. In one mare, examinations on four consecutive days were required before the presence of the foetus was detected and this together with a history of irregular cyclic behaviour had led to a suspicion of the presence of a pyometra (See Photos 4.17). It was found that in these cases, moving the transducer to a more lateral position and sliding it down the side of the uterus so the sound beam was angled obliquely through the uterus helped penetrate the more ventral areas and that if sufficient time was allowed, the sound waves appeared to stimulate the foetus and its movements made it easier to detect. Alternatively, the use of a 5 MHz transducer would give better penetration in these situations although the resolution is not as good.

In Corrie, once her pregnancy had continued past day 175, the foetus dropped in the abdomen and so only fluid and membranes were visible on transrectal ultrasound, even using a 5 MHz transducer. (See Photo 4.18a). An attempt was made to scan Corrie's foetus at day 200 by the transabdominal route using a 3.5 MHz sector transducer (Dynamic Imaging). Because the midline area of her abdomen immediately cranial to the mammary gland had little hair, clipping was not necessary but ultrasound gel was rubbed well into the area to provide a good contact between the transducer and the skin.

The uterus was easily identified using this method with its endometrial folds, fluid contents and floating foetal membranes (See Photo 4.18b). Initially a foetal extremity was visible as a hyperechoic area and this was probably due to the foetus being inactive at that point and lying in the ventral uterus close to the abdominal wall. Again, the ultrasound waves appeared to stimulate the foetus, producing spontaneous movement and in this case the foetus moved out of the field of the scanner before photos could be taken.

Photo 4.1a - Jenny c. Undersized 5 mm embryonic vesicle at day 13 post ovulation, prior to early embryonic death.

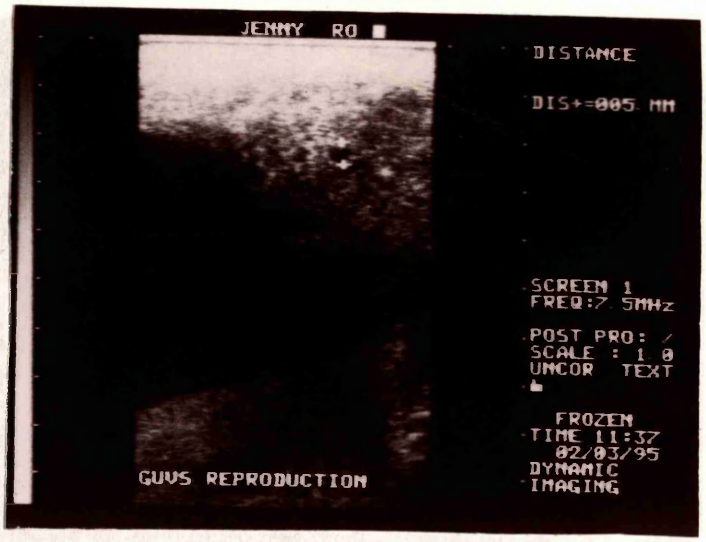


Photo 4.1b - Jenny d. Undersized 5 mm vesicle at day 19 post ovulation prior to early embryonic death.

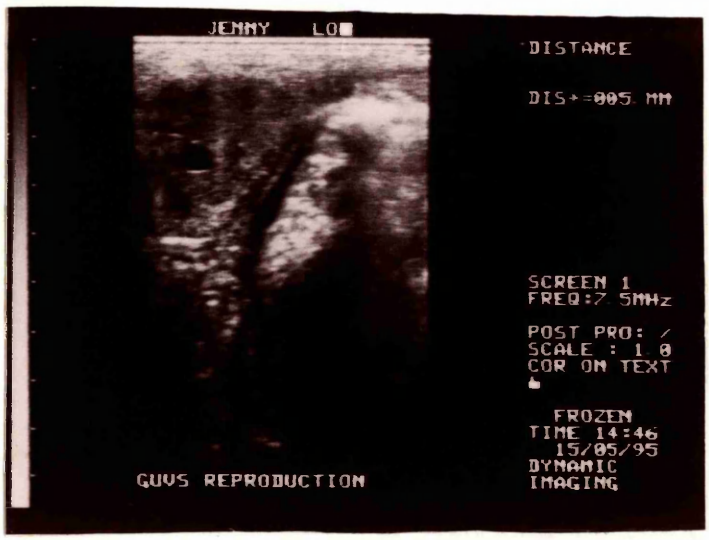


Photo 4.1c - Jenny f. Normal sized 14 day old vesicle at subsequent pregnancy

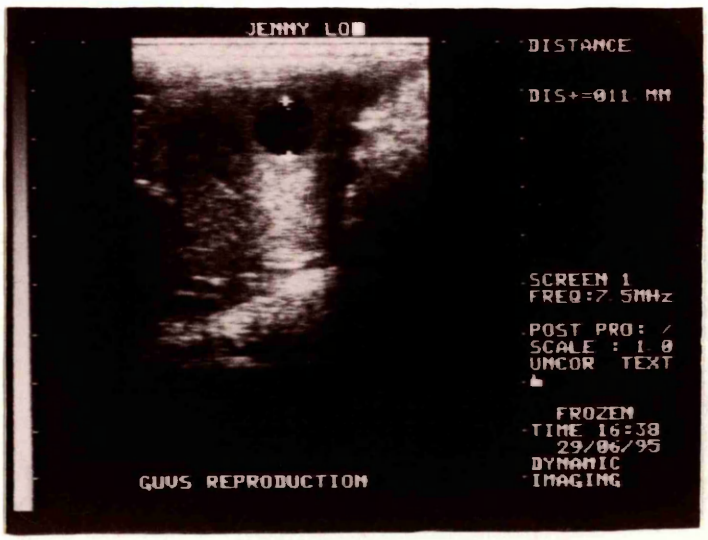


Photo 4.2 - Laura b. Appearance of conceptus at last examination before being lost at 19-24 days old.

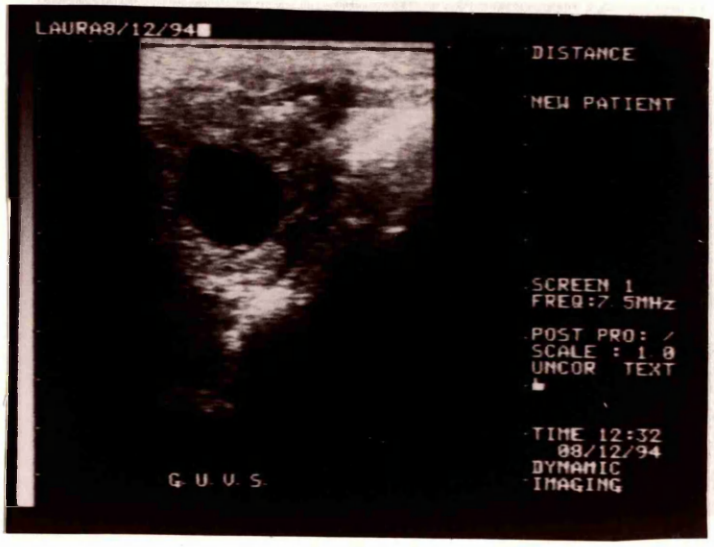


Photo 4.3a - Poppy e. Twin vesicles of different ages and sizes (20 mm and 24 mm). (Markers indicate the smaller of the two)

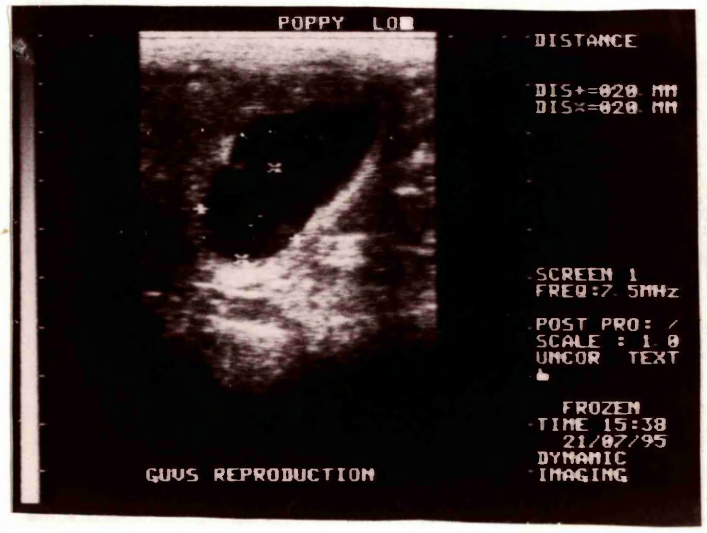


Photo 4.3b - Poppy e One large follicle in left ovary, present at AI which produced twin embryos

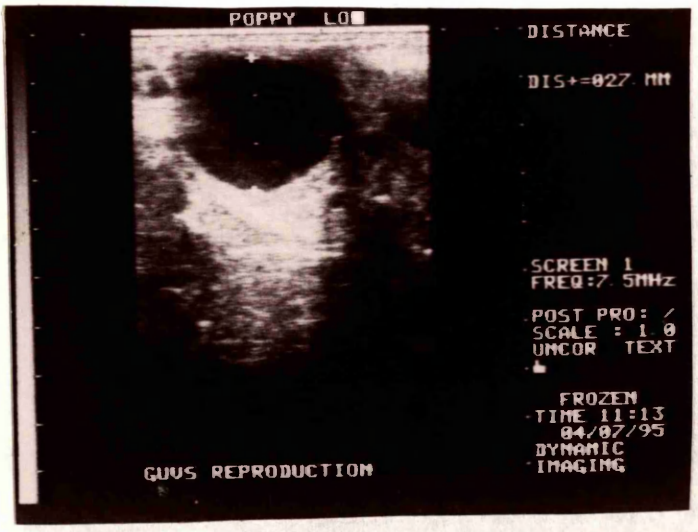


Photo 4.3c - Poppy e

2 CLs (indicated by

markers) present in

left ovary after end

of oestrus. Ventral

one is more hyperechoic

so is probably the younger

of the two

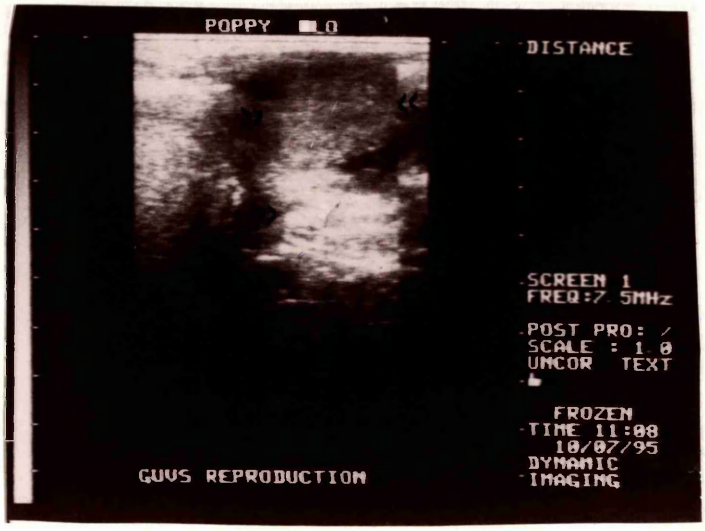


Photo 4.3d - Poppy e.

1 vesicle remaining

at day 21 - 23 after

other resorbed

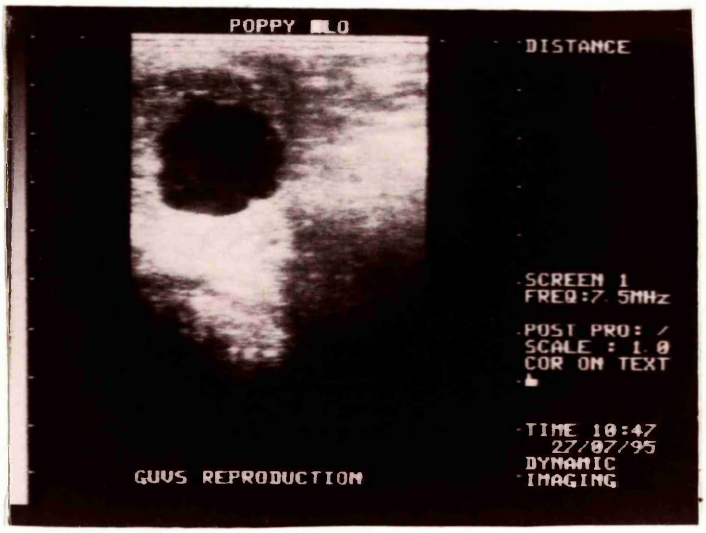


Photo 4.4a - Misty h.

One CL in left ovary

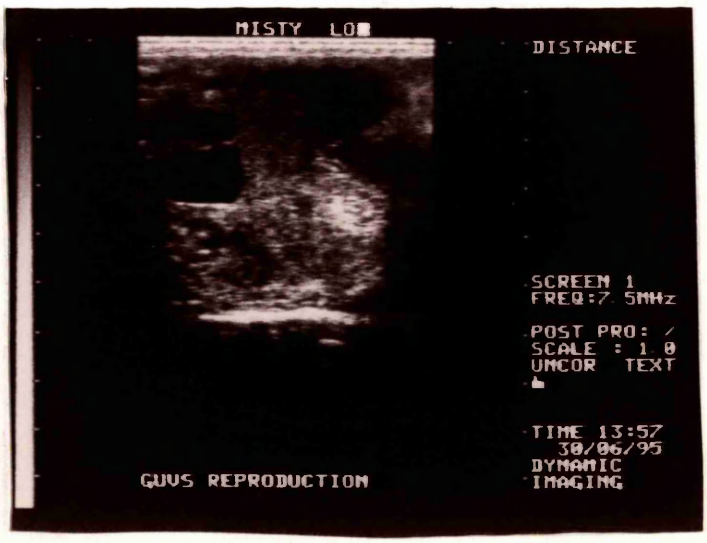


Photo 4.4b - Misty h.

Other CL in right ovary

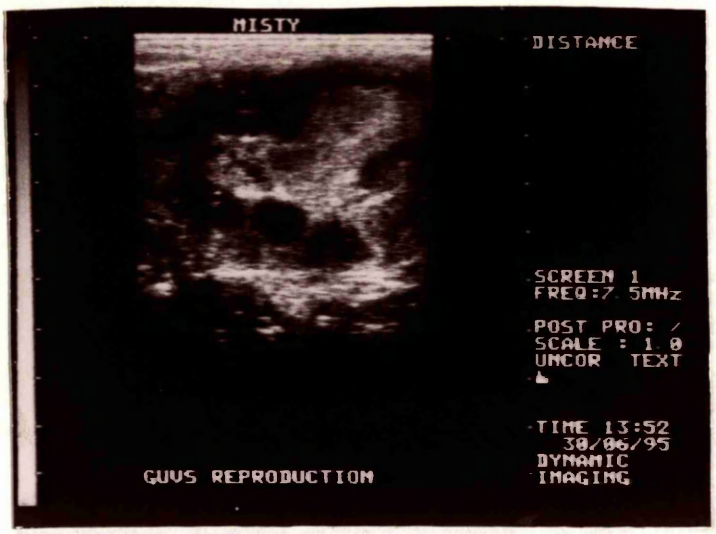


Photo 4.4c - Misty h.

Different view of CL in right ovary that gives the impression it is two and not one CL

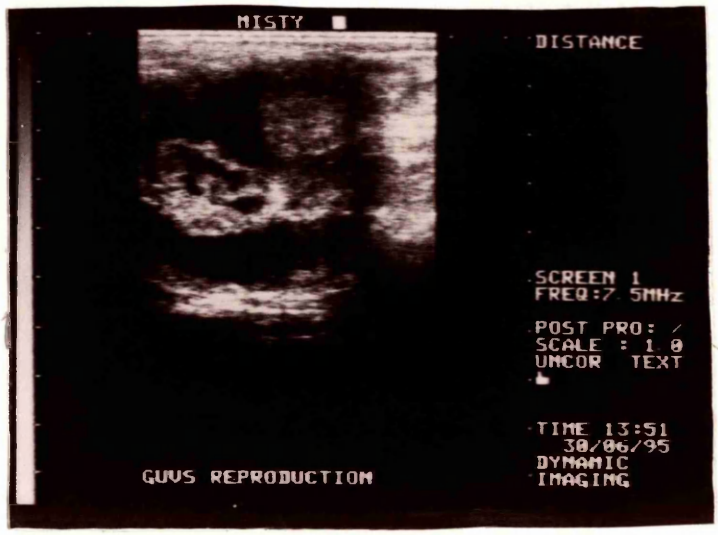


Photo 4.4d - Misty h.

1 vesicle remaining at day 16 - 18

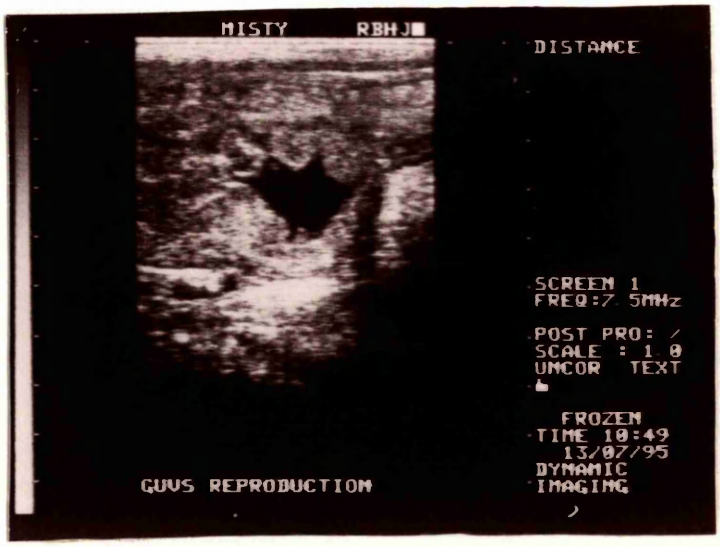


Photo 4.5 - Corrie c. Conceptus at time of first prostaglandin injection on day 39

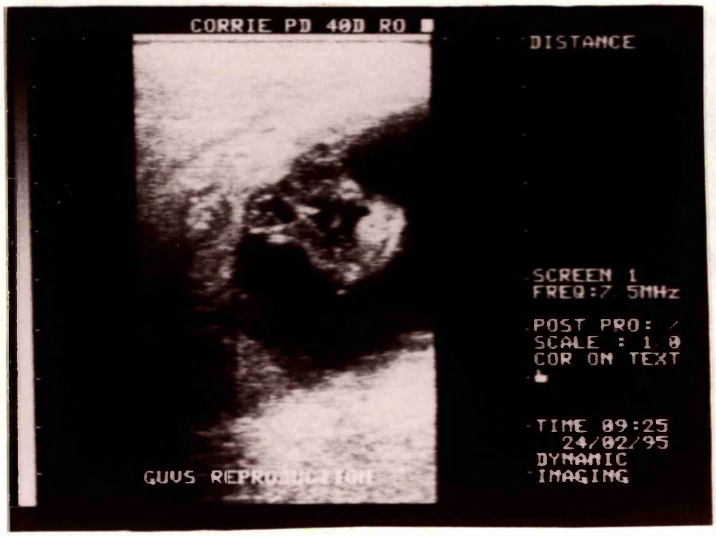


Photo 4.6a - Greedy e. Conceptus at time of first prostaglandin injection on day 39

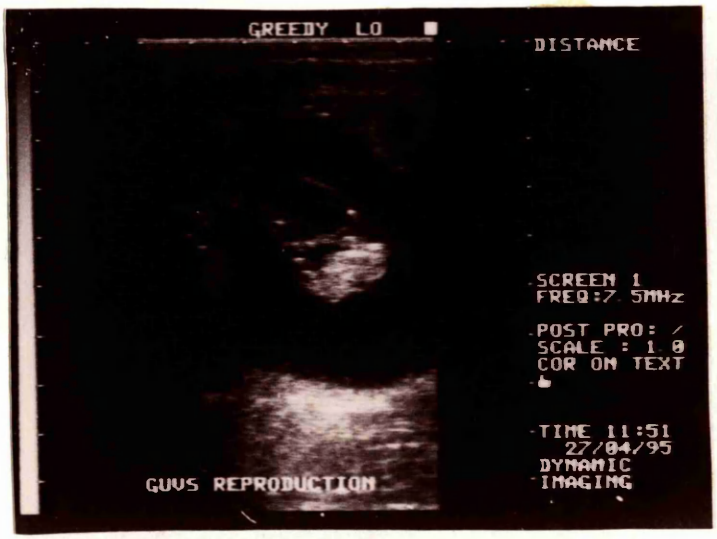


Photo 4.6b - Greedy f. Conceptus on day 44. No heartbeat detected, membranes detached and foetus floating freely in uterine lumen showing changes in position

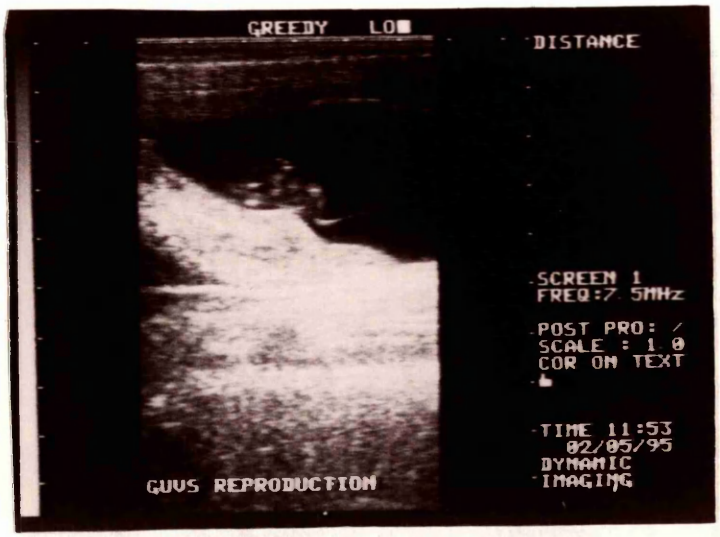
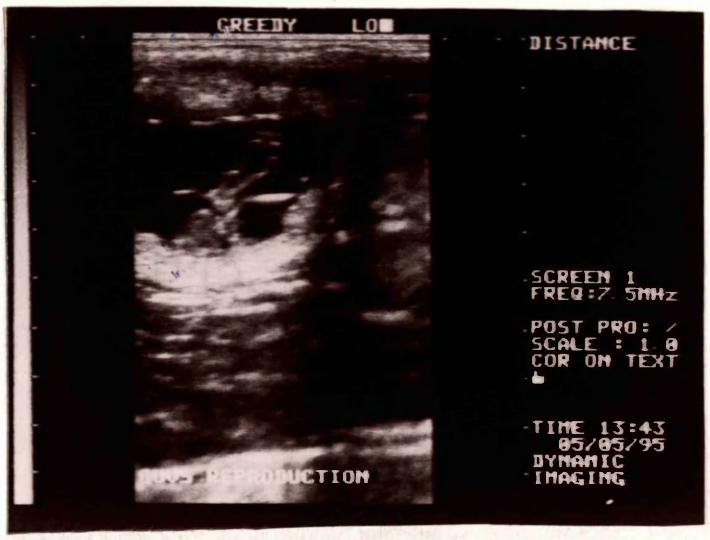


Photo 4.6c & d - Greedy f. Conceptus on days 47 and 50. No heartbeat detected, membranes detached and foetus floating freely in uterine lumen showing changes in position



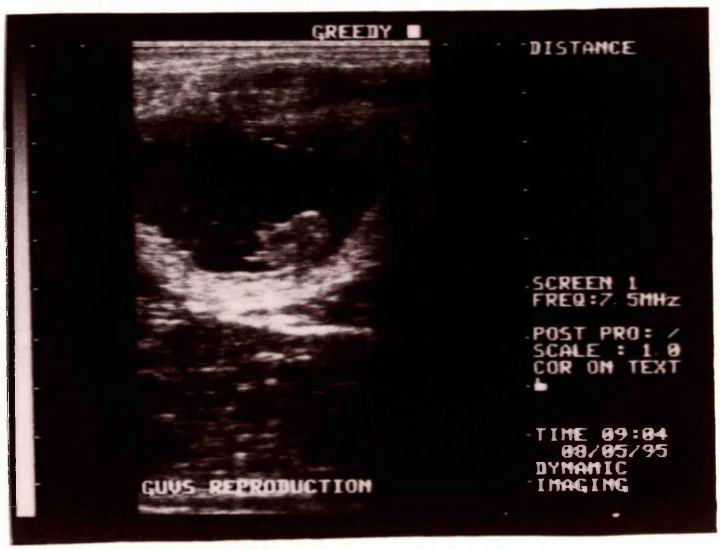


Photo 4.6e - Greedy f. Day 82. Free fluid in uterine lumen but no sign of foetus

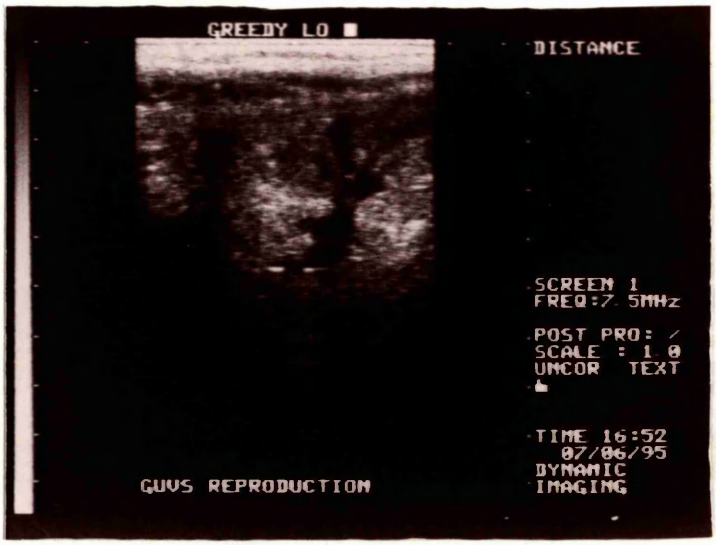


Photo 4.7 - Greedy g. Largest follicle present in left ovary during prolonged oestrus

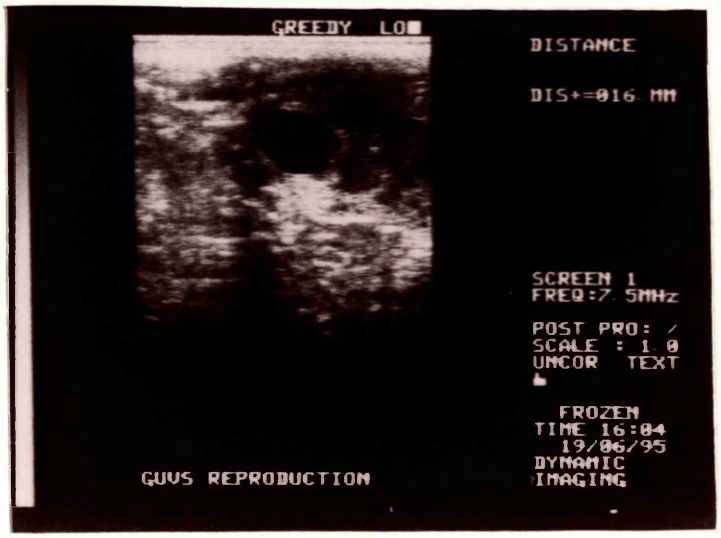


Photo 4.8 - Appearance of granulosa cell tumour following prostaglandin administration. Increase in amount of dense tissue compared to fluid filled areas

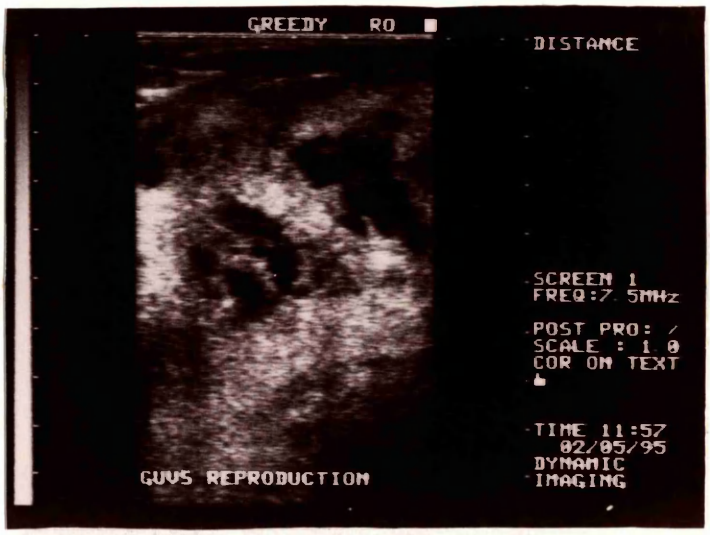


Photo 4.9a - Misty f. 12 day old vesicle before flushing (indicated by markers)

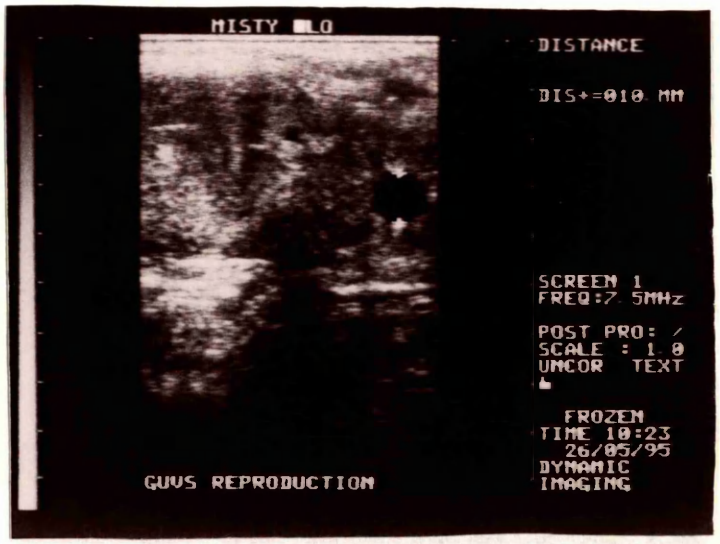


Photo 4.9b - Misty f. Vesicle during flushing floating free in the dorsal area of the fluid

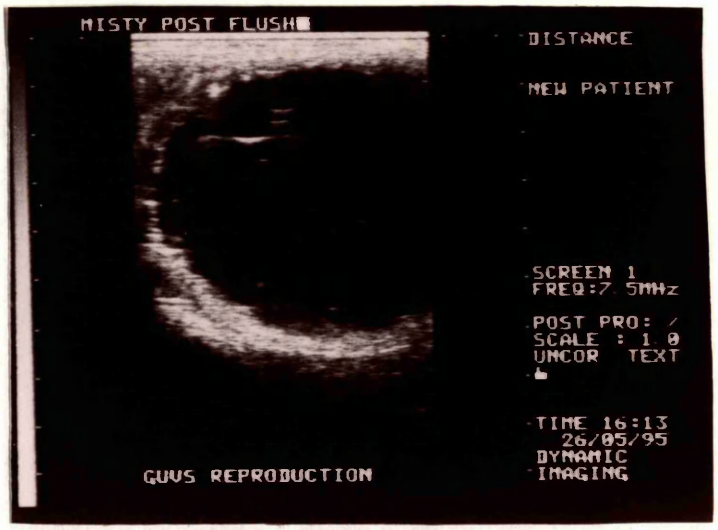


Photo 4.10a - Poppy c. Day 21 vesicle before fluid introduced

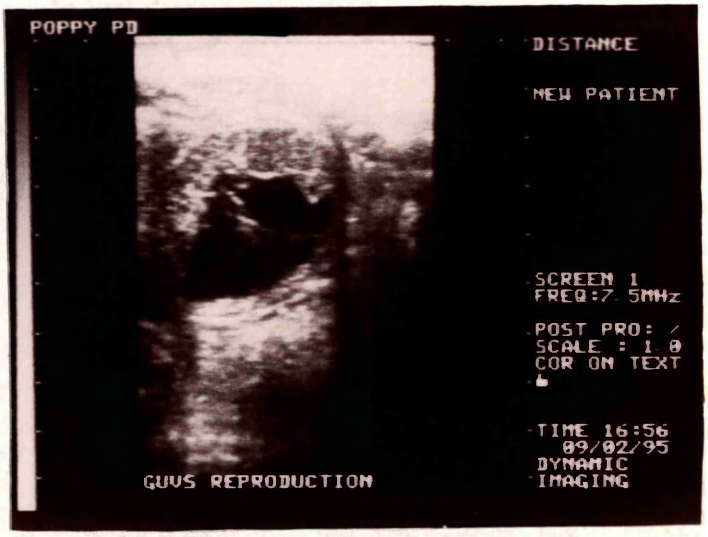


Photo 4.10b - Poppy c. Outline of vesicle visible in ventral area of fluid accumulation in right body horn junction after introduction of fluid.

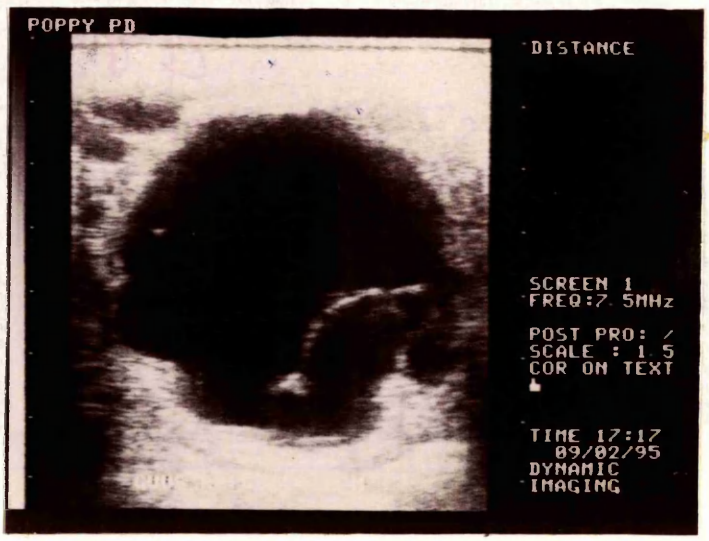


Photo 4.11a - Filly f.

Day 14 -15 conceptus

before flushing

(indicated by markers)

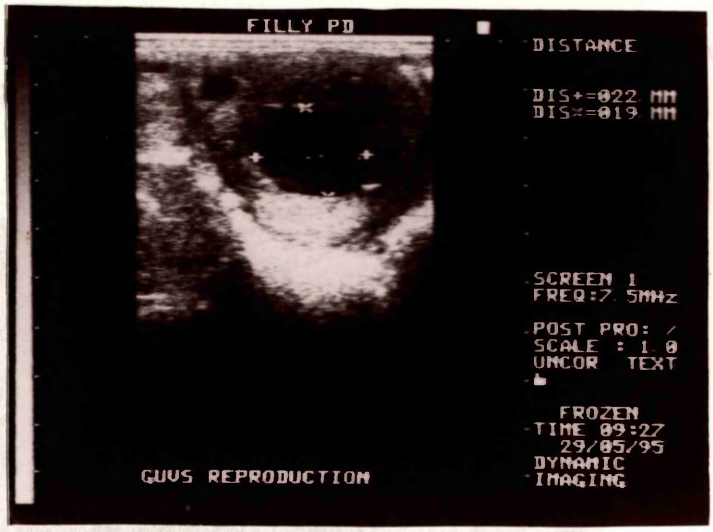


Photo 4.11b - Filly f. Same conceptus after retrieval. Consist entirely of yolk sac and embryo proper not visible. Capsule lost during collection

Photo 4.12a - Jenny f. Day 17 conceptus after retrieval. Consists entirely of yolk sac and capsule ruptured but visible. Embryo proper visible

Photo 4.12b - Jenny f. Close up of embryo revealing neural canal and somite formation

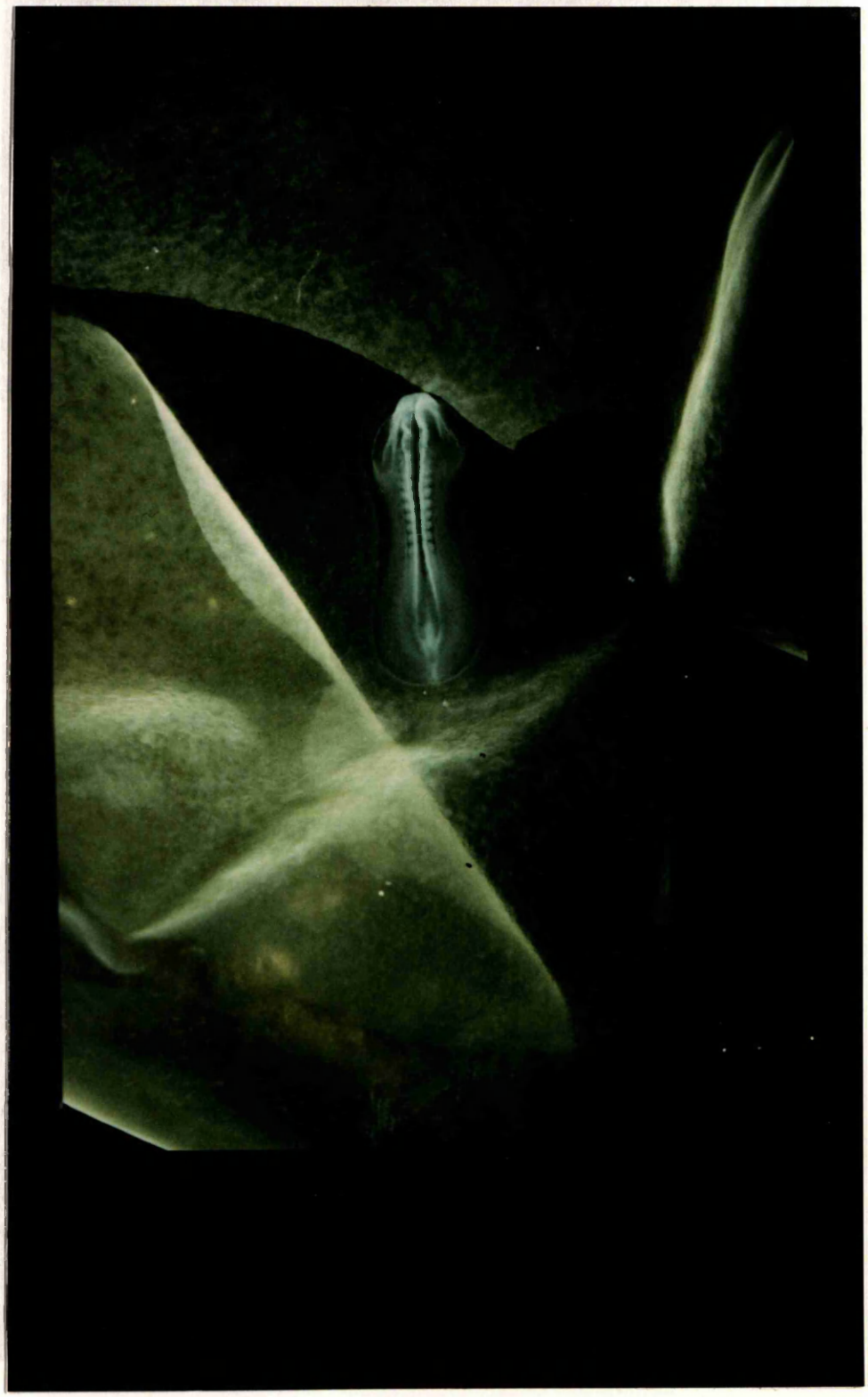


Photo 4.13b - Misty h. Same conceptus after retrieval. Allantois developing and neural fold of embryo prominent

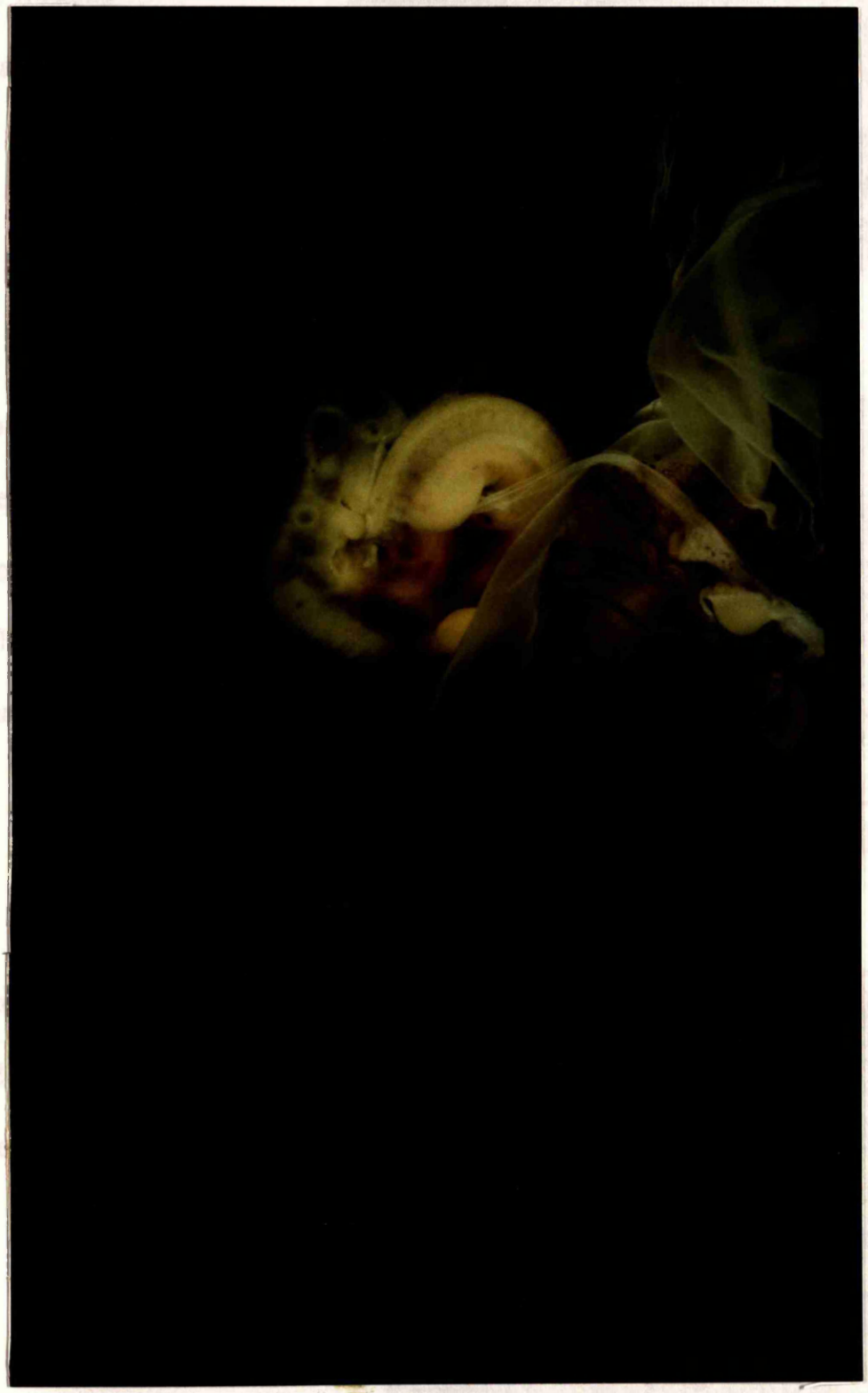


Photo 4.13a - Misty h.

Day 25 conceptus

before flushing

(indicated by markers)

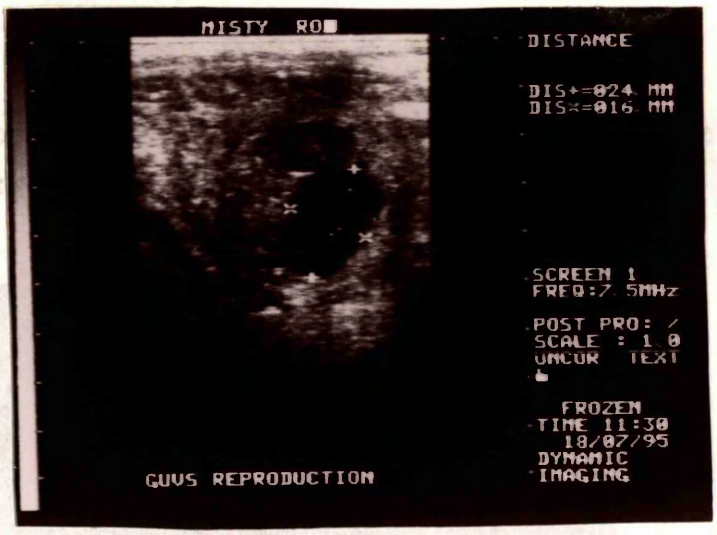


Photo 4.13c - Misty h.

Marked follicular

development 3 days

post flushing

(indicated by markers)

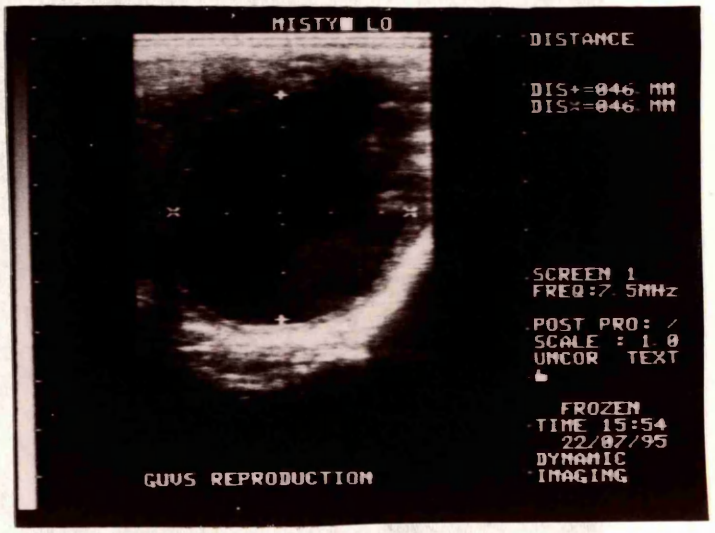


Photo 4.14 - Meg i.

Day 21 - 23 conceptus

before unsuccessful

flush

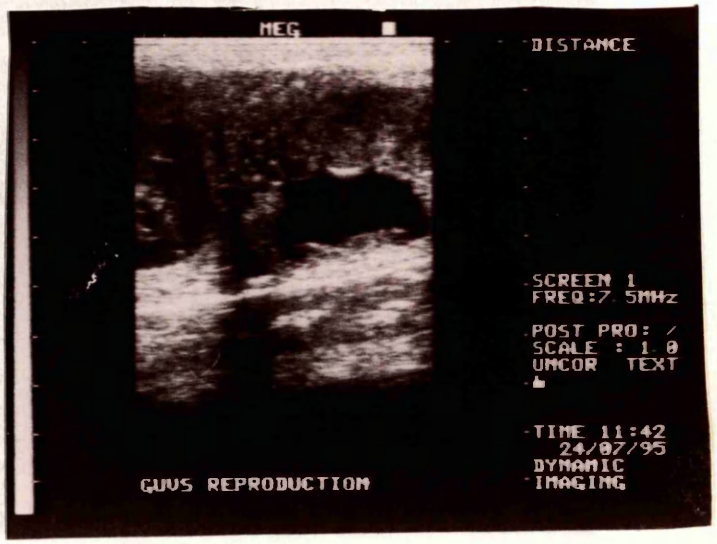


Photo 4.15 - Suzy h. Day 22 embryo after retrieval. No capsule present by this stage but neural fold etc. in embryo well developed

Photo 4.16a - Greedy e. Marked follicular development associated with eCG stimulation

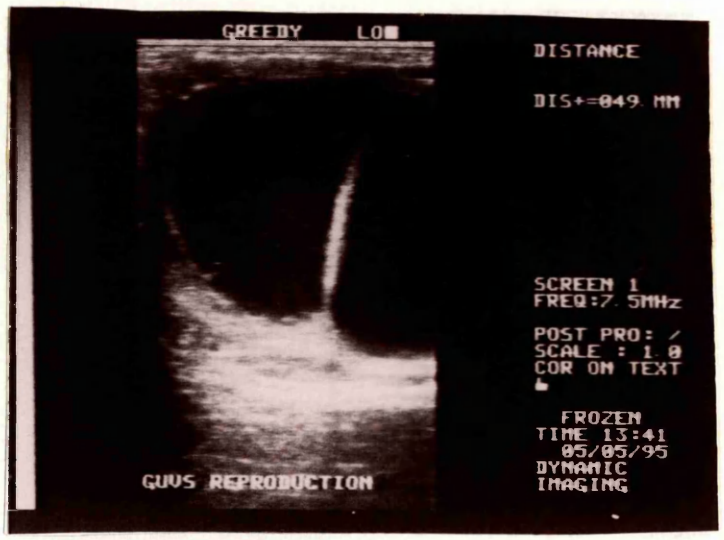


Photo 4.16b - Greedy e. Anovulatory follicle undergoing spontaneous luteinisation in association with eCG stimulation

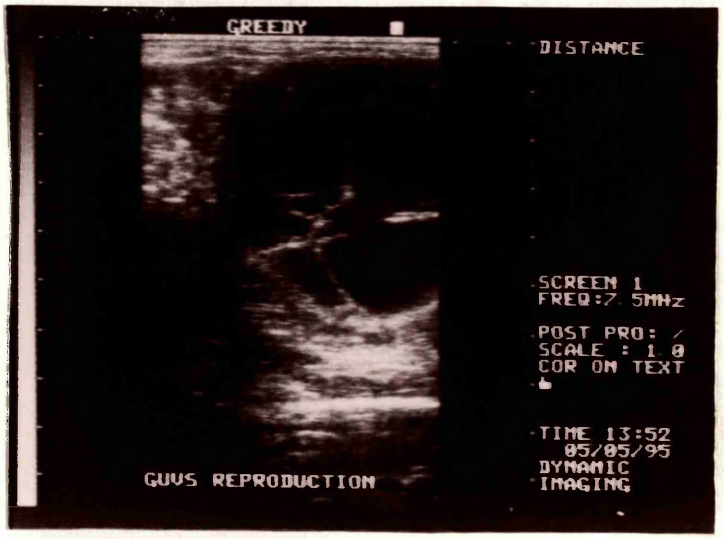


Photo 4.17- Fluid filled uterus containing scintillating particles suggesting the presence of debris. No foetus visible but 60 days pregnant

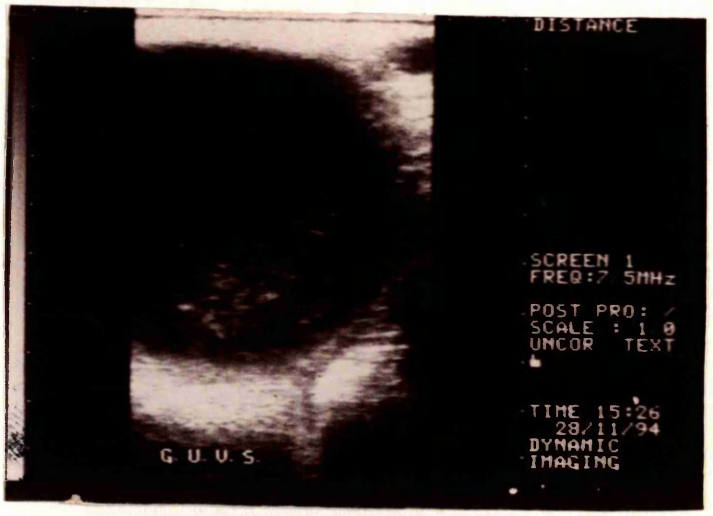


Photo 4.18a - Foetal membranes visible on ultrasound at day 175 but no foetus visible

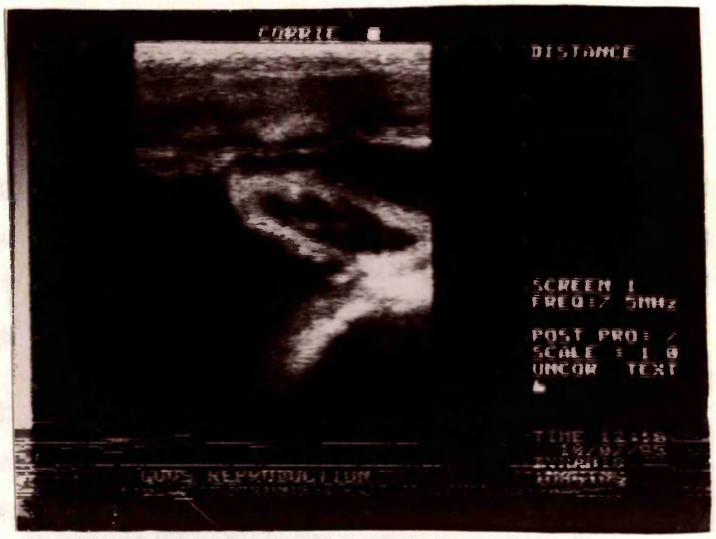
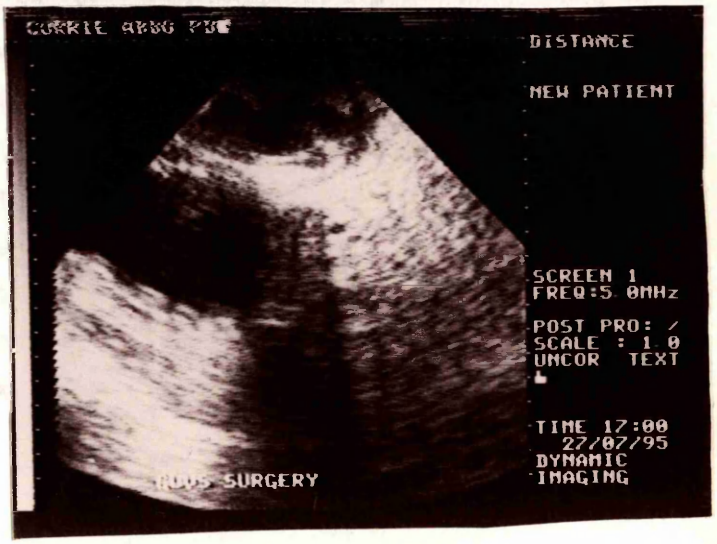


Photo 4.18b - Foetal membranes and endometrial folds visible on transabdominal ultrasound examination but no foetus visible



4.4 Discussion

The pregnancies in this study occurred from November to June, confirming that ovulations taking place over the winter were fertile. This was in agreement with the findings of Colquhoun (1984). Corrie is now well advanced through a normal pregnancy having conceived at the start of January.

Ginther (1983a) and Pascoe (1982) reported that the conceptus lodged in the right body horn junction in 56-67% of maiden and 59% of barren mares. The 100% incidence of right sided pregnancies in the present study does not coincide with these findings. Pascoe (1982) also found that in 80% of lactating mares, the vesicle became fixed in the previously non-gravid horn (Pascoe, 1982). This was probably due to the diameter of the previously gravid horn still being larger than that of the non-gravid one at the time of fixation and so the vesicle is preferentially trapped in the narrower space. The longer the interval between parturition and conception, the smaller this proportion became suggesting that as the uterus returned to normal it had less effect on the site of fixation (Pascoe, 1982). Since there is no information available regarding the side of the previous pregnancy in the three mares weaned during the present study, it is impossible to determine what effect, if any, this had. However, unless all three had previous left sided pregnancies, it seemed likely that they were all far enough post partum for there to be no effect of the previously gravid horn.

Embryonic Loss

Pregnancy-loss rates of approximately 18% have been reported by Ginther (1992) when initial pregnancy detection is carried out at day 20, decreasing to 12% at day 40. Since the pregnancies in the present study were detected at day 11-14, this could explain the slightly higher incidence of 21.5%. Although all three occurred during the winter months, other mares also conceived at this time and maintained their pregnancies, so it seems unlikely that the time of year was the cause. Woods et al. (1985) also reported no seasonal effect on the rate of early embryonic death in the mare, although their study did not commence until February.

Woods et al. (1985) reported that the first four weeks of gestation had the highest incidence of early embryonic death, although only those that occurred after the embryo became visible on ultrasound were readily detected. Both Laura and Jenny lost their pregnancies during this period. Many causes of embryonic death in the

mare have been suggested including endocrine factors, uterine environment, embryonic factors, stress and age of the mare (McKinnon and Voss, 1993).

The similarities in appearance between Jenny's vesicles just prior to embryonic death suggested that some factor associated with either Jenny's uterine environment or both of the embryos retarded their growth at a similar stage. Ginther et al. (1985) found that 62% of pregnancies with undersized vesicles underwent early embryonic death. Because of their size, they also did not become fixed in a body horn junction but remained mobile past the time of normal fixation and this occurred in both of Jenny's cases. The embryo is thought to secrete oestrogens which help produce the characteristic increase in tone associated with pregnancy and therefore also contribute to its own fixation (Hayes and Ginther, 1986 ; Zavy et al., 1979).

In Jenny, the lack of increase in tone beyond that associated with the same stage of dioestrus and the failure of fixation could therefore have reflected the abnormal state of the embryos. However, since this increase in tone is not consistent in all mares and Jenny's embryos were both lost before the time at which maximum tone would have occurred, this observation may not be of any significance.

Shideler et al. (1982) reported a decrease in progesterone concentrations prior to pregnancy loss, which suggests the occurrence of luteolysis. This is usually associated with endometritis (Ginther, 1992) and occurs in 71% of mares which lose their pregnancy before day 20, causing them to return to oestrus (Ginther et al., 1985). However, this was not the case with either Jenny or Laura whose progesterone concentrations remained elevated until the spontaneous regression of the CL at day 61 (Jenny c) and day 75 (Laura b) respectively.

The gradual decline in progesterone concentrations over these periods suggested progressive ageing and regression of the CLs. Only then did they both return to oestrus. Ginther (1992) suggests that this phenomenon, which occurs in the remaining 29% of mares which lose their pregnancy before day 20 (Ginther et al., 1985), is associated with a healthy uterine environment, although this has not yet been directly studied. There was no evidence of uterine pathology on ultrasound or clinical examination in either Jenny or Laura and because the embryo was present beyond days 13 and 14, the luteolytic mechanism was blocked by maternal recognition and therefore the primary CL was maintained. This coincides with findings of Kooistra and Ginther (1976) who reported that mares who lost their conceptus before day 35 only maintained high progesterone concentrations for the natural lifespan of the primary CL.

The term 'pseudopregnancy' has been defined by Ginther et al. (1985) as the 'development and maintenance of turgid uterine tone and the corpus luteum after embryo loss' and it will last for a similar period of time to that of the prolonged dioestruses as discussed in Chapter One. This is due to the cause being the same, namely the failure to lyse the CL, therefore both conditions have a lifespan similar to that of a normal CL and will produce the same progesterone profile (Jenny b compared to Jenny c). This is known as a Type 1 pseudopregnancy and that it is produced solely by the maintenance of the CL is demonstrated by Jenny returning to oestrus following her second case of early embryonic death due to a single luteolytic dose of prostaglandin on day 20 in the same way the prolonged dioestruses in Chapter One were ended.

As discussed in Chapter One, Jenny also demonstrated two idiopathic prolongations of the CL earlier in the study (Jenny a & b). This may reflect some defect in her endometrium which interfered with the induction of luteolysis and therefore may also have been capable of contributing to the embryonic deaths. Since the characteristic tone associated with pregnancy did not develop in either case, she was not pseudopregnant by the definition of Ginther et al. (1985). It is possible that the prolongation of the CL occurred spontaneously and was not due to maternal recognition, corresponding with the underdevelopment of the embryos.

The cause of the previous embryonic losses in Jenny appeared to be temporary and spontaneously resolved allowing her to maintain her next pregnancy until flushing. Whether she would have carried the conceptus to term however, is not known. Woods et al. (1985) reported that 65% of mares which underwent early embryonic death conceived again during the same breeding season but of these, 40% also lost this subsequent pregnancy. It was suggested that this was due either to embryonic defects or the uterine environment not being capable of supporting a pregnancy.

It was not known whether Laura failed to conceive again throughout the rest of the study or she did conceive again but lost the embryo before it became detectable on ultrasound. She is however, a young, maiden mare and as such should be expected to have a better uterine environment and higher pregnancy rate than older mares (McKinnon and Voss, 1993 ; Woods et al., 1985). That she failed to maintain a pregnancy beyond day 23 is therefore surprising and further investigation would be indicated if she was to be used for breeding purposes.

Prolonged dioestruses can occur in inseminated or mated mares which do not have an embryo present at the time of maternal recognition (Greedy d, Lady i). In theory they could also occur in mares which conceived but underwent early embryonic death before ultrasound detection of the vesicle, although this would be difficult to

confirm. Since the same initial progesterone profiles can therefore be produced by mares in a wide variety of reproductive states, it is useful to use ultrasound examination to ensure mares are actually pregnant if they fail to return to oestrus at the expected time. This avoids wasting time during the breeding season.

Twin Pregnancies

The incidence of twin pregnancies in the Thoroughbred is reported to be 1-2% as based on an analyses of the stud books (Osbourne, 1975), although they are reported to be more prone to conceiving twins than other breeds. The reason for this is presumably due their increased incidence of multiple ovulations (Ginther, 1982) since twin embryos in the mare are dizygotic and therefore must arise from more than one ovum. There has only been one report of identical twins in a mare which occurred due to the splitting of a single fertilised ovum. The occurrence of twins is also reported to have a high degree of repeatability within individual mares and also in family lines (Ginther, 1982).

The high value calculated for the incidence of twin pregnancies found in the present study was probably due to the relatively small number of pregnancies produced. Both of the mares which produced twins were part Thoroughbred which corresponded with the findings (Ginther, 1982).

Twin pregnancies are undesirable in the mare since they are one of the main causes of abortion, second only to endometritis. In approximately 60% of cases, a live single foal is delivered at term while 31% lose both pregnancies and only 9% carry twins to term. Of these, 64.5% produce two dead foals, 21% produce one live foal and 14.%, two live foals. Recently, the incidence of twin pregnancies has decreased in Thoroughbreds due to the use of ultrasound in detecting twin conceptuses at an early stage and action being taken to either terminate the whole pregnancy or destroy one of the conceptuses (Ginther, 1982).

Ginther (1984a) reported that 64% of mares with twin pregnancies will resorb one conceptus spontaneously and this corresponded with the 100% rate in the present study. An earlier report (Ginther, 1982) suggested that twins conceived due to a synchronous, double ovulation were more likely to undergo spontaneous reduction than those due to an asynchronous double ovulation. This report also observed that in 76% of twin pregnancies there was only one detected ovulation with the second one occurring undetected later in the same oestrus or early in dioestrus. Failure to

re-examine Poppy after the first ovulation would explain why the second ovulation went undetected.

Ginther (1984a) also observed that 70% of twins will undergo unilateral fixation. They became fixed in the same body horn junction due to one vesicle becoming lodged and then impeding the passage of the other. When this occurred, the chances of one being spontaneously resorbed rose greatly (89% v 11%) (Ginther 1984b). In Poppy's case, the occurrence of unilateral fixation meant an increased chance of one vesicle being resorbed. However, in Misty's case, it was not known if the second vesicle became fixed at all. Since it was still mobile at day 12-14 when fixation should normally have occurred, this suggested that it was not going to became fixed but would undergo early embryonic death. However, according to Ginther (1982), since these twins arose due to a synchronous, double ovulation there was a greater chance of one being resorbed.

The third instance of insemination in association with a multiple ovulation resulted in a single conceptus detected on ultrasound. Ginther (1982) reported that all other ova produced by multiple ovulations had the same potential to become fertilised which means that there is an increased conception rate associated with multiple ovulations. It was impossible to know whether twins arose in this case with one being resorbed before it had become old enough to be imaged using ultrasound whether there was only one conceptus present throughout. If the three mares in the present study had not been examined until 21 days post conception, they would all have appeared to have normal single pregnancies.

Pregnancy Termination

Ginther (1985b) found that mares administered a luteolytic dose of prostaglandin on day 21 of gestation lost their embryo within two days of treatment and ovulated within nine days. Poppy reacted in a similar manner to prostaglandin administration on day 18-20 of gestation demonstrating that at this time, pregnancy is maintained only by the corpus luteum. That this treatment had no effect on her fertility is demonstrated by her conceiving again at the induced oestrus.

According to the manufacturer's instructions, a single administration of 5 mg Dinoprost or 250 mcg Cloprostenol will induce abortion up until day 35 of gestation. However, after this the effect is more variable and repeated injections may be necessary. This is due to the persistence of the endometrial cups in the

absence of the foetus. They continue to produce eCG causing accessory CL formation and resurgence of progesterone concentrations, until they involute several months later (Squires et al. 1980).

That prostaglandin administration did cause lysis of luteal tissue was demonstrated by the marked decline in Corrie's plasma progesterone concentrations. However, the conceptus survived despite concentrations falling to 2.5ng/ml. This corresponds with the findings of Schideler et al. (1982) who reported that concentrations as low as 2.5 ng/ml could occur on occasions throughout this stage of gestation without loss of the conceptus but if concentrations remained consistently below 2 ng/ml, only 17% of conceptuses would survive. However, Squires et al. (1980) reported one mare with progesterone concentrations less than 2 ng/ml for 48 hours following prostaglandin treatment and another with concentrations less than 1 ng/ml for five days, which did not abort.

Greedy's progesterone concentrations declined more markedly after her initial prostaglandin treatment than did Corrie's. Since she has only one functional ovary, this suggests that the functional capacity of her luteal tissue was less than that of Corrie's. This reduction was sufficient to cause the death of the foetus which was not in agreement with the findings of Squires et al. (1980) previously mentioned.

Initially the foetus appeared normal on ultrasound examination and it was the absence of a foetal heartbeat which indicated that the termination had been successful. Following it's death, the foetus remained visible on ultrasound for several days although it's appearance was disorganised and it became mobile within the uterus. This coincided with the findings of Ginther (1985b) who reported that in three mares administered prostaglandin on day 30 of gestation, the intact embryonic vesicle became dislodged. This was thought to be due to the decrease in progesterone concentrations and therefore uterine tone, rather than from direct stimulation of uterine contractions by the prostaglandin.

The eventual fate of the Greedy's foetal tissue was unclear. Her cervix remained closed throughout this period in a similar manner to the mares in a study carried out by Ginther (1985b). He reported that resorption must have been responsible for elimination of the foetal fluids and since the embryonic tissues were no longer visible on ultrasound once the fluid was removed, the fate of the rest of the conceptus was not known. In contrast, in the study performed by Squires et al. (1980), abortion of mares at day 70 resulted in the foetuses being expelled out through the cervix.

Greedy's continuing eCG production was in agreement with the findings of Squires et al. (1980) who reported that the if a pregnancy was lost after the endometrial cups had formed, the cups remained in the endometrium and continued to function despite the death of the foetus. The eCG they produced supported the CL past its natural lifespan of 40-60 days, as seen in association with prolonged dioestruses and embryonic losses before day 30. The mare remained under the influence of progesterone until the endometrial cups were rejected around day 70 - 100, causing an end to eCG production and therefore regression of the secondary and accessory CLs formed and maintained by it.

Progesterone and eCG concentrations returned to basal concentrations by day 120 allowing the mare to return to oestrus. This was therefore another form of pseudopregnancy, known as Type II. The presence of an embryo up to day 30 was necessary for the development of endometrial cups and eCG but after this, the progesterone concentrations remained high until day 120 whether a conceptus was present or not (Squires et al., 1980).

Greedy's increase in progesterone concentration associated with this period of pseudopregnancy was short lived which may have been due to a lack of reserve ovarian tissue to produce more luteal tissue or a decline in eCG support for new luteal tissue formation. However, her eCG concentrations were high enough to prevent her returning to oestrus until prostaglandin was administered again. Unfortunately, her behaviour following this treatment and the causes of it remain unclear due to the end of the study being reached.

Meagher et al.(1977) reported that granulosa cell tumours were identified and removed from two mares during gestation without loss of the pregnancy. Although the stage of gestation at which these tumours were removed was not recorded it does suggest that it is possible for a mare to maintain a pregnancy through the ovarian dependant stages with only one functional ovary. However, in Greedy's case, since luteolysis was induced using prostaglandin, it may be that there was not enough reserve ovarian tissue to allow formation of more luteal tissue before the pregnancy was lost.

The change in appearance of Greedy's tumour that was noticed at this time may have been due to part of it consisting of tissue with prostaglandin receptors. This may conceivably have altered the functional state of the tumour causing it to produce oestrogens or similar substances responsible for the prolonged oestrous behaviour in the absence of follicular development seen at the end of the study. It was interesting to note that three years ago, when her tumour was first diagnosed,

she displayed nymphomaniac behaviour for prolonged periods in a similar manner to this

Hershman and Douglas (1979) reported that maternal recognition occurred between days 12 and 16 in ponies and that cycle lengths were not significantly extended by removal of the embryo on day 10-14. However, this did not correspond with the findings of Betteridge et al. (1985) who observed extended cycles in mares from which embryos were removed before day 14. Misty was flushed before maternal recognition was complete at day 12 and she returned to oestrus confirming that prostaglandin release did occur causing luteolysis. This suggested that either the procedure itself caused prostaglandin release that the conceptus could not prevent or that the saline infusion affected the embryos ability to prevent prostaglandin release. This could have occurred due to damage to the conceptus or by the alteration of the uterine environment.

Poppy was flushed after maternal recognition was complete and the primary CL had been maintained. Her return to oestrus again suggested that some part of the procedure stimulated the release of prostaglandin causing luteolysis and also that the uterus must still be capable of prostaglandin release despite its previous inhibition at the time of maternal recognition.

Prostaglandin release, as measured by PGFM concentrations in the general circulation, is reported to occur most uniformly in conjunction with cervical dilation and uterine manipulation (Betteridge et al., 1985). This report also found that as the luteal phase progressed, prostaglandin release was less likely and less marked after flushing if an embryo was present producing extended cycles in mares flushed after 14-16 days of gestation. Most studies do not conduct flushing past day 16 so there is little with which to compare the findings of the present study. However, Poppy and Misty's return to oestrus may reflect some part of the procedure used in the present study which causes a more marked prostaglandin release than that of Betteridge et al (1985) or Hershman and Douglas (1979). For example, the use of normal, unbuffered saline may have caused uterine irritation, or the failure to remove the fluid after infusion may have produced uterine distension or prolonged the contact between the saline and the endometrium.

An interesting finding was reported by Betteridge et al. (1985). Four mares flushed on day 6.5-9.5 without recovering embryos were subsequently found to be pregnant and yielded normal conceptuses when flushed again on day 24.5-27.5. No information was given regarding the flushing procedure, medium or volume so again comparisons cannot be made with the present study. However, this did suggest that

embryos can survive the flushing process and changes to the uterine environment without being damaged or their ability to instigate maternal recognition being affected. Older embryos may be more susceptible to environmental changes or the uterus itself may become more sensitive to the flushing procedure thus explaining why the day 6.5-9.5 conceptuses in the report of Betteridge et al. (1985) survived the flushing process while the day 12 and 21 embryos in the present study did not.

Embryo Retrieval

Sirois and Betteridge (1988) reported that the collapse of the trophoblastic membrane within the intact capsule, as seen on retrieval of Filly's embryo, occurred most often with collections performed after day 13.5. They suggested this could be due to trauma during collection, since the acellular capsule was more resistant to external stresses than the cellular membranes within.

It could also be due to the saline used in the retrieval procedure having a higher osmolarity than the uterine fluid and therefore causing fluid to be drawn out of the conceptus. However, since the saline used was isotonic, this would suggest that the uterine fluid was hypotonic. A similar occurrence was observed in canine embryos when they were placed in a medium which was of a higher osmolarity than the uterine fluid of the bitch (Renton et al., 1991). This proves that the capsule must allow the passage of fluids, although because it is rigid, it maintained its shape during collection.

Betteridge et al. (1982) described an attachment between trophoblastic microvilli and the capsule and Betteridge (1989) speculated that this could diminish with time, thus increasing the chances of a retracted blastocyst within a spherical capsule at this stage.

Jenny's embryo was 2-3 days older at collection the trophoblastic membrane did not collapse away from the capsule. According to Sirois and Betteridge (1988), this could be due to less trauma during the collection procedure but could also reflect a closer tonicity between the concentration of saline infused and that of the conceptus and uterine fluid. Renton et al.(1991) found that by diluting the medium with an equal quantity of distilled water, the structural changes in the canine conceptus were almost totally reversible. This suggested that the osmolarity of the mares uterine fluid had altered in this short period of time.

Betteridge (1989) speculated that there was another period of attachment between the trophoblast cells and the capsule after day 14, since the capsule was less easily removed from older conceptuses. There was no sign of any attachment between the A localised fluid accumulation was observed in the body horn junction of Misty's uterus after infusion of the first litre of saline. Its presence in this area around the conceptus was presumably due to the increased tone and interlocking endometrial folds of the uterus trapping the fluid and preventing its passage to other areas which is in agreement with the findings of Enders and Liu (1991).

A second flush was required in this instance and this corresponded with the findings of Sirois and Betteridge (1988) who reported an increased number of flushes being necessary with older embryos. This was due to the greater difficulty of making the larger conceptuses enter the recovery catheter which was also observed in the present study. However, the study conducted by Sirois and Betteridge (1988) did not refer to any embryos older than 16.5 days.

Misty took longer than expected to return to oestrus after this retrieval which suggested that as the pregnancy progressed, either the procedure failed to stimulate prostaglandin release or the uterus was incapable of inducing luteolysis. However, Kooistra and Ginther (1976) caused a return to oestrus in mares at a similar stage of gestation by the injection of cochicine and saline into the uterus, which suggested that the uterus was still capable of prostaglandin release. Also, Poppy's spontaneous return to oestrus following the introduction of saline into the uterus at day 21 suggested that Misty would probably have returned to oestrus spontaneously if left.

Hayes and Ginther (1986) reported that uterine tone was at its maximum around day 25 of gestation and so this was probably the most difficult time to dislodge and retrieve an embryo. Since both Meg and Poppy were approaching this stage of pregnancy when retrieval was attempted, this could have accounted for the failure which resulted. However Suzy's embryo was successfully retrieved at a similar stage although the rapid and complete expulsion of all the saline as well as the embryo was in contrast to the events seen in Meg and Poppy. Uterine contractions are still occurring at this stage as the embryo has just undergone orientation which may explain the occurrence of such rapid and complete expulsion of the fluid, or it may have been individual mare variation.

A recent report (Meadows et al., 1995) described the retrieval of a conceptus 28 days after mating by first removing the foetal fluid by videoendoscopic aspiration. The collapsed vesicle was then flushed out of the uterus via a transcervical catheter. This approach may have been useful in the present study for this period of time

For various reasons, there was not an opportunity to repeat these procedures in any of the mares. Therefore, it was not known which observations made during the retrieval procedures were associated with the stage of pregnancy and which with individual mare variation. Also, the effect of this procedure on future fertility would have to be considered although Sirois and Betteridge (1988) reported no negative effects of their similar procedure on the subsequent fertility of the mare.

Ginther (1984a) found that 70% of twins will undergo unilateral fixation due to one becoming fixed and impeding the passage of the other and that 64% of mares with twins will spontaneously resorb one. The chances of this occurring are much greater when the vesicles are fixed unilaterally (89% v 11%) (Ginther, 1984b).

Pregnancy Monitoring

The hormone profiles produced by Corrie and Greedy up until the time of prostaglandin administration were similar to those widely reported during pregnancy (Holtan et al., 1975 ; Nett et al., 1975). The cross reaction between eCG and the LH radioimmunoassay observed in the present study coincided with the findings of Nett et al. (1975) who also noted a similar occurrence due to the similarity in structure between the two hormones.

The wide fluctuations in eCG concentrations between subsequent samples seen in the present study suggests an episodic secretion of eCG. However, Thompson et al. (1982) reported that eCG was secreted in a tonic, non-episodic manner and was constant over 24 hours. Any short term variations in that study were due to random assay variations rather than rapid fluctuations in eCG secretion, as shown by the trends not being repeatable when the samples in were reassayed. The samples in the present study would have to be reassayed to confirm that the fluctuations observed were real and not due to assay variation. However, since these samples were days rather than hours apart, they may represent long term variations in secretion rather than short term ones. Squires et al. (1980) also demonstrated fluctuations in LH between daily samples.

It has been suggested that this procedure may be useful in late pregnancy to establish the well being of a foetus in a high risk pregnancy by assessments such as foetal size, heart rate, movement, placental thickness, allantoic fluid volume estimation and appearance etc. (McGladdery, 1995). There is still much work to be

done in this area to establish normal ranges but it will probably prove to be a very useful non-invasive technique.

General Discussion

The main aim of this study was to produce equine embryos of different gestational ages for inclusion in a study of the early embryological development of the equine eye. Since there are many factors involved in the successful production of pregnancy in the mare, many aspects of reproduction in the mare were by necessity also included in this study.

The first section of this study dealt with the reproductive cycles of the mares and although most of the findings corresponded with previously published data, it did highlight the range of variation seen within even a small group of mares such as this. It also demonstrated the need to combine information from various sources in order to determine the reproductive state of each of the mares at a given time, as well as some of the problems associated with not treating mares as individuals, the use of group teasing and the performance of some of the examinations by inexperienced operators. In general, the information obtained from daily teasing, rectal palpation, ultrasound examination and plasma progesterone and LH assays, correlated well with each other and also with the reproductive stage of the mare. However, some of the mares did display behavioural anomalies and further work would have involved investigation of these in more detail.

It would be interesting to investigate the role of prostaglandin in the occurrence of both shortened and lengthened dioestrous intervals by measuring plasma concentrations of PGFM, the main metabolite of PGF2a, during the luteal phases of the individual mares in the present study which repeatedly displayed these anomalies. Its measurement throughout prolonged dioestruses may also determine its role in the termination of these phenomenon. An investigation into the relationship between spontaneous prolongation of the CL and the occurrence of early embryonic death would also be of interest as two mares in the present study exhibited both on different occasions.

Likewise, measurement of plasma oestrogen concentrations throughout Lady's silent and erratic oestruses would be of interest. This may have given an indication as to whether they were caused by insufficient oestrogen production, incorrect timing of oestrogen production or poor sensitivity of Lady's behavioural centres to the hormone.

Increasing the frequency of ultrasound examinations during oestrous periods would have allowed more sequential evaluation of the appearance of preovulatory follicles

and may have allowed the actual timing of ovulation to be predicted more accurately.

The information obtained from the LH assay in this study was limited due to the infrequency of the blood sampling. Further work would have increased the frequency of sampling markedly during oestrus, to allow the LH profile detected by this particular assay in association with ovulation during both normal and silent oestruses, to be determined The measurement of LH concentrations throughout dioestrous intervals in the mares known to have a high incidence of dioestrous ovulations would determine whether this assay could detect an LH peak associated with such ovulations. Therefore, this would indicate whether the absence of these peaks during the present study was artefactual due to poor timing of the sampling or was due to LH peaks either not occurring, or not being detectable using this particular assay.

Greedy's normal cyclic behaviour in the presence of a granulosa cell tumour did not correspond with previous reports. This may be due to most granulosa cell tumours being diagnosed as a result of some form of abnormal reproductive behaviour such as nymphomania or virilism, or the failure of the mare to become pregnant. Initially Greedy did present with these signs but unlike all the mares in previous reports which were treated by ovarioectomy, due to financial constraints at the time, Greedy's tumour was left intact and she was used for undergraduate teaching.

Her tumour had obviously become non-functional, allowing her to return to normal cyclicity and become pregnant but due to the study ending, it was not clear how the administration of prostaglandin to terminate the pregnancy had affected the functional state of the tumour. It would have been of interest to have monitored Greedy's plasma oestrogen and testosterone concentrations along with her progesterone and LH/eCG during the period of her pseudopregnancy and subsequent prolonged oestrus, to determine if the tumour had returned to a functional state. If so, it may have been responsible for the behaviour she displayed during this period.

Poppy's display of 'mouth clapping' during group teasing over the summer was novel and there has only been one other report of it occurring in a mare (Ginther, 1992).

The second chapter investigated the accuracy and reliability of the commercially available Target Progesterone Assay Kit (Biometallics, Princeton, NJ, USA) when

compared with the Coat-A-Count Progesterone Radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA) which produced the results used in Chapter One. The accuracy and reliability of the Coat-A-Count assay was demonstrated by inter and intra assay variations of less than 10% and the results it produced corresponded well with the mares' reproductive behaviour as determined by the other methods used in the present study. These were therefore accepted as the correct progesterone results to which the Target results were then compared.

Although the Target kit was most appropriate for use in a veterinary practice situation, due to the speed and ease with which it was performed and its suitability for single samples, it was not found to be as reliable or accurate as the Coat-A-Count assay. The most accurate interpretation of the Target results were in a qualitative manner, giving either a high or a low concentration, rather than in the semi-quantitative manner which the manufacturer's intended.

Further work in this area would have investigated ways to improve the accuracy of the Target kit. There was little information provided with the assay regarding its components and the reasoning behind its methodology and it was possible that it had been adapted from an assay used to determine progesterone concentrations in a different species. Since the kit had a tendency to produce results higher than those of the Coat-A-Count, manipulation of its procedure may have produced more accurate results. For example, decreasing the plasma volume added or its contact time with the antibody or increasing the enzyme volume added or its contact time with the antibody may have had an effect on the outcome and further work would have investigated this.

The repeatedly low results obtained for one mare in particular suggested some matrix effect was occurring and further investigation of this would have been of interest to determine the possibility of the existence of particular mares for which this assay would be of no use.

The manufacturers recommended the use of the Target kit to detect progesterone insufficiency in early pregnancy. It would have been of interest to monitor mares' plasma progesterone concentrations throughout pregnancy to determine if the Target kit detects the placental progestagens produced during the last half of gestation. This would reflect the specificity of the assay and since the Coat-A-Count assay demonstrated high specificity for progesterone, it may be that the Target kit could identify increased progestagen concentrations in late pregnant mares while the Coat-A-Count could not. Using prostaglandin to partially lyse luteal tissue during the ovarian dependant stages of pregnancy would determine whether the Target kit

detects such declines in circulating concentrations and therefore if it is of use in determining progesterone insufficiency as the manufacturers claim.

Chapter three involved the artificial insemination of mares and demonstrated that good pregnancy rates can be achieved without an intensive approach being necessary. Good record keeping allowed accurate results to be calculated and problems with the procedures to be identified early.

There were few studies with which to compare the results of this chapter since most previous studies have evaluated fresh semen or chilled semen stored for less than 24 hours before insemination., The finding that the age of the semen and the interval between insemination and ovulation did not have any effect on the outcome of the insemination in the present study was surprising but more inseminations would need to be performed to confirm this. However, the inaccuracy of %PMS and %Live in predicting the outcome of the insemination was in keeping with the findings of other studies.

Further work in this area would involve the evaluation of chilled, extended stallion semen in a manner similar to that performed for fresh semen by Wood et al. (1990). This would involve the use of standardised procedures, insemination volumes and concentrations and the performance of a sufficient number of inseminations to be statistically significant. Inseminations would be carried out at various intervals before ovulation and with different ages of semen to determine the effects these would have on the resulting pregnancy rates. The same criterion would then be examined using semen from different stallions to determine the effect of stallion variation in the outcome and therefore the possibility that some stallions may be more suitable than others for this type of procedure. This information would be particularly useful since many insemination programmes now involve the use of transported chilled semen.

Chapter four discussed the outcome of all the pregnancies achieved in the study and whether embryos were successfully retrieved. There were no previous reports regarding the retrieval of embryos via the transcervical route this late in pregnancy, with which to compare the findings of the present study.

An insufficient number of collections were performed to evaluate the effect of such a procedure on the subsequent fertility of the mare and also the effect of individual mare variations in uterine tone and shape or the effect of the age of the embryo on

the subsequent retrieval rate. This would be the subject of further work to allow modification of the technique to improve retrieval rates and reduce any deleterious effects on the mare.

Many aspects of reproduction in the mare are incorporated into this study and there are many areas where further investigation would be merited. However, the main aim was to develop a technique to retrieve embryos from mares at day 12-28 of gestation for inclusion in another study investigating the early embryological development of the equine eye. The successful retrieval of four embryos between these gestational ages indicated that the study was a success.

Appendix A - Assay Procedures

All plasma samples and assay components were warmed to room temperature and mixed thoroughly prior to use.

1/ Coat-A-Count Progesterone Radioimmunoassay.

a/ Two plain (uncoated) tubes were labelled T (total counts) and another two NSB (non-specific binding).

Fourteen progesterone antibody coated tubes were labelled A to G in duplicate. Additional coated tubes were labelled in duplicate for each of the unknown and control samples.

b/ 100µL of zero standard was pipetted into the NSB and A tubes. 100µl of each of the other standards, the unknown samples and the control samples were pipetted into the correspondingly labelled tubes.

The standards used in each of the assays were as follows:

Calibrators	Assay 1-2	Assay 3-5
A(MB)	0	0
B	0.1	0.5
C	0.5	1.0
D	2.0	2.0
E	10	5.0
F	20	10
G	40	20

The standards were altered in assay 3-5 to produce a more accurate curve in the low concentration range.

The manufacturer's results demonstrated by using parallelism that the assay maintained good linearity under dilution so the 1.0ng/ml standard was prepared by diluting the 2.0ng/ml standard 1:1 with zero standard and the 5.0ng/ml by diluting the 10ng/ml standard 1:1 with zero standard.

c/ 1ml of I^{125} labelled progesterone was added to every tube with no more than 10 minutes elapsing between the first and last tubes.

d/ All the tubes were incubated at room temperature for a minimum of 3 hours.

e/ Using a foam decanting rack, all except the T tubes were inverted and allowed to drain for 2-3 minutes until all visible moisture had been removed.

f/ The tubes were then counted for 1 minute each in a gamma counter.

2/ Target Progesterone ELISA

a/ 4 drops of the unknown sample was placed in the centre of the tub.

b/ After 2 minutes, 3 drops of enzyme conjugate from the red cap bottle was added to the tub.

c/ After exactly 1 minute, the tub was filled with wash solution from the white cap bottle and this was allowed to drain completely into the tub.

d/ Fresh substrate solution was prepared in the empty blue cap mixing bottle by adding one dropper filled to the mark each of substrate A and substrate B. This was mixed thoroughly before 3 drops were added to each tub. The substrate solution was then discarded and the bottle rinsed.

e/ After 7 minutes, the colour change in each tub was recorded.

3/ LH Double Antibody Radioimmunoassay

a/ Assay buffer was made by mixing 2.5g of B.S.A, 1g of sodium azide, 500µl of triton X-100 and 100ml of 0.5M phosphate buffer pH 7.4. This was made up to 1

litre and stored at 4°C. If not used within 1 week it was discarded and fresh solution made up.

b/ The standards for the assay were prepared using purified pituitary equine LH kindly donated by Dr. A. F. Parlow, Director of the Pituitary Hormone and Antisera Centre, Harbor-UCLA Medical Centre, Torrance, CA., USA. The concentration of the cold standard was 100 ng/ml and it was stored in 300µl aliquots.

To one aliquot of standard, 300µl of assay buffer was added to give 600µl of 50 ng/ml standard solution. Six tubes were labelled 25, 12.5, 6.25, 3.12, 1.56 and 0.78 and to each was added 500µl of assay buffer. 500µl of the 50 ng/ml standard solution was added to the tube marked 25 and mixed well. 500µl of the resulting solution was added to the tube marked 12.5, mixed and then this procedure repeated until all the tubes contained 500µl of different concentrations of standard with the exception of the 0.78 tube which contained 1000µl.

c/ Two tubes were labelled T (total counts) and two NSB (non-specific binding). Another twelve tubes were labelled for each of the standards in duplicate along with additional ones labelled in duplicate for each of the unknown and control samples.

d/ 100µl of assay buffer was pipetted into the NSB and zero standard tubes. 100µl of each of the standards, the unknown samples and the control samples were pipetted into the correspondingly labelled tubes.

e/ 100µl of LH free plasma (foal plasma) was added to each of the standards and 100µl of assay buffer was added to each of the unknown samples, the control samples and the NSB tubes.

f/ The first antibody was rabbit anti-rat LH (CSU120), kindly donated by Dr. G. D. Niswender, Colorade State University, Fort Collins, Colorado 80523. It was used at a dilution of 1:12 000 and 100µl was added to all the tubes except the TC and NSB tubes. The tubes were then incubated overnight at 4°C.

g/ 100µl of I^{125} labelled ovine LH was added to all the tubes which were again incubated at 4°C overnight.

f/ 100µl of the second antibody, which was donkey anti-rabbit, used at a dilution of 1:200, and 100µl of normal rabbit serum was added to all of the tubes except the TC tubes. Both of these were kindly donated by the Scottish Antibody Production Unit, Victoria Hospital, Glasgow, Scotland. The tubes were then incubated overnight at 4°C.

g/ The tubes were spun in a refrigerated centrifuge at around 2000 rpm for 30 minutes. They were then placed in an ice bath to keep them cool and the supernatant aspirated before being read in a gamma counter.

APPENDIX B - Results from Coat-A-Count samples which were assayed more than once.

MARE	SAMPLE DATE	RUN 1	RUN 2	RUN 3
GEMMA	05-Apr	0.05	0.01	0.01
GREEDY	11-Jan	0.42	0.18	
GREEDY	08-Mar	3.29	3.67	
GREEDY	16-Mar	0	0	
JENNY	21-Oct	6.48	6.9	
JENNY	07-Feb	3.86	3.88	
JENNY	16-Mar	7.17	7.17	8.7
LADY	01-Nov	2.54	2.9	
LADY	24-Nov	1.53	1.87	
LADY	08-Mar	0.12	0.08	
LADY	16-Mar	2.95	2.96	2.7
MEG	16-Mar	0	0	0.05
MISTY	05-Feb	16.35	15.6	
POPPY	06-Oct	3.98	3.6	
POPPY	23-Mar	6.04	7.53	6.97
POPPY	05-Apr	0.41	0.27	0.48
SUZY	06-Dec	0.27	0.27	
SUZY	14-Dec	7.09	9.48	
SUZY	20-Dec	0.9	0.8	
SUZY	19-Jan	1.34	1.51	
SUZY	30-Jun	7.5	6.6	
SUZY	26-Jun	4.6	4.1	
SUZY	19-Jun	9	6	

APPENDIX C - Target and Coat-A-Count results which were not in agreement

COAT - A - COUNT	TARGET
0.10931	INT
0.17379	INT
0.1775	INT
0.3823	INT
0.44387	INT
0.7478	INT
0.83279	INT
1.39	HIGH
1.5391	HIGH
2.8416	HIGH
3.5588	HIGH
3.5938	HIGH
3.6261	HIGH
3.6864	HIGH
3.8464	HIGH
3.8595	LOW
4.3123	LOW
6.48	LOW
9.1347	INT

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