EFFECTS OF FOLLICULAR ASPIRATION ON THE BOVINE OESTROUS CYCLE

by

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DECLARATION

I hereby declare that this work is original, was carried out by the undersigned, except where acknowledged, and has not previously been presented in any form to any Institution for an award of a degree.

Georgios Amiridis

ΑΦΙΕΡΩΣΗ

Στους γονείς μου Σωκράτη και Γεωργία - τους ανθρώπους που με εισήγαγαν στη ζωή και μόχθησαν και στερήθηκαν για τη μόρφωσή μου

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SUMMARY

Increasing numbers of reports in the past eight years have described oocyte recovery from cattle. Most of these studies focused on the oocyte recovery rate and the quality of the harvested cumulus oocyte complexes. The present thesis deals with the impact of the ultrasound guided follicular aspiration on the hormonal profiles of the cow as well as with the effects of the applied technique on the follicular dynamics and the fate of the aspirated follicles.

Once weekly follicular aspiration did not extend the interoestrous intervals of the cows, provided the last aspiration was not performed after day 18 of the oestrous cycle. In the present study the overall oocyte recovery rate (30.5%) was comparable with the average found in the literature. An increased number of follicles emerged after aspiration regardless of the stage of the cycle. Plasma oestradiol concentration decreased dramatically soon after aspiration and was maintained at low levels dependent on the stage of the oestrous cycle. Ablation of all ultrasonically visible follicles resulted in a small increase in plasma LH concentration regardless of the stage of the oestrous cycle, but this increase was not statistically significant from either the pre-aspiration concentrations or from that measured in the control animals.

Selective aspiration of the dominant follicle resulted in a significant decrease in plasma oestradiol concentration, and a significant increase in plasma FSH concentration at all three stages of the luteal phase. However, ablation of the dominant follicle did not appear to affect LH secretion very significantly.

Repeated aspirations during the early luteal phase resulted in the formation of an accessory luteal structure, within seven days of aspiration. The accessory structure had the macroscopical and histological appearance typical of a corpus luteum and contained higher amounts of oestradiol and lower amounts of progesterone than the cyclic corpus luteum. Furthermore, aromatase activity and FSH receptors were detectable only in the accessory structure.

The methodology used in the present research offers an alternative model for study of a: the interactions between ovarian steroids and the pituitary gonadotrophins, and b: the role of the dominant follicle at different stages of the oestrous cycle.

Appearance of an accessory luteal structure, although of very low incidence, indicates that under a certain hormonal milieu follicular rupture is sufficient to induce luteinisation without an LH peak.

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Chapter 1

INTRODUCTION

1.INTRODUCTION

1.1 ANATOMY OF THE REPRODUCTIVE SYSTEM

The reproductive system of the female animal is composed of the external and the internal genital organs. The external genitalia consists of the vulva, the clitoris and the vagina, whereas the ovaries, the oviducts, the uterus and the cervix form the internal genitalia.

1.1a. Vulva and clitoris.

The vulva separates the vaginal vestibule from the outside world. It consists of two vertical lips (labia minora) dorsally and ventrally connected. The vulva changes in appearance with maturity, as well as, with the stage of the oestrous cycle, being swollen and moist during oestrus and soft and dry during dioestrus. The clitoris is located in the floor of the vulva. It is the vestigial female analogue to the male penis having a similar structure. It is composed of erectile tissue covered by stratified epithelium and being supplied with sensory nerve endings.

1.1b. The vagina.

The vagina represents the tubular connection between the vulva and the cervix. The caudal portion of the organ serves both a reproductive function and as the external urethral orifice. The vaginal wall consists of a surface epithelium, mucosa, muscular coat and serosa. The muscular layer is not very developed, compared to more cranial regions of the tract. The vaginal epithelium is stratified with very few mucus cells which are scattered around, particularly in the area next to the cervix. Although the vagina contains no glands, the vaginal wall is kept moist due to fluid

derived from different sources such as transudate from the vaginal wall, vulvar secretions and cervical mucus. Because of the communication with the outside world, the vaginal flora is a dynamic mixture of anaerobic and aerobic micro-organisms with new strains constantly being introduced from the outside via the vulva.

1.1c. The cervix.

This is the sphincter that separates the external from the internal genitalia. In the cow, the cervix protrudes slightly into the vagina. The organ is characterised by a thick wall comprising transverse folds (or annular rings) which restrict the cervical lumen. Under the influence of progesterone, during the luteal phase, the cervical cells produce a thick, sticky mucus that seals the lumen, forming a definite barrier between the uterus and the vagina. Cervical mucus is less viscous during oestrus and hangs as a string of clear mucus from the vulva. As part of its barrier function, the cervix serves to facilitate sperm transport and to select viable from non viable spermatozoa. It may also act as a sperm reservoir, since sperm viability in the cervix is relatively prolonged compared to other parts of the reproductive tract (Hafez, 1992).

1.1d. The uterus.

The bovine uterus consists of two horns and a very short body. Both sides of the uterus are attached to the pelvic and abdominal wall by the broad ligaments. The uterine wall comprises three distinct layers, the endometrium, the myometrium and the perimetrium. The endometrial epithelial cells change in shape and function during the oestrous cycle. The endometrium contains many coiled uterine glands, the structural complexity and secretory activity of which changes with the steroidal enviroment during the oestrous cycle. During oestrus the uterine fluid provides an

ideal environment for survival and capacitation of sperm. After fertilisation and before implantation it favours the cleavage of the early blastocyst, and the nourishment of the early embryo for some weeks preceding implantation (Pineda, 1988). Such changes in endometrial function rely on cyclic priming by oestrogens, followed by progesterone dominance and changes in hormone receptors and enzyme expression. The myometrium is the muscular layer and is responsible for uterine contractility. Myometrial activity is maximal around oestrus and minimal throughout dioestrus in the non pregnant cow, this condition being maintained until term if pregnancy ensues. The perimetrium represents the outer layer of the internal genitalia, being continuous with the peritoneum. The blood supply to the uterus varies according to the stage of the cycle, and reaches its zenith as pregnancy progresses. Functions of the uterus include the conveyance of sperm towards the fertilisation site in the oviducts, regulation of corpus luteum longevity and provision of a favourable environment and life support for the embryo and fetus and, finally, fetal expulsion at parturition.

1.1e. The oviducts.

These are the organs that connect the ovary to the uterus. The oviduct can be divided into three segments. The funnel-shaped end or infundibulum which embraces the ovary, the main tubular part, called the ampulla and the portion of the organ connected to the uterus called the isthmus. The infundibulum may envelop the ovary and is lined with ciliated epithelium and edged with fine extrusions called fimbriae. These serve to transport the egg from the surface of the ovary through the infundibulum to the ampulla where fertilisation occurs. Histologically the tubular wall consists, from inside to outside of: a mucous membrane with secretory function, a muscular layer and a serous coat. The mucosa is made of primary, secondary and tertiary folds and is covered in an epithelium of

columnar cells containing ciliated and non-ciliated cells. The percentage of ciliated epithelial cells decreases from the infundibulum towards the isthmus. The movements of the cilia in combination with the contractions of the muscularis, convey the egg and the spermatozoa in opposite directions at the same time.

1.1f. The ovary.

The ovary is the female gonad assigned with two functions: (a) the production of oocytes and (b) the secretion of various steroid (progesterone, oestrogens) and protein (inhibin, activin, follistatin) hormones. In the cow, the ovary weighs 10-20 g and has an almond or ovoid shape, unless large follicles or a corpus luteum protrude from the surface. The ovary is supported by the mesovarium, a part of the broad ligament. Structurally, the ovary consists of the peripheral parenchyma, where gametogenesis takes place and the central zona vasculosum consisting of the medulla and the stroma. The ovarian medulla is composed of loose connective tissue and is crossed by several nerves, lymphatics, blood vessels and smooth muscles. Histologically the ovarian stroma comprises poorly differentiated embryonic mesenchymal like cells, which undergo morphologic changes during reproductive life. Superficially the ovary is covered by an epithelium of cuboidal or low columnar cells, known as the germinal epithelium. Beneath the germinal epithelium lies the tunica albuginea, which consists of dense fibrous connective tissue. The ovary receives blood from the ovarian artery, which branches directly off the aorta. In the cow, the ovarian vein is large compared to the corresponding artery. Perhaps this is explained by the fact that a fraction of uterine blood circulates to the ovary where it is important in regulating luteal function. The blood flow varies during the different stages of the cycle, being highest in the luteal phase and decreasing at luteolysis to a minimum around ovulation.

Gonadal differentiation into an ovary takes place at about 45 days of gestation (Marion & Gier, 1971) when primordial cells together with supporting epithelial cells form the cortical sex cords. After that stage, the primordial cells multiply and are transformed to oogonia. Oogonia enter the meiotic prophase and differentiate into primary oocytes. This stage of development ends before or just after birth. The nucleus of the primary oocyte remains arrested in the dictyate stage until follicular maturation resumes after puberty (McDonald & Pineda, 1989). There are three distinct categories of follicles; primordial, growing and vesicular (Erickson, 1966). The primordial follicle consists of an oocyte surrounded by a single layer of flattened granulosa cells. There are some indications that parenchymal theca cells are also involved at this stage of development (Hirshfield, 1991). At this stage, the zona pellucida appears (Baker, 1971), initially as a thick extracellular coat that surrounds the primary oocyte, later increasing in width as the oocyte develops. Later stage growing follicles can easily be distinguished from primary follicles, by the presence of many layers of cuboidal granulosa cells (Hulsof, 1992). By day 270 of pregnancy, vesicular follicles can be seen in fetal calf ovaries (Erickson, 1966). Third category vesicular follicles consist of a primary oocyte, several layers of granulosa and thecal cells, and a large fluid-filled cavity (antrum). In the neonatal heifer ovary, antral follicles are present. It is not clear whether follicular growth and regression occurs in waves in young calves, but there is evidence that this happens in prepubertal heifers (Evans et.al., 1992). The time taken for a small antral follicle (2 mm) to reach the preovulatory size is relatively short, perhaps five to six days (Savio et al.,1993). During this growth phase granulosa cells proliferate and differentiate into two subpopulations: the first surrounds the follicular wall in contact with the basement membrane and the second forms the cumulus cells which enclose the oocyte. Gap junctions are observed between the two types of granulosa cells and between the oocyte and the cumulus cells. This type of junction

seems to play an important role in terms of transportation of small molecules, ions and nutrients from the basement membrane towards the antrum. The follicular wall comprises two layers of theca cells together with granulosa cells. The outer layer of theca cells (the theca externa) is formed by myoid-type cells. These cells contain actin and myosin which are believed to play a role in the follicular contractility. The theca interna layer is highly vascular and its cells are rich in fibrocytes and epitheloid cells, which contain granules and cytoplasmic organelles in an increasing number as the follicle matures.

The corpus luteum (CL) is a transient endocrine gland with limited lifespan in the cyclic non pregnant animal. Following ovulation, there is haemorrhage into the follicular cavity and the blood clots. This and the wall of the ruptured follicle, now devoid of the oocyte and the follicular fluid, is referred to as corpus haemorrhagicum. The blood clot is the substrate for the quick proliferation and differentiation of the granulosa and theca cells, which give rise to luteal cells. Histologically, there are two types of luteal cells, large and small. It is believed that the large luteal cells originate from the granulosa cells and the small ones from the theca interna cells (Alila & Hansel, 1984). The growth of the CL is rapid with the maximum size being attained by days 14-15, after which it degenerates rapidly in non-pregnant cycles. The appearance of the CL changes according to the stage of the cycle. Between days 1 and 4 its surface is red and its diameter varies from 0.5-1.5cm. Between days 5-10 the colour becomes red or brown with visible vasculature on its surface and it typically has a diameter from 1.6-2cm. Between days 11-17 the colour changes to yellow or orange without any red remaining. On days 18-20 there is no visible vasculature and the CL colour varies from orange to brick red (Ireland et al.,1980). If a viable embryo is not present in the uterus, the CL undergoes degeneration and regresses.

1.2. Oestrous cycle

The oestrous cycle refers to the functional and structural changes which take place in the reproductive system of the female animal between two succesive oestrus periods and ovulations. The length of the oestrous cycle averages 21days in cows and 20 days in heifers, with a normal range of 18-24 days. The oestrous cycle is conventionally divided into four phases: proestrus, oestrus, metestrus and diestrus.

Proestrus.

Proestrus is the 2-3 day period preceding oestrus i.e.generally from days 19-21 of the cycle. At this stage, the cow shows increased sexual activity and may ride other cows or assess their receptivity by sniffing them and resting her chin on their loins, but she never stands to be mounted. On the ovary one, or rarely two, preovulatory follicles can be detected and the CL of the previous cycle has already been regressing for several days. Both internal and external genitalia show increased mucosal vascularity associated with turgidity and enhanced activity of uterine cervical and vaginal secretory cells.

Oestrus.

This is the period of the sexual receptivity and its manifestation is the only accurate external criterion for the determination of oestrous cycle length (Arthur et al., 1993). The cow is restless and very active but will stand to be mounted only during this stage. During oestrus, the preovulatory follicle reaches its maximum diameter and secretes rising levels of oestrogens which induce behavioural oestrus. Copious transparent mucus is secreted which may hang from the vulva and adhere to the tail and the flanks. The cervix is relaxed and dilated, so that one or two fingers can be inserted into the cervical os. The surge in plasma concentrations of the

gonadotrophin hormones occurs during this stage, bringing about ovulation and the formation of a new corpus luteum.The duration of oestrus varies according to the breed, management and environmental factors (Allrich, 1993).

Metoestrus.

This is the 2-3 day period immediately after oestrus during which ovulation takes place, generally about 10-12 hours after the end of the standing heat or some 24 hours after the LH surge (Bernard et al., 1983). Other events include formation of the new CL from the granulosa and theca cells of the ruptured follicle. Metoestrus is characterised by declining secretory activity of the uterine, vaginal and cervical glands along with a rapid reduction in vascularity of the vagina and the cervix. Sometimes a bloody, vaginal mucous discharge occurs which if observed, confirms metoestrus.

Dioestrus.

Dioestrus is the period of the cycle between oestrus but owing to the brevity of pro-and metoestrus in the cow, this generally equates to the period of progesterone dominance. The CL reaches its maximum size by day 7 and secretes large amounts of progesterone. A periodic emergence of follicles starts, with some selected follicles reaching almost 2 cm in diameter but failing to ovulate. The uterus is soft with very little contractility, the cervix is firmly constricted and the endometrium is covered by a meagre secretion from the uterine glands. This phase lasts from day 3-4 to day18-19 of the cycle.

1.2.1 Oestrus synchronization

The use of animals with synchronized cycles is very common in experimental and practical husbundry breeding protocols. In addition, oestrus synchronization of both donor and recipient is critical for success in embryo transfer programmes (Ashworth 1992). Synchronisation of oestrus in a group of cows may be achieved by several means including prostaglandins (PG); progestagen ear implants (Crestar); intravaginal progesterone-releasing devices with an attached oestradiol benzoate capsule (PRID); or controlled internal drug release (CIDR) devices.

PGs are given intramuscularly either as single or double injections 10-12 days appart. PGs are effective in oestrus induction between days 5-15 of the cycle when the CL is responsive. The double injection protocol is more effective than the single injection since it catches animals which failed to respond to the first injection and 80-90% of the animals are in oestrus within 48 to 72 hours of the second injection (Diskin & Sreenan 1993).

PRIDS are inserted in the vagina for 12 days. They can be regarded as luteolytic and progestational and are effective regardless of the stage of the cycle. Crestar contains norgestomet and insertion is normally accompanied by a luteolytic injection of oestradiol valerate. The implant remains in place under the skin of the ear for 9-10 days and it is effective at all stages of the cycle (Tregaskes et al., 1994). Progestagens induce an oestrus response between 24-48 hours after their removal in 85-95% of the treated animals (Tregaskes et al., 1994). However, combination of progestagens and prostaglandins is the method of choice for various research programmes. This regimen comprises the insertion of either PRID or CIDR or Crestar and a luteolytic PG injection administered two days before the removal of the progestagen device (Sinclair et al., 1992; Broadbent et al., 1992).

1.3 Endocrinology of the oestrous cycle

The cascade of structural and functional changes in the reproductive tract that take place during each oestrous cycle is regulated by interactions between a variety of hormones, but mainly those of the hypothalamus and the hypophysis (pituitary gland), the ovary and the uterus. In some species, the pineal gland has been demonstrated to play an important role in terms of reproductive seasonality, but this does not seem to apply for the bovine.

1.3.1 Hormones of the hypothalamus.

Several hormones have been found to be synthesized and secreted by the hypothalamus. All affect the function of the hypophysis and the list includes gonadotrophin releasing hormone, thyrotrophin releasing hormone, growth hormone releasing hormone, somatostatin and corticotrophin releasing hormone, (Hafez 1992). The hypothalamic hormone most directly related to reproduction is the gonadotrophin releasing hormone (GnRH). GnRH is a decapeptide, which is stored in the medial basal hypothalamus. Specific hypothalamic neurones respond to both neural and hormonal signals and synthesize GnRH, which is secreted into the hypophyseal portal system for transfer to the anterior pituitary, where it stimulates LH and FSH synthesis and release (Schally et al., 1971). Regulation of pituitary GnRH receptor synthesis varies between species and at different stages of the oestrous cycle but maximum receptor numbers are observed prior to the LH surge (Marian et al.,1981). It is believed that GnRH receptor synthesis in the pituitary is controlled by the ovarian hormones (Marian et al.,1981). More specifically, oestradiol and inhibin appear to enhance the number of GnRH

receptors in ovine pituitary cell cultures (Gregg & Nett, 1989; Laws et al., 1990), while progesterone has the reverse action (Laws et al.,1990). However, Wang et al.(1989) showed that inhibin down-regulated the synthesis of the GnRH receptor in rat pituitary cells.

Although considerable research has been carried out concerning the pattern of GnRH secretion in the ewe (Clarke et al., 1987 and 1989; Karsch et al., 1987 and 1992; Karsch 1995; Laws et al., 1990), this has been difficult to achieve in the bovine (Peters 1985) but it seems reasonable to assume that the pattern of GnRH secretion in sheep and cattle would be similar.

Thus, increasing plasma oestradiol concentration during the late luteal phase is associated with a decrease in GnRH pulse amplitude and increased pulse frequency. Suppression of the GnRH pulse amplitude is only transient though and a very strong surge of GnRH occurs which probably induces the LH surge. However, the GnRH surge lasts about ten hours longer than than the LH surge (Karsch et al.,1992). It is postulated that during the follicular phase, high levels of oestradiol cause activation of the hypothalamic GnRH neurosecretory system prior to ovulation (Clarke and Cummins 1985, Caraty et al., 1989). The mechanism, by which oestradiol exerts this powerful feedback action on the hypothalamus remains enigmatic, since hypothalamic secretory neurones do not appear to contain oestrogen receptors (Karsch et al., 1995).

1.3.2 Hormones of the pituitary.

1.3.2.1 The gonadotrophins

The adenohypophysis (anterior pituitary) mainly consists of two types of secretory cells, basophils which include the gonadotrophs and acidophils which include the lactotrophs. Gonadotrophins follicle stimulating hormone (FSH) and luteinizing hormone (LH) are synthesized in the gonadotrophs where they are stored in the form of cellular granules. Lactotrophs synthesize, store and secrete prolactin. In addition to these hormones, the adenohypophysis also secretes growth hormone (GH), corticotrophin (ACTH) and thyrotrophin (TSH) (Hafez, 1993).

FSH and LH are the main gonadotrophins. Prolactin has a gonadotrophic role in the rat but it seems unlikely to have a similar action in the cow (McDonald & Pineda 1989). Both FSH and LH are glycoproteins comprising α and β subunits with combined molecular weight of approximately 30000 daltons and half lives of 35 min for LH and 300min for FSH (Bousfield et al., 1994; McDonald and Pineda 1989).The α subunit is identical for both gonadotrophins within a species, while the β subunit is unique for each hormone and specifies the biological activity (Ward et al.,1991).

GnRH appears to be the primary stimulus for both FSH and LH synthesis by the gonadotrophs. However, GnRH causes only a transient release of gonadotrophins, since the secretory cells become insensitive to prolonged GnRH stimulation (Davidson et al., 1994). Ovarian steroids provide a negative feedback control of gonadotrophin secretion by acting at the hypothalamic level to suppress GnRH release (Fink 1988). Moreover, the mode of GnRH secretion seems to ensure the selective secretion of the gonadotrophins, with a pulsatile signal preserving the responsiveness of the basophils while the amplitude and the frequency of the

GnRH pulses determine FSH or LH secretion(McIntosh & McIntosh, 1983, Haisenleder et al., 1993). An earlier report by Foster et al. (1980) also showed selective gonadotrophin secretion after GnRH challenge in cows. Nevertheless the mode of GnRH secretion appears not to be the sole modulator of differential LH and FSH secretion and it has been found that the proportion of basophils that contain both gonadotrophins varies from 40-75%. The FSH β subunit is undetectable in 5-35% of cells (Childs et al., 1992), although other workers put this at 1% (Dada et al.,1983), whereas the LH β subunit is undetectable in at least 11% gonadotrphs (Childs et al., 1992). This supports the concept of the existence of two distinct gonadotroph subpopulations, that respond selectively to either GnRH (Denef et al., 1980) and/or other stimuli for the gonadotrophin secretion (Data et al., 1983, Katayama et al., 1991). Even the secretory granules might contain one or both gonadotrophins (Kile & Nett, 1994). In an attemt to deprive the gonadotrophs of the pulsatile GnRH signal the pituitary gland was transplanted under the renal capsule in rats (De Paolo et al., 1992). Under these conditions the gonadotrophs secreted very small amounts of LH but substantial amounts of FSH. This phenomenon provides the basis for the idea that FSH secretion is independent of GnRH. Further indirect evidence of this is the fact that the preovulatory surge of the two hormones is not exactly contemporaneous.

1.3.2.2 Control of gonadotrophin secretion.

Both gonadotrophins are secreted in pulsatile manner with frequency and amplitude varying according to the stage of the oestrous cycle (Schallenberger et al., 1985). Gonadotrophin secretion is under the influence of various positive and negative feedback controls acting between the central nervous system and the hormones of the hypothalamus and the ovary. As mentioned above although FSH

and LH are both released by the same releasing hormone, the pattern of secretion of each is such that one could assume that separate mechanisms modulate the secretion of each hormone (Foster et al., 1980). Wolfe et al., (1992) demonstrated that low doses of oestradiol had little effect on LH pulse amplitude but enhanced mean circulating FSH concentration. Higher doses suppressed the amplitude of plasma LH secretory episodes and the mean concentration of FSH. Treatment of ovariectomized heifers and ewes with oestradiol suppresses the LH release for a period lasting from 12 to 20 hours and this is followed by a preovulatory- like LH surge (Bolt et al., 1990; Caraty et al.,1989). This oestradiol-induced LH surge can be blocked by the administration of progestagens (Beck & Convey 1977; Schoenemann et al., 1985; Bolt et al.,1990). The regulatory role of the gonadal steroids on gonadotrophin secretion therefore changes according to the length of exposure to the steroid (Fink, 1988). Experiments in the rat have revealed that progesterone exerts a positive followed by a negative feedback during the first hours of proestrus (Aiyer et al., 1974).

The inhibitory effect of progesterone on LH pulse frequency, without any effect on pulse amplitude, is well documented in many studies (Baird, 1978; Rahe et al., 1980; Ireland & Roche, 1982; Walters & Schallenberger, 1984; Stumph et al.,1993). Oestradiol, either alone or in combination with progesterone decreases both amplitude and frequency of LH. In addition, in ovariectomized ewes, oestradiol reduces the GnRH-induced secretion of LH (Tamanini et al., 1986). However progesterone-oestradiol combination has been found to be more effective in suppressing LH secretion in both heifers and ewes than oestradiol alone (Beck et al., 1976; Goodman et al., 1980). Since oestradiol stimulates the synthesis of pituitary GnRH receptors (Moss et al., 1981; Shoenemann et al., 1985) it seems unlikely that oestradiol exerts its inhibitory action on LH through the GnRH receptor. Ovariectomized heifers have been found to respond to exogenous GnRH

by secreting large quantities of LH regardless of the steroid pretreatment. In the same animals progestagens blocked the oestradiol-induced LH surge, presumably due to reduced secretion of endogenous GnRH (Bolt et al., 1990; Shoenemann et al., 1985). Presently it is well documented that the ovarian peptide inhibin selectively suppresses FSH secretion in many species (Tsonis et al., 1986; Findlay & Clarke 1987; Taya et al., 1991; Baird et al.,1991; O'Shea et al., 1991) and it is believed that oestradiol and inhibin synergistically suppress FSH secretion. This combined inhibitory feedback action on the pituitary is mediated through a decrease of FSH β subunit mRNA, which follows the inhibition of expression of the gene encoding the FSH β subunit (Mercer et al.,1987; Beard et al., 1989). Although oestradiol and inhibin are the main regulators of FSH secretion, the relative role of each hormone remains unclear. Results of studies investigating the separate effects of either inhibin or oestradiol are perhaps not very reliable, since the two hormones are never alone in the blood circulation. The model of Baird et al., (1991) suggests that inhibin suppresses FSH secretion in the long term while oestradiol contributes to acute regulation of FSH.

1.3.3 Ovarian hormones

The endocrine functions of the ovary include the synthesis and release of a variety of steroid and peptide hormones. The steroid hormones are progesterone, oestrogens and androgens,. The peptide hormones include inhibin, activin, follistatin, oocyte maturation inhibitor (OMI) and relaxin. Steroids and some of the peptides play key roles in the changes that take place in the genital tract and other parts of the body at different stages of the oestrous cycle.

1.3.3.1 Steroid hormones.

Cholesterol is a common substrate for all steroid hormones. The source of cholesterol can be either the blood, in which cholesterol circulates in the form of low and high density lipoproteins (LDL-HDL), or intracellular stores of cholesterol derived from *de novo* synthesis. Follicular granulosa and theca cells can take up cholesterol in both LDL and HDL forms. LDL binds to the cell membrane receptor and is internalized through coated pits. The lipoprotein is subsequently degenerated in lysosomes and the free cholesterol is either used for steroidogenesis or is stored in the form of cholesterol ester (Yoshiba & Veldhuis 1990). The process for HDL utilization is quite different since cholesterol is taken up without a complete internalization of the lipoprotein (Yoshiba & Veldhuis, 1990). There is much species and steroidogenic cell variation regarding of cholesterol source that is utilized (Gore-Langton & Armstrong 1988).

The first conversion of cholesterol takes place in the mitochondria. This is a catalytic reaction that results in pregnenolone production. Cytochrome P-450 side chain cleavage enzyme ($P450_{scc}$) catalyses this reaction (Waterman & Simson, 1985; Richards et al., 1987; Rodgers et al., 1987). $P450_{scc}$ is present in greatest concentration in the corpus luteum with less in the preovulatory follicle and least in the small antral follicles (Zlotkin et al., 1986). All subsequent stages in steroidogenesis take place in the smooth endoplasmatic reticulum.

A key step in progesterone synthesis is the conversion of pregnenolone to progesterone. This reaction is catalysed by the 3β-hydroxysteroid-dehydrogenase (3βHSD). This conversion starts in the theca cells (O'Shea et al., 1980), increases in magnitude in parallel with the degree of follicular maturation and is further increased in luteal tissue (O'Shea et al., 1980; Couet et al., 1990). In the theca cells

of the developing follicle, pregnenolone or progesterone are converted to androgens after the enzymatic expression of the 17α-hydroxylase cytochrome (P-$450_{17}\alpha$). This enzyme has been found in abundance in bovine antral follicles but is almost undetectable in the corpus luteum (Rodgers et al., 1986,1987). In granulosa cells, androgens are subsequently aromatized to oestrogens by the microsomal aromatase cytochrome P-450 (P-450_{arom}) (Rodgers et al., 1987; Voss & Fortune, 1993). The lack of this enzyme in the corpora lutea of domestic ruminants may expain the absence of oestradiol synthesis in this tissue (Savard, 1973). The mRNA levels of P-450_{arom} in the preovulatory follicle are positively related to the rate of follicular maturation but a dramatic decrease (almost 94%) levels occurs approximately 20 hours after the LH surge (Voss and Fortune 1993) indicating a loss of oestradiol biosynthetic potential.

1.3.3.2 Factors affecting steroidogenesis.

Theca and granulosa cells respond to gonadotrophin stimuli from very early stages of follicular development (Richards, 1980). At the preantral stage, theca cells contain only LH receptors, whilst granulosa cells possess only FSH receptors (Richards, 1980). During folliculogenesis, FSH induces the appearence of granulosa cell LH receptors. The theca cells respond to LH by activation of P-450_{scc} and 17α- hydroxylase enzymes, initiating the production of androgens. Results from *in vitro* studies led to development of the two cell model for oestradiol synthesis. according to which theca cells are stimulated by LH and produce androgens and these are utilized by granulosa cells for oestrogen synthesis in response to FSH stimulation (Evans et al., 1981; Armstrong et al., 1981; Gore-Langton & Armstrong 1988). Both FSH and LH increase the uptake of lipoproteins, the mobilization of cholesterol and the conversion of pregnenolone to

progesterone (Hsueh et al., 1984; Erickson et al., 1985). FSH enhances the steroidogenic performance of granulosa cells at a number of different sites. Culture of granulosa and theca cells *in vitro* causes spontaneous luteinization as revealed by a shift from oestrogen and androgen production to progesterone synthesis in both cell types. Treatment with gonadotrophins does not prevent such luteinization, but on the contrary, increases progesterone synthesis (Hsueh et al., 1984; Fortune & Quirk, 1988; Saumande, 1991). However, Bernston et al . (1995), demonstrated that treatment of follicular cells with very low doses of gonadotrophins prevents the luteinization *in vitro*. The authors concluded that progesterone production from granulosa cells is gonadotrophin dose dependent. The stimulatory action of LH on granulosa cells for progesterone secretion has been found to be amplified when cells are cultured in the presence of either HDL or LDL, with LDL being more effective (Yoshiba &Veldhuis, 1990; O'Shaughnessy et al.,1990).

Steroidogenesis takes place in the small and large cells of the corpus luteum. However, the two cell types respond in different ways to various stimuli. Small luteal cells in culture are extremely sensitive to LH administration, whereas large luteal cells are quite insensitive to the same dose (Alila et al., 1988; Farin et al., 1989), but can double progesterone production at higher doses (Hansel et al., 1991). Surprisingly, the natural prostaglandin $PGF_2\alpha$ led to an increased progesterone production when added to luteal cells in culture (Hixon & Hansel, 1979). Nevertheless this increased progesterone production after the addition of prostaglandin has been proved to be limited to the small luteal cells. Furthermore, $PGF_2\alpha$ has been found to inhibit LH-induced progesterone secretion (Alila et al., 1988)

1.3.3.3 The physiological role of ovarian steroids.

As mentioned above, the main steroid hormones secreted by the ovary are oestradiol and progesterone. The physiological role of these hormones is not restricted to the reproductive system but extends to other parts of the body such as the brain.

Oestrogens

Oestrogens in general and oestradiol 17β, in particular, are produced by the theca interna and granulosa cells under the synergistic effects of FSH and LH. Perhaps their main actions are concerned with the maintenance of the functional structure of the female genitalia. To this end, oestrogens promote growth of the endometrial glands, induce secretory activity of the oviducts and stimulate ductal and alveolar development of the mammary gland. However, the uterus is the organ where oestrogens bring about the most profound changes such as promoting vascularization, myometrial activity, mucosal development and salt retention. Oestrogens are also responsible for behavioural changes such as manifestation of sexual receptivity in the female.

Progesterone

Progesterone is secreted by small and large cells of the corpus luteum after LH stimulation as well as by the placenta and the adrenal gland. The primary role of progesterone is to establish a favourable uterine environment for implantation of the early embryo and maintenance of pregnancy, by increasing the secretory activity of glands in the endometrium and by reducing uterine contractility. In addition, progesterone is attributed with causing maternal immunosuppresion,

important in preventing rejection of the embryo which bears paternal antigens. The effects of progesterone on the reproductive tract can only be expressed after previous exposure to oestrogens to stimulate receptor formation.

1.3.3.4 Ovarian peptide hormones.

Inhibin

The concept of a gonadal factor regulating pituitary gonadotrophin secretion is not new (McCullagh, 1932). The putative non-steroidal component of the follicular fluid able to selectively regulate FSH secretion has been called inhibin. But it was not until 1985 when several independent research groups were able to isolate inhibin from bovine and porcine follicular fluid (Robertson et al., 1985; Rivier et al., 1985; Miyamoto et al., 1985). According to Burger (1988) "inhibin is a glycoprotein hormone consisting of two dissimilar disulphide-linked subunits termed α- and β- which inhibits pituitary gonadotrophin production and/or secretion, preferentially that of FSH". The main, if not the only, source of bioactive inhibin in the female animal is the granulosa cell of the ovarian follicle (Demoulin et al., 1987; Findlay et al., 1991). From studies in the ewe it has been demonstrated that small antral follicles as well as large non-oestrogenic ones produce a significant proportion of the circulating inhibin (Baird et al., 1991). There is some evidence that granulosa cells are capable of secreting inhibin before they attain the capacity for oestrogen synthesis (Tsukamoto et al., 1986). Androgens (androstenedione and testosterone) seem to stimulate the production of inhibin from granulosa cells, while progesterone inhibits inhibin synthesis. FSH selectively enhances inhibin synthesis from healthy antral follicles but has no effect on the granulosa cells from atretic follicles. Neither prolactin nor LH seem to influence

inhibin secretion *in vivo* (Demoulin et al., 1987; Campbell et al., 1989). However, *in vitro* studies have provided some evidence that low concentrations of LH increase inhibin secretion from rat granulosa cells after prior FSH exposure (Zhiwan et al., 1988). In the cow, preovulatory dominant follicles secrete large amounts of inhibin, while the non-ovulatory dominant follicle of each follicular wave secretes only moderate amounts of inhibin.

The contribution of inhibin to FSH suppresion has been documented after administration of either steroid-free follicular fluid (Ireland et al., 1983; Beard et al., 1989) or highly purified inhibin (Beard et al., 1988). It is well documented that oestradiol also plays a role but the interaction of the two hormones resulted in greater suppression of FSH than did either hormone alone (Mann et al., 1990). Oestradiol and inhibin exert their effects probably due to their inhibitory feedback on the pituitary by suppressing FSH-β mRNA (Carroll et al., 1989; Beard et al., 1989). Only the α-β dimer of inhibin is biologically active. This puts some obstacles to the determination of the active form, since most of the RIAs that are currently in use, assay both the biologically active form plus the inactive α-subunit (Knight et al., 1991; Baird et al., 1991). Only recently, Price et al. (1995) reported the development of an ELISA specific for the α- β dimer of inhibin and showed that the bioactive form of inhibin is almost undetectable in the peripheral blood of nonsuperovulated heifers. However, the pattern of immunoreactive (ir.) inhibin secretion is similar to that of oestradiol and increases in parallel with the growth of a dominant follicle during the follicular and early luteal phases (Taya et al., 1991).

Follistatin and activin.

Follistatin is an ovarian peptide with a role similar to that of inhibin and it too has been isolated from follicular fluid (Robertson et al., 1987). Although it was suggested that follicular fluid was the source of follistatin, ovariectomy led to a

non-significant reduction of follistatin in the peripheral circulation, while its concentration in the ovarian vein was significantly lower. It is postulated that the adrenal-pituitary axis may also produce follistatin (Findlay et al., 1992).

Activin, the third follicular peptide, stimulates FSH secretion from rat pituitary tissue without any effect on LH release (Schwall et al., 1988).

The role of the gonadal peptides (especially activin-follistatin) as regulators of gonadotrophin secretion remains unclear, but there is evidence of their role at the gonadal level (Findlay et al., 1991).

1.3.4 HORMONE PATTERNS DURING THE OESTROUS CYCLE.

Division of the bovine oestrous cycle into four phases (early, mid and late luteal and a follicular phase) is conventional to facilitate description of the sequential events of the cycle.

1.3.4.1 The early luteal phase.

Early in the luteal phase, the plasma concentration of both progesterone and oestradiol is very low since neither the corpus luteum is fully developed, nor is the first dominant follicle fully functional, although it has already been selected. At this stage, LH is secreted in a pattern which is characterized by high frequency pulses of low amplitude (Rahe et al.,1980; Walters et al., 1984; Rhodes et al., 1995). Both estradiol and progesterone play a role in the negative feedback control of LH secretion in the cow. It has been demonstrated in ovariectomized ewes that the replacement of either progesterone or oestradiol alone cannot return LH to its

precastration concentration, but this can be achieved by combined administration of both hormones (Karsch et al., 1980). Treatment of ovariectomized heifers with progesterone decreased LH pulse frequency, while oestradiol only suppressed pulse amplitude (Price & Webb, 1988; Stumph et al., 1993). On the other hand, oestradiol secretion appears to be regulated by the LH pulse frequency and in the early luteal phase, oestradiol concentration is maximal during the growth phase of the first dominant follicle, which coincides with a period of high frequency pulsatile LH release (Rhodes et al., 1995).

1.3.4.2 The mid luteal phase.

In the cow, FSH is secreted in a pulsatile manner unlike in the ewe (Walters & Schallenberger, 1984; Clarke et al., 1986). During both early and mid luteal phases, all LH pulses are associated with an FSH pulse. However, in the mid luteal phase, about 41% more FSH than LH pulses are observed (Walters et al.,1984) and a temporal relationship exists between pulses of LH, FSH and oestradiol. Every gonadotrophin pulse is followed by a distinct oestradiol pulse after a delay of one hour. Nevertheless, it is suggested that there is a closer association between LH and oestradiol pulses, since oestradiol pulses don't follow separate FSH pulses. Moreover, almost all combined FSH/LH pulses and separate FSH pulses are concomitant with or are followed by a progesterone pulse (Walters et al., 1984). During this phase, the mode of LH secretion is characterized by an increased pulse amplitude and a decreased pulse frequency (Rahe et al., 1980; Walters et al., 1984; Schallenberger et al., 1985a; Kaneko et al; 1991). LH is essential for normal luteal function, especially during the mid luteal phase. Withdrawal of the LH support by hypophysectomy, causes a rapid cessation of progesterone secretion in the ewe

(Baird, 1992). However, McNeilly et al., (1992) reported that an absence of LH pulsatility did not affect the progesterone secretion in the ewe. In the cow, Cupp and co-workers (1995) failed to correlate pulsatile secretion of gonadotrophins with pulses of either progesterone or oestradiol, which prompted them to suggest that a local regulator of progesterone might be activated in the corpus luteum.

FSH pulse frequency is higher during the early luteal phase when compared to the mid luteal (Walters et al., 1984). It has been hypothesized, that the reduction in FSH pulse frequency might be enabled by progesterone negative feedback on the hypothalamus (Goodman & Karsch 1980). Basal plasma FSH concentration is increased during days 1-3 and 10-12. This increase of FSH occurs after the suppression of the oestradiol basal secretion, which blunts the negative feedback on FSH (Adams et al., 1992; Sunderland et al.,1994).

1.3.4.3 The late luteal phase.

After luteolysis, the declining plasma progesterone concentration removes an inhibitory effect on LH pulse frequency allowing it to increase (Rahe et al., 1980; Walters et al., 1984). This leads to increased secretion of oestradiol during the subsequent follicular phase. The elevated oestradiol concentration, causes firstly, a transient decrease in FSH release (Butler et al., 1983; Schallenberger et al., 1984) and secondly, triggers a massive and sustained surge of GnRH from the hypothalamus (Karsch et al., 1992) which initiates the preovulatory LH surge. During the preovulatory period in the cow, oestradiol is not secreted in continously increasing concentrations, but in a wave-like manner, each wave lasting several hours. Oestradiol needs to be suppressed for a few hours in order to elicit the preovulatory positive feedback on FSH release (Walters & Schallenberger 1984). Surges in plasma concentrations of FSH and LH occur concurrently, the so-called

gonadotrophin surge, and trigger the process of ovulation and luteinization. A second FSH surge takes place approximately 24 hours later, probably due to reduced oestradiol and inhibin concentrations as a result of ovulation.

1.4 OVULATION

The process of ovulation involves the physical rupture of the distal wall of the follicle, which is brought about by a cascade of biochemical and structural changes. The fibroblasts of the tunica albuginea and theca externa become elongated and dissociated from one another, while the theca interna cells remain relatively unchanged. Electron microscopy studies have revealed that the follicular surface epithelial cells become necrotic and vacuolated (Espey & Lipner, 1994). The granulosa cells undergo hypertrophy with an accumulation of lipid granules in their cytoplasm indicating a shift from oestrogen to progesterone secretion.

Shortly before ovulation, a stigma appears on the surface of the ovary. This is a protrusion of the follicle through the dissociating epithelial cells. Proteolytic enzymes, such as plasminogen activator from the granulosa cells and collagenase from the fibroblasts, are secreted after the gonadotrophin surge and these digest the follicular wall (Reich et al., 1985). The rupture of the follicle is facilitated by ovarian contractility which gradually increases 2-3 hours before ovulation, reaching its peak around the time of rupture. It is believed that prostaglandins such as $PGF_{2\alpha}$ contribute to the enhanced ovarian contractility, since the follicular fluid concentration of the $PGF_{2\alpha}$ increases as the ovarian contractions become more frequent (Hafez, 1992). It has been proposed that, ovulation resembles an inflammatory reaction (Espey, 1994) and substantial hyperaemia is observed within

a few hours of the gonadotrophin surge, probably due to vasoactive agents such as histamine, kinins and prostaglandins (Tanaka et al., 1989).

The oocyte and the cells of the cumulus separate from the pedicle and float freely in the follicular fluid. The LH surge triggers the resumption of meiosis in the oocyte, a process which had been blocked since before puberty. This nuclear maturation involves breakdown of the germinal vesicle and extrusion of the first polar body (Pineda 1988).

1.5 CORPUS LUTEUM

1.5.1 Corpus luteum formation and development

After the preovulatory surge, a series of morphological and biochemical changes in theca interna and granulosa cells initiate the process of luteinization. Following ovulation, blood vessels from the theca interna invade the cavity of the ruptured follicle, probably as a result of an angiogenic factor that must be secreted soon after ovulation. In the intact follicle, the basement membrane acts as a barrier to prevent penetration of the thecal vasculature into the granulosa layer. Shortly after ovulation, this membrane breaks down and allows invasion of the granulosa layer by endothelial cells, fibroblasts and macrophages (Cavender & Murdoch 1988; O'Shea et al., 1980). Apart from their structural and functional role in the new corpus luteum, these cells serve as a source of angiogenic factors. The invasion of the granulosa layer by capillaries from the thecal vasculature is mediated by a dramatic proliferation of the endothelial cells (Gaede et al., 1985). Numerous

angiogenic factors are believed to contribute to vascularisation of the corpus luteum such as fibroblast growth factors (Gospodarowitz et al., 1985), heparin-binding growth factors (Grazul-Bilska et al., 1992), angiogenin, transforming growth factor and tumor necrosis factor α (Klangsburn & D'Amore 1991).

Several distinct cell types are found within the new corpus luteum. These include granulosa and theca cells, fibroblasts, endothelial cells and pericytes (Hansel et al., 1991; Niswender & Nett, 1994). Following ovulation, the follicular wall undergoes a dramatic infolding, which presumably facilitates the remodeling of the tissue by permitting migration of fibroblasts, endothelial cells and theca cells inwards towards the central region of the cavity (O'Shea et al., 1980). There is no evidence of mitotic proliferation of already luteinized granulosa cells, but mitosis is observed in fibroblasts, theca and endothelial cells. It is now clear that small and large luteal cells originate from theca and granulosa cells respectively (Alila & Hansel, 1984; O'Shea 1987). However, the tranformation of granulosa cells into large luteal cells is a gradual process. Thus, in early luteal phase, monoclonal antibodies raised against granulosa cell membrane preparations bound to small luteal cells and it was concluded that these were granulosa cells that had not yet enlarged (Alila & Hansel 1984). In addition, it has been proposed that stem cells of the corpus luteum may initially diferentiate into small luteal cells and subsequently into large luteal cells (Niswender et al., 1985).

Small luteal cells are characterised by a peripherally located, cup-shaped nucleus with a densely stained nucleoplasm and mitochondria arranged in an arch, while the nucleus of the large luteal cells is located centrally and has distinct nucleoli and mitochondria surrounding the nucleus (Hansel at al., 1991). In terms of absolute cell numbers, comprising the new corpus luteum, the granulosa cells never exceed 10% of the total. (Hansel et al., 1973, 1991; O'Shea et al., 1989).

The main hormone produced by the corpus luteum is progesterone. The pathways of progesterone biosynthesis have been described in the section on steroidogenesis. Studies with highly purified luteal cells preparations have revealed that different mechanisms regulate the progesterone biosynthesis in the two luteal cell types. During the mid luteal phase, small and large luteal cells probably synergize to promote progesterone secretion, whereas luteal cell interactions may be inhibitory during luteolysis (Hansel et al., 1991). Basal progesterone secretion is greater in large than in small luteal cells. However, treatment with LH causes a substantial increase in progesterone secretion from small luteal cells in culture, but not from the large luteal cells (Hansel et al., 1991). The same response was observed after stimulation with forskolin, cholera toxin or dibutyryl cAMP (Wiltbank, 1994). Protein kinase A and B are two enzymes that regulate luteal progesterone production. However, protein kinase A stimulates progesterone synthesis from the small luteal cells with little, if any, effect on the large luteal cells (Hoyer & Kong, 1989), whereas protein kinase C acts as an inhibitor of steroidogenesis on the large luteal cells (Wiltbank et al., 1989).

There is empirical evidence that progesterone secretion is partially independent of LH stimulation (McNeilly et al., 1992; Peters et al., 1993) because approximately 80% of the progesterone produced from the corpus luteum derives from the large luteal cells (Niswender 1985) which only express a small responsiveness to LH (Hansel et al., 1991).

In addition to progesterone, a number of non-steroidal substances are produced from the corpus luteum which may have either endocrine or paracrine actions. Oxytocin is the most important example (Wathes et al., 1983). Oxytocin can be located in the secretory granules of the large luteal cells (Fields et al., 1992), but it appears to be synthesized primarly by the granulosa cells after the LH surge (Schams et al., 1985). The exact physiological role of oxytocin is still not

elucidated (Luck, 1989) but it appears to stimulate progesterone production *in vitro* (Miyamoto & Schams, 1991) and probably stimulates contraction of the oviductal and uterine smooth muscles (Luck, 1989). Moreover, it is likely that oxytocin contributes to luteolysis (Silvia et al., 1991) as will be shown below.

The luteal cells are also known to produce prostaglandins, more so during the early luteal phase compared to the mid luteal phase (Rodgers et al., 1988). It is believed that prostaglandins of the E and I series stimulate whilst those of the F series inhibit secretion of progesterone.

1.5.2 Corpus luteum regression

In situations when a viable embryo is not present in the uterus, the corpus luteum undergoes luteolysis and regresses. The concept that the uterus is the source of the luteolytic signal in the cow was first demonstrated by Wiltbank and Casida (1956) since hysterectomy prolonged the lifespan of the corpus luteum. Ten years later, Babcock (1966) suggested that a prostaglandin from the uterus is the substance that causes luteolysis. It now generally accepted that $PGF_{2\alpha}$ is the main luteolytic factor in the cow. Indomethacin, a potent inhibitor of $PGF_{2\alpha}$ synthesis, has been found to extend the lifespan of the corpus luteum when injected into the uterus (Lewis & Waren, 1977). Several mechanisms have been proposed to explain the luteolytic action of the $PGF_{2\alpha}$ such as: constriction of ovarian vessels and ischaemia of the luteal tissue (Niswender et al., 1976), reduction of LH receptors in the luteal cells (Behram et al., 1978), interference with the progesterone biosynthesis (Torday et al., 1980) and direct cytotoxic effects on the luteal cells (Niswender et al., 1985).

$PGF_{2\alpha}$ can act directly on the large luteal cells, since they possess $PGF_{2\alpha}$ receptors (Hansel et al., 1991). However, after the administration of a luteolytic dose of $PGF_{2\alpha}$, a significant decrease in the number of small luteal cells was observed, followed by degeneration of the large luteal cells (Braden et al., 1988). The reason for the small cell and capillary cell loss in the regressing corpus luteum is not understood, but it might be attributed either to alterations in blood flow, or to the production of deleterious factors by the degenerating large cells, or to the direct effect of the prostaglandin (Wiltbank & Niswender, 1992). Treatment of luteal tissue with $PGF_{2\alpha}$ resulted in increased progesterone production (Hansel et al., 1973) but further studies have revealed that $PGF_{2\alpha}$ selectively suppresses LH-stimulated progesterone synthesis, without any effects on basal progesterone production (Pate & Condon, 1984). Prostaglandins do not seem to affect either the binding or the uptake of lipoproteins by the luteal cells (Pate & Condon, 1989), but they might inhibit conversion of cholesterol to progesterone (Grusenmeyers & Pate 1992). Furthermore, it is believed that $PGF_{2\alpha}$ causes degeneration and death of the large luteal cells due to the sustained influx of calcium (Wiltbank et al., 1989).

Although $PGF_{2\alpha}$ appears to initiate the process of luteolysis, additional factors must be involved. The role of oxytocin must be considered, since endometrial oxytocin receptors are required for the secretion of $PGF_{2\alpha}$ (Milvae & Hansel 1980; Schams, 1987). It has been suggested that oxytocin interacts with endometrial oxytocin receptors and triggers $PGF_{2\alpha}$ release, which in turn induces further oxytocin release and hence amplifies secretion of prostaglandin from the uterus (Schams, 1987). Progesterone inhibits the development of endometrial oxytocin receptors, while oestradiol stimulates both oxytocin receptors and $PGF_{2\alpha}$ secretion (Lamming & Mann, 1995).

Regression of the corpus luteum possibly occurs by the process of apoptosis with some types of immune cells also participating (Pate, 1994).

1.6 FOLLICULAR DYNAMICS

1.6.1 Follicular waves.

It has been known since the work of Rajakoski (1960) that follicular development during the bovine oestrous cycle is not a continous process, rather it occurs in wave-like pattern. However, until recently the study of this phenomenon has been based either on examination of abattoir material (Marion et al., 1968), or by following individual follicles by laparoscopy after dye marking or cauterization (Matton et al., 1981). Another approach has been the ovariectomy performed on particular days of the cycle (Ireland & Roche 1983). Using these techniques it was concluded that the follicular development in cow occurs in two or three waves or in a continous follicular turnover (Spicer & Echternkamp 1986). The use of real-time ultrasonography has shed more light on this matter and has resolved a number of controversies about follicular dynamics in cattle.

Firstly by Pierson and Ginther (1984) it was concluded that during the bovine oestrous cycle two follicles develop to ovulatory size, the first during dioestrus and the second during the follicular phase (Pierson & Ginther 1987). In a later study the same authors reported that in heifers follicles develop in either two or three waves (Ginther et al.,1989b). Other research groups suggest that in most heifers three follicular waves occur (Ireland and Roche 1987) with two waves been exception (Savio et al., 1988). Alternatively, heifers exhibit either two or three follicular waves but the three wave pattern is more commonly observed (Sirois & Fortune 1988; Fortune et al., 1988).

1.6.2 Recruitment and selection of the dominant follicle.

Each follicular wave is initiated by synchronous growth of a group (cohort) of small follicles (<5mm). This is the phase of recruitment, when the follicles enter the gonadotrophin-dependent stage (Driancourt et al., 1987). It has been reported that FSH is associated with this follicular recruitment since a rise in plasma FSH concentration has been observed one to two days before the emergence of the follicular wave (Adams et al., 1992). However, it is now known that follicular waves take place during early pregnancy in a hormonal millieu of basal LH and FSH secretion (Ginther et al., 1989a; Savio et al., 1990).

After recruitment, one follicle of the cohort gradually out grows the others. This becomes the dominant follicle and it prevents the growth of its subordinates and also suppresses the development of other small follicles (Ireland & Roche 1987; Ireland 1987; Fortune et al., 1991). The factors which regulate selection of the dominant follicle are uncertain but the decline of FSH concentration just before hand might be critical (Adams et al., 1992). It is apparent that, both attainment and loss of dominance are closely related to changes in the hormonal environment. Since an artificially prolonged luteal phase increases the number of follicular waves in heifers, it can be hypothesized that waves normally occur under basal gonadotrophin concentrations (Sirois & Fortune 1990). In addition, greater development of the first dominant follicle early in the luteal phase compared with the subsequent anovulatory dominant follicles later in dioestrus, is attributed to the low progesterone concentration during its growth (Adams et al., 1992a).

Morphologically, the dominant follicle can be defined as the largest follicle present in either ovary. However, functional dominance has two distinct aspects. Firstly, the dominant follicle must be capable of suppressing its subordinates and secondly, it must ovulate after appropriate hormonal stimulation. Such characteristics are apparent for the natural preovulatory follicle of the cycle (Fortune 1993). It has also been shown that dominant follicles of the first follicular wave can ovulate following administration of a luteolytic dose of $PGF_{2\alpha}$, on day seven, when the follicle has attained its maximum size, or even when the follicle is still in the growing phase (Savio et al., 1990a). However, after similar follicles had been static for five days the injection of $PGF_{2\alpha}$ induced ovulation of the smaller dominant follicle of the next wave (Fortune et al., 1991). Therefore, follicle size is not the only criterion to determine dominance (Fortune et al.,1991; Lavoire & Fortune 1990; Kastelic and Ginther 1991). An intrafollicular ratio of oestradiol:progesterone content greater than one can be used to distiguish healthy from atretic follicles. In addition, the number of LH receptors increases while the number of FSH receptors decreases in the healthy dominant follicle (Ireland & Roche 1982a, 1983, 1987).

1.6.3 The follicular dominance.

Factors that lead to dominance of one follicle in the cow and suppression of its the subordinates are still obscure. There is support for the view that the decline of FSH is a crucial component in the selection and exertion of dominance (Adams et al., 1992; Stock & Fortune 1993).

According to Hillier (1994) each follicle has a threshold which must be exceeded before the preovulatory development can start. The follicle with the most FSH-

responsive granulosa cells (lower FSH threshold), becomes the dominant follicle by secreting oestradiol and inhibin which cause a suppression of FSH. Thus, FSH circulates in concentrations insufficient to sustain development of the subordinate follicles which have higher FSH requirements, and inevitably, they undergo atresia. Increased sensitivity of the dominant follicle to FSH could be related to the secretion of autocrine factors such as insulin-like growth factor (IGF_1) which is found in high concentrations in large follicles (Echtenkamp et al., 1990). It is likely that oestradiol interacts with FSH to stimulate IGF_1 synthesis. FSH stimulates IGF_1 binding to granulosa cells and increases aromatization and further oestradiol secretion as well as increasing the number of LH receptors (Adashi et al., 1985a, b).

Nevertheless, other studies provide compelling evidence for an alternative mechanism of follicular dominance, mediated by the secretion of follicular factors which directly suppress the subordinate follicles. Thus, inhibin-free bovine follicular fluid inhibited follicular development in cows without significantly altering FSH secretion (Law et al., 1992).

1.6.4. Follicular atresia

Atresia is the fate of most ovarian follicles, since only a very small proportion of those that begin to grow will survive to the preovulatory stage. In an early study in cattle Erickson (1966) found that the population of primordial follicles remained unchanged until the fifth year of age thereafter declining to zero in the 20th year. During atresia in the mouse follicle the changes in the granulosa layer take place in three progressive stages (Byskov 1974). Initially, approximately 20% of the granulosa cells undergo pyknosis and fragmentation. In the second stage, the

proportion of the pyknotic cells increases and leukocytes and cytotoxic T lymphocytes invade the granulosa layer (Bucovsky et al., 1984). In the third stage, the follicle is already shrunken but comprises only 5% pyknotic cells, probably because of the increased clearance of dead granulosa cells by macrophages. Although the exact mechanisms leading to follicular degeneration remain unclear, it has been hypothesized that FSH may be the factor involved in programmed cell death in human follicles (Faddy et al., 1992). Macroscopically, growing follicles are more likely to protrude from the surface of the ovary, while regressing ones are mainly embedded within the ovarian tissue (Spicer et al., 1987). In general, atretic follicles are characterized by the loss of aromatase activity in their granulosa cells which results in markedly decreased oestradiol synthesis (McNatty et al., 1984). High levels of progesterone are also found in atretic follicles and this derives from the granulosa cells, which produce progesterone after the loss of aromatase activity (Henderson et al., 1987). Theca cells retain their LH receptors even with increasing atresia but they are not capable of secreting androgens in response to LH stimulation (Henderson et al., 1984). The loss of LH receptors from granulosa cells is only detected in advanced atresia (Ireland & Roche 1982a).

1.7 ULTRASONOGRAPHY

During the last decade, the use of ultrasonography in veterinary medicine has proven a very helpful aid in both research and clinical fields. As Ginther (1995) states "Ultrasonography is the most profound technological advance in the field of

animal research and clinical reproduction since the introduction of rectal palpation and radioimmunoassay for circulating hormones".

Ultrasound is a sound with frequency much higher than most humans can hear. The range of audible sound is below 20000 cycles per second (20 KHz), ultrasound used for medical purposes is of very high frequency usually ranging between 1-10 MHz. The principle of the ultrasonography is the utilisation of the echo. A simplified example of this principle is the technique for sea bed scanning used by ships.

1.7.1 Physical principals-image interpretation

The B-mode, real time scanners is the type most commonly used in the study of veterinary reproduction. B-mode refers to the brightness modality, while real time implies that a moving display which is continuously updated can be produced on the screen.

The basic production unit of ultrasound waves is the piezo electric crystal. The main physical property of these very thin crystals is to convert electrical energy into mechanical ultrasound waves and, subsequently, to reconvert the mechanical energy, from the reflected ultrasound beam into electrical energy. The appropriate electric signal is transmitted to the piezoelectric material through electrodes, which cover the crystal from both sides. The crystal responds to the electric stimulus by expansion and contraction, according to the frequency of the electric signal. This 'vibration' generates the ultrasound wave, which propagates in two directions. The transmission of the beam is restricted with a damping material on one side of the crystal and thus, only one wave propagates in the tissue.

When the reflected proportion of this wave returns to the crystal, the generated electric signal is measured on the electrodes. The electrical signal is subsequently transferred to the scanner. In the scanner, the signal is amplified and projected on the screen in the form of dots. The brightness of each dot is positively related to the strength of the returned echo, while the position of the dot on the screen represents the distance of the reflective surface from the transducer. Thus, the image of the organ is created on the screen by the influx of the returning echoes, each one carrying certain information about the distance and the density of the surface on which it was reflected.

According to its density, the image of the reflecting tissue appears on the screen in grey shades, varying from white to black. Liquids do not reflect sounds, hence they appear black on the screen and are characterised as nonechogenic. Less dense tissue produces an image in different shades of grey (echogenic). Dense tissues reflect much of the beam, producing a white image (hyper-echogenic).

As the ultrasound waves travel through the tissues, they inevitably undergo a weakening, known as attenuation, which results to a decreased capacity to penetrate. Attenuation is positively related to the frequency of the ultrasound pulse. In the same medium, low frequency pulses result in deeper penetration. The factors that contribute to attenuation are: the refraction, the scatter and the absorption.

Refraction is the diversion of the beam, which usually occurs when the interface between the media is not perpendicular to the axis of the beam. Refraction often results in the false location of the image of the scanned structure.

Scattering happens when the beam is reflected on very small particles, or on an irregular interface.

Absorption refers to the conversion of the mechanical energy of the beam to heat, when the beam is trapped between tissues. The biological effects of absorption are inconsiderable, since ultrasonography utilises very low energy levels.

Resolution, the feature that characterises an accurate ultrasound scan, is the ability of the instrument to distinguish between two reflectors located close to each other. Resolution is specifically defined as axial and lateral, depending on the relative location of the two reflectors with respect to the beam axis. Thus, in the axial resolution the reflectors are located along the axis of the beam, while in the lateral at right angles. The axial resolution is related to the frequency of the transducer. The smallest detectable distance between the two reflectors is half pulse length. Therefore, the higher the frequency, the more detailed the image can be.
High frequency transducers (5.0 and 7.5 MHz) provide very good resolution but they propagate in limited depth. Therefore they are used for detailed studies of structures located close to the transducer, e.g. ultrasonographic study of ovarian structures and early pregnancy diagnosis. Low frequency transducers (3.0 MHz) penetrate deeper in the tissues but gives less resolution compared to the high frequency.

1.7.2 Artifacts

The correct interpretation of the ultrasound image is a difficult task, which requires a deep understanding of, the physical properties of the sound, the complexity of the tissue, and the relationships between tissues and echoes. The ultrasonic image is never the exact representation of the scanned structure, since the tissues through which the waves are transmitted, are not homogenous media. This results in the appearance of artifactual images, which may be misleading. However, some of

these errors are easily recognisable and even useful in enhancing the accurate interpretation.

Reverberation artifacts occur when the echoes oscillate between two strongly reflective interfaces, until the wave is completely attenuated. This artifact is characterised by the appearance of a number of parallel lines on the screen, representing the creation of multiple echoes from a single pulse, after repeated bounces of the pulse between the reflector and the probe. Small, highly reflectable particles produce a reverberation of regular arrangement, which is called the comet-tail artifact. Gas filled bowel segments are a very common reason for the creation of this artifact. In fluid filled structures, reverberation can cause a false representation of an internal structure.

Large, strongly reflective interfaces can produce the **mirror-image artifact**, which is related to the false interpretation of the location of an organ. The ultrasound pulse reaches the target organ after reflection on a large interface and returns to the probe along the same way. The scanner, assuming that the echo returned to the probe in a straight line, calculates the distance and places the organ beyond its actual position.

Acoustic shadowing is created in a way similar to the shadow that occurs behind a light barrier. Thus, acoustic shadowing appears when most of the ultrasound beam is either reflected back to the transducer by a dense structure such as a bone, or undergoes reflection or refraction.

Acoustic enhancement or through transmission artifact, occurs when the ultrasound beam traverses a structure of very low attenuation, such as fluid filled structures. On the screen, enhancement appears as an area of increased brightness, distal to the structure of the low impedance. This artifact can be exploited to differentiate cystic structures from hypoechoic masses, such as abscesses, granulomas or tumours.

1.7.3 The use of ultrasonography in bovine reproduction

Sixteen year have elapsed since the first report of the use of ultrasonography as an aid in Veterinary Reproduction, for the examination of the genital tract of the mare (Palmer & Driancourt 1980). Since then, sonography has provided a substantial contribution to the better understanding of the reproductive events in various species, and has proven a powerful tool for the veterinary practitioner.

In cattle, it has been shown that ultrasonography is successfully applicable for many research and diagnostic purposes. Visualisation of the ovaries per rectum provided information about the follicular population and the changes in their growth patterns (Edmondson et al., 1986; Pierson & Ginther 1987; Pierson et al., 1988; Kastelic et al., 1991; Fortune 1993), presence of functional corpora lutea (Kito et al., 1986), early pregnancy diagnosis (Boyd et al., 1988; Willemse & Taverne 1989), early embryonic loss (Kastelic et al., 1988) and the characteristics of pathological conditions of the reproductive tract (Kahn & Leidl 1987; Edmondson et al., 1986).

In recent years an increasing number of publications have reported oocyte collection from the live cow by means of ultrasound guided follicular aspiration. Development of protocols for repeated recovery of oocytes at frequent intervals has become an active area of research (Callesen et al., 1987; Pieterse et al., 1988; Walton et al., 1993; Kruip et al., 1993; Gibbons et al., 1994; Scott et al., 1994)

1.7.3.1 The sonographic appearance of the uterus

The sonographic image of the non pregnant uterus changes according to the stage of the oestrus cycle. During the periovulatory period the endometrial folds can be

easily distinguished as echogenic structures projecting in the lumen of the uterus. The reduced echogenicity of the uterus during this period is attributed to the accumulation of fluid, due to increased secretions from the uterine glands. The diameter of the uterine horns varies considerably throughout the cycle. The maximum diameter is attained during the periovulatory period, and the minimum during metoestrus. During dioestrus there is no fluid in the uterine lumen, but the endometrium can be still distinguished from the more echogenic myometrium (Kähn 1994).

1.7.3.2 Ovarian structures.

The follicles.

A circular anechoic cavity is the characteristic feature of the ovarian follicle. The follicular wall is not easy to identify because although the follicular wall is approximately 2mm thick, its echogenicity does not differ substantially from the surrounding ovarian stroma. Most recent studies of follicular populations utilise a 7.5 MHz transducer which gives good resolution and allows the identification of follicles as small as 2-3mm. The use of 5 MHz transducers leads to underestimation of the number of follicles smaller than 3mm (Kähn 1994). The resolution of a 3.5 MHz probe is said not to allow detection of follicles smaller than 10mm (Pierson & Ginther 1984). Blood vessels are likely to be confused with small follicles, but scanned from different angles, unlike the follicles, they alter their circumscribed appearance (Pieterse 1989). Preovulatory follicles appear as gradually enlarged structures, eventually attaining a diameter more than 2cm (Fortune et al., 1991). However, ovulatory follicles of 10mm have been reported to result in successful pregnancy (Kähn 1994). The retrospective detection of

ovulation was almost impossible for early researchers, hence, the only way to confirm ovulation was by the disappearance of the preovulatory follicle detected previously (Kähn 1994). However, using high quality equipment the detection of corpus haemoragicum was feasible 12 hours after ovulation (Omran 1989, Robertson et al., 1993).

Since ultrasonography is not an invasive technique, it can be used for daily monitoring of the ovarian population. Pierson and Ginther (1984) were the first researchers who, using sonography, counted the number of follicles throughout the cycle. Based on their observations they concluded that two follicular waves occur in cattle. Since then, many reports have been published related to the study of the follicular dynamics in the cow (Sirois & Fortune 1988; Quirk et al., 1986; Savio et al., 1988; Fortune et al., 1988). Although follicular dynamics have been extensively studied, there is still some controversy whether two or three waves occur in the cow. It is generally accepted that the first follicular wave begins around day 2 and consists of a group of 3-6 follicles ≥5mm. In the following days one follicle (the dominant) grows larger, while the rest regress. Around day 6 the dominant follicle attains maximum diameter and stays in the plateau phase until day 10. Meanwhile, the second wave emerges on day 9. This means that although the dominant follicle of the first wave is the largest in both ovaries until day 13, after day 10-11 it loses its dominance (Fortune et al., 1991). The second dominant follicle reaches maximum size by day 15 and it is detectable until the end of the cycle. However, from day 16 it starts regressing and until day 18 is the largest follicle on both ovaries. (Sirois & Fortune 1988). The ovulatory follicle is first detectable on day 16 and its size is 5-6mm. It has been shown that the dominant follicles of each cycle are detectable for approximately 11.5, 7.5, and 6 days in the first , second and third wave respectively (Savio et al., 1988).

Corpora lutea.

The corpora haemorrhagica are recognised as poorly echogenic areas, with grey (echogenic) spots, occupying the area of the collapsed follicle. Early corpora lutea can be discerned as light echogenic areas two to four days post-ovulation. Sometimes, the regressed corpus luteum of the previous cycle can be visualised together with the new developing corpus luteum. In general, old corpora lutea having lost their vascularization appear lighter, resembling the appearance of the ovarian stroma. An active corpus luteum can grow as big as 30mm in diameter by day 16 of the cycle (Pieterse 1989). The sonographic image of the corpus luteum represents a well defined, circular or ovoid granular structure, having different a echogenicity from the surrounding ovarian stroma (Pierson & Ginther 1984). The lower echogenicity of the structure, when compared to the ovarian stroma, is attributed to the extensive capillary network of the corpus luteum (Kähn 1994). In some cases, in the central area of the structure, a thin hyperechoic line appears, representing the connective tissue that is located in the middle of the corpus luteum. Another common finding among corpora lutea is a central fluid filled cavity. In a study of cycling cows, it was found that 37.2% of the corpora lutea had a central cavity or lacuna. This fact did not deprive the corpora lutea of their normal functions (Kito et al., 1986).

1.7.3.3 Pregnancy diagnosis.

Early pregnancy diagnosis is a crucial factor for an increased herd fertility. The success of the traditional rectal palpation of the uterus is highly dependent upon operator experience and it is difficult to be accurate before the 40th day after service or artificial insemination.

Using a 7.5 MHz rectal transducer, the first indications of pregnancy can be obtained as early as day 9 after insemination, by visualisation of a fluid-filled vesicle in the uterine horn. The embryo is visible by day 13 and a positive diagnosis for a live embryo can be made on day 22 after the detection of the heart beat (Boyd et al., 1988). However, a study, comprising pregnant and non-served heifers, revealed that on day 10 the accuracy of pregnancy diagnosis was not better than a guess (50%), on day 18 increased to 85%, and on day 22 was 100% accurate (Kastelic et al., 1991). The first detection of anatomical or functional characteristics such as the heart beat (d22), spinal column (d28) placentomes (d35), hooves (d44), can be used as a criterion for accurate determination of the foetal age (Pierson & Ginther 1984a; Boyd et al., 1988; Curran et al., 1992).

In addition to pregnancy diagnosis and embryo viability examinations, determination of the foetal sex can be carried out by means of ultrasound imaging. The ultrasound sexing is either based on the relative position of the genital tubercle from the umbilicus, which in male foetuses is located very close to the umbilicus, while in the females is just ventral to the tail (Curran 1992;), or on the identification of the scrotal swelling in males and mammary glands on females (Muller & Wittkowski 1986). In both cases sex determination was found to be accurate after the 60th day of pregnancy.

1.7.3.4 Diagnosis of pathological conditions of the reproductive tract.

Indisputable diagnoses for various pathological conditions of the reproductive tract can be made by means of ultrasound scanning, based on abnormal appearance of the organ under examination

Ovarian cysts.

Sonographically ovarian cysts resemble large ovarian follicles. They appear as large non echogenic structures greater than 3-4cm (Kähn & Leidl 1989). Depending on the thickness of wall, cysts can be divided into two categories: luteinised, with a luteal wall 3-4mm thick, and follicular, with a thin wall (Kähn & Leidl 1989). The presence of a cyst is usually associated with either anoestrus or nymphomania (Pieterse 1989), although cysts have been detected on ovaries together with a functional corpus luteum, and in other cases they have been found during the early stages of pregnancy (Kähn 1994).

Pyometra is characterised by the accumulation of high quantities of fluid in the uterus. The sonographic appearance of the fluid depends on its consistency. Various echogenic particles float in the fluid, which may represent uterine debris or clusters of leukocytes, giving the image of snowy patches. The uterine wall in some cases is thickened while in other remains thin, categorising pyometra as thick or thin walled.

Endometritis. Depending on the severity of the case, endometritis is characterised by the presence of different volumes of fluid in the lumen of the uterus. In mild cases a few fluid-filled pockets can be detected, whilst in severe cases large quantities of fluid are present distending the uterus and both horns.

Maceration. In this case many small echoic spots, representing pieces of tissues from the degenerating foetus, can be seen in the amniotic sac, while the allantoic fluid remains clear. The determination of the foetal contour is difficult, since the echoic particles reduce the contrast between the foetus and the surrounding amniotic fluid.

Mummification. The mummified foetus gives an ultrasonic image of a highly echogenic mass in the lumen of the uterus, in the absence of any foetal fluid. No

organs or parts of the foetus can be identified. A thickened uterine wall is easily discerned in most cases (Khän & Leidl 1989; Fissore et al., 1986).

1.7.4 Ultrasound guided follicular aspiration.

In humans, oocyte collection by means of ultrasound guided follicular aspiration was first described in 1985 (Dellenbach et al., 1985). Two years later Callesen and co-workers published a report of the application of the same technique in cattle, claiming that the method had many advantages over the laparoscopic procedures then in use (Callesen et al., 1987). In recent years, there have been an increasing number of reports from different research groups dealing with oocyte recovery from the live cow. The procedure is not invasive for the cow, relative simple, easy to learn, fast and very efficient.

For more than 20 years superovulation has been used for early embryo collection from valuable cows. The disadvantages of superovulation are, first, that the donor cow has to be of normal fertility and second, superovulation results in a marked increase in the calving intervals of the donor cow (Bak et al., 1989). Ultrasound guided ovum pick-up (OPU) can overcome these problems and seems to be a potent alternative to superovulation. Initially, the technique was used in combination with superovulation of the donor cow (Pieterse et al., 1988; van der Schams et al., 1991) but later it was found that the recovery rate of oocytes was similar or even better in non-stimulated animals (Pieterse et al., 1991; Pieterse et al., 1991a; Simon et al., 1993; Scott et al., 1994; Bungartz et al., 1995; Bols et al 1995). The recovery ranges from 2-3 up to 25 oocytes per cow per session (Pieterse 1988; van der Schams et al., 1991). Most of the OPU protocols comprise

once weekly aspirations. However, it has been shown that twice weekly sessions can result in a higher recovery rate and subsequently more transferable embryos (Gibbons et al., 1994; Walton et al., 1993). Furthermore, it has been reported that follicular aspirations at 48 hours intervals resulted in increased number of harvested oocytes, but apparently compromised quality (Simon et al., 1993). The highest recovery rate has been achieved during the early luteal phase (days 3-4) and from follicles smaller than 10 mm (Pieterse et al., 1991). Oocytes recovered by means of follicular aspiration are capable of normal development and the yield of morulae and blastocysts is comparable to oocytes from abattoir material (Bungartz et al., 1995; Gibbons et al., 1994). According to Pieterse et al., (1991a) 30 transferable embryos may be produced every year from each donor cow. It has been demonstrated that the OPU with or without gonadotrophin treatment, can be applied in pregnant cows, without any side effects on pregnancy (Meintjes et al., 1993; Burgartz et al., 1995). Prepubertal and peripubertal heifers have been used as oocyte donors. The fertilization rate of both prepubertal and peripubertal heifer oocytes was comparable to that of adult cows. However, the developmental competence of the prepubertal oocyte appeared to be much lower. Only 1.6% developed to the stage of blastocyst, whilst 60% of the peripubertal oocytes reached the same stage (Looney et al., 1995).

Finally, the ablation of follicular fluid from selected follicles provides an alternative methodology for the study of hormonal interactions during the different stages of the oestrous cycle. Using this methodology, it has been shown that in the mare, aspiration of small to medium size follicles resulted in a higher increase in plasma progesterone, FSH and LH than did the aspiration of large follicles (Hinrichs et al., 1991).

Objectives of the study

There has been much interest in recent years in harvesting cattle oocytes by ultrasound-guided needle puncture and aspiration of the entire follicular contents. Such procedures were developed to allow oocyte recovery for in vitro maturation and fertilisation, followed by embryo transfer. Little attention was paid to the natural course of the reproductive cycle in the donor animals but preliminary work by our research group indicated certain aberrations of cyclic activity, were in some cases linked to the spontaneous appearance of a luteal structure after aspiration.

In these original studies there was little opportunity to study the oestrous cycles and reproductive endocrinology of the cows deprived of oocytes by follicular aspiration. The present study was initiated to explore some of these areas in more detail.

During preliminary work, techniques for repeated ultrasound guided aspiration of all visible follicles were developed and oocyte quality was assessed as described in Chapter 3.2, while Chapter 3.3 describes the effects of this repeated multiple aspiration procedure on interoestrus intervals and on circulating hormone concentrations. Later it was suspected that it was aspiration of the dominant follicle that might cause formation of an accessory luteal stucture. This hypothesis was tested and the results of dominant follicle aspiration described in Chapter 3.4, together with the endocrine consequences of dominant follicle ablation. Results of detailed functional and morphological studies of an accessory corpus luteum which arose during this work are also described in Chapter 3.4.

Chapter 2

MATERIALS & METHODS

2. MATERIALS AND METHODS

2.1 Experimental animals.

Non-pregnant, dry British Holstein-Friesian cows were selected from near-by dairy farms based on fitness, identification of an active corpus luteum and the absence of abnormalities in the genital tract. Eight cows were used for each of the two phases of the experiment: numbers 4, 9, 61, 96, 100, 131, 156 and 158 for the study of the effects of the multiple aspiration, and 47, 61, 68, 96, 97, 100, 131 and 156 for study of aspiration of just the dominant follicle. Two cows from the former group were reused in later cycles. Throughout the experimental period, the animals were tethered in double stalls at Netherton Farm, Glasgow Veterinary School. The cows had access to automatic drinkers and were fed silage or hay *ad libitum*, supplemented with 2-4 kg cattle concentrates depending on the liveweight.

2.1.1. Oestrus synchronisation

Once the cows were transferred to the farm, they received two intramuscular injections of the prostaglandin cloprostenol (500 mg Estrumate, Coopers Animal Health, Crewe Hall, Cheshire, UK), given 11 days apart. After the second injection the animals were observed for oestrus behaviour, for 20 to 30 minutes twice daily between 09-10 am and 05-06 pm. A cow was designated in heat when she was seen to stand to be mounted by a fellow cow. Subsequently, ovarian activity was assessed by transrectal ultrasonography and the day of ovulation was defined as

day 0. Alternatively, progestagen ear implants were used for oestrus synchronisation (Norgestomet 3mg, Crestar, Intervet, Science Park, Milton Road, Cambridge, UK). The accompanying oestradiol valerate injection was never used in our synchronization protocol. The implants stayed *in situ* for 9 or 10 days. The day before the ear implant removal, an Estrumate injection (500mg) was given to cause regression of the corpus luteum.

2.1.2. Blood collection.

During the day before the aspiration, an indwelling catheter was inserted into the jugular vein of the cows. The cannulation system was a modification of that described for dogs by Jeffcoate (1992), consisting of 30cm long polythene tubing (Portex Ltd, Kent, UK) with external and internal diameters of 2mm and 1mm respectively. The cannula was disinfected in 0.1% chlorexhidine gluconate solution (Hibitane 5%, Zeneca, Cheshire, UK), and it was introduced into the vein through a 14 g hypodermic needle. A blunt 20g needle, stoppered with the tip of a 1ml syringe, was attached to the free end of the cannula swathed by waterproof tape. The cannula was then sandwiched between two pieces of elastic self adhesive bandage (Elastoplast, Smith and Nephew Ltd, Hull, UK), and secured with contact adhesive to the hair of the neck of the cow. A 10ml test sample could then be withdrawn after discarding saline and diluted blood. To maintain patency between samples, the cannula was flushed firstly with 3ml 0.9% sodium chloride saline (Aquapharm-Animal Care Ltd, York, UK.) and then with 0.5ml heparinised saline containing 200IU/ml heparin (Heparin, Sigma, St.Louis USA) to prevent blood coagulation. The plasma was immediately separated by centrifugation at 2000rpm for 20 minutes and it was stored at -20°C before hormone analysis.

2.2 Ultrasonography

Competence in the use of ultrasound scanning equipment started with initial training on abattoir specimens in water bath. Further training was carried out by daily scanning of a group of four cows, over a period of three cycles. Attention was focused on the early identification of the corpus luteum and the detection and measurement of the small follicles.

2.2.1 . Transrectal ultrasound scanning.

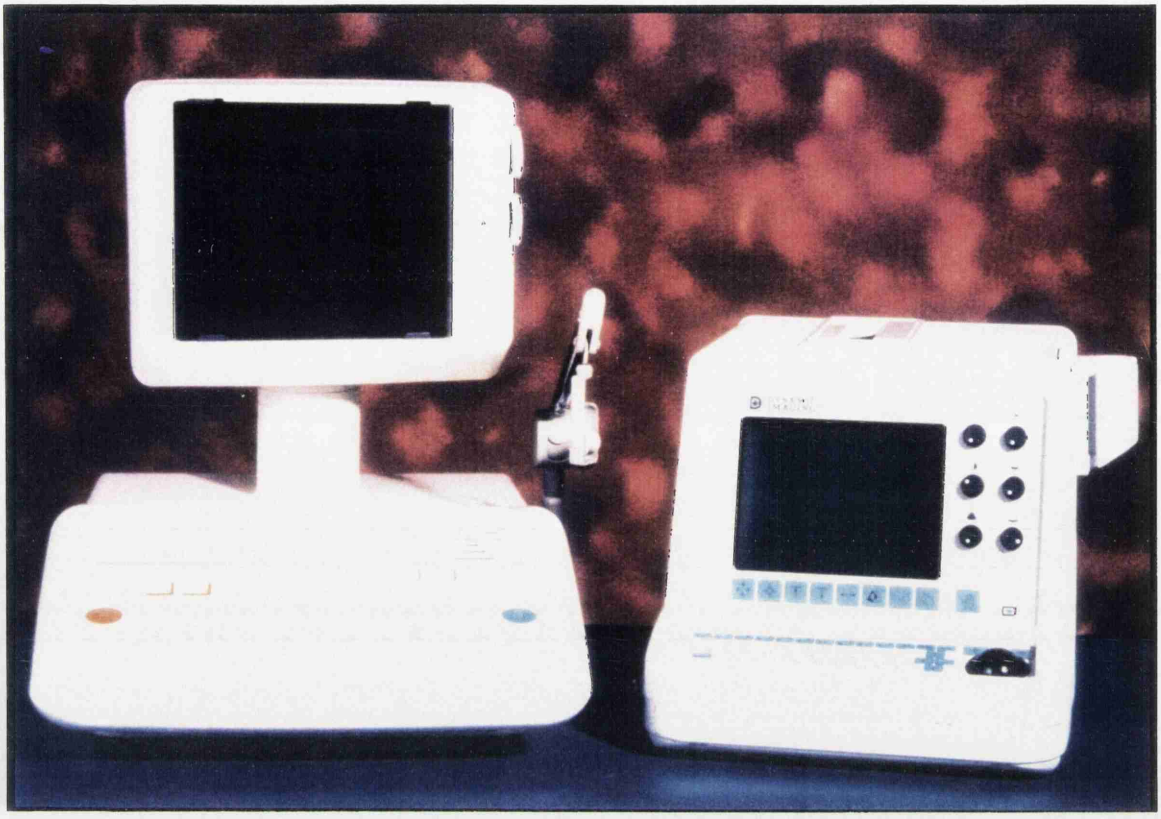
For transrectal ultrasonography the same type of scanner was used throughout the study. Initially this was a B-mode, real-time, portable Concept LC (Dynamic Imaging, Livingston, Scotland, UK) which was later replaced by the latest model Concept MC. Both models were fitted with a 7.5 MHz transducer and provided freezing, zoom and structure measurement facilities, as well as a screen splitting function, which enabled the sonographer to compare two different views of the same structure. The scanner was connected to a video recorder for retrospective study of ovarian status. In some cases, when a high quality image was needed, a Toshiba Capasee scanner (model SSA-220-A; Toshiba Medical System, Manor Royal, Crawley) equipped with a 7.5 MHz microconvex curvilinear rectal transducer was used (fig. 1).

Throughout the transrectal ultrasound examination the animal was kept in a cattle crush. Restriction of the lateral movements of the cow was necessary to facilitate the attainment of good quality images and to minimise the possibility of accidental damage to the ultrasound and videotape equipment.

After having evacuated the rectum of any faecal matter, the transducer was covered with coupling gel (Lubrel Arnolds Vet. Products Ltd, Shrewsbury,UK)

Figure 1. Equipment used for ultrasound guided follicular aspiration and transrectal scanning.
Upper: Real time B - mode scanners, Toshiba Capasee (left) and Concept MC (right)

Lower: Transducers used for rectal scanning
7.5 MHz linear array transducer (left) and 7.5 MHz curvilinear transducer (right)



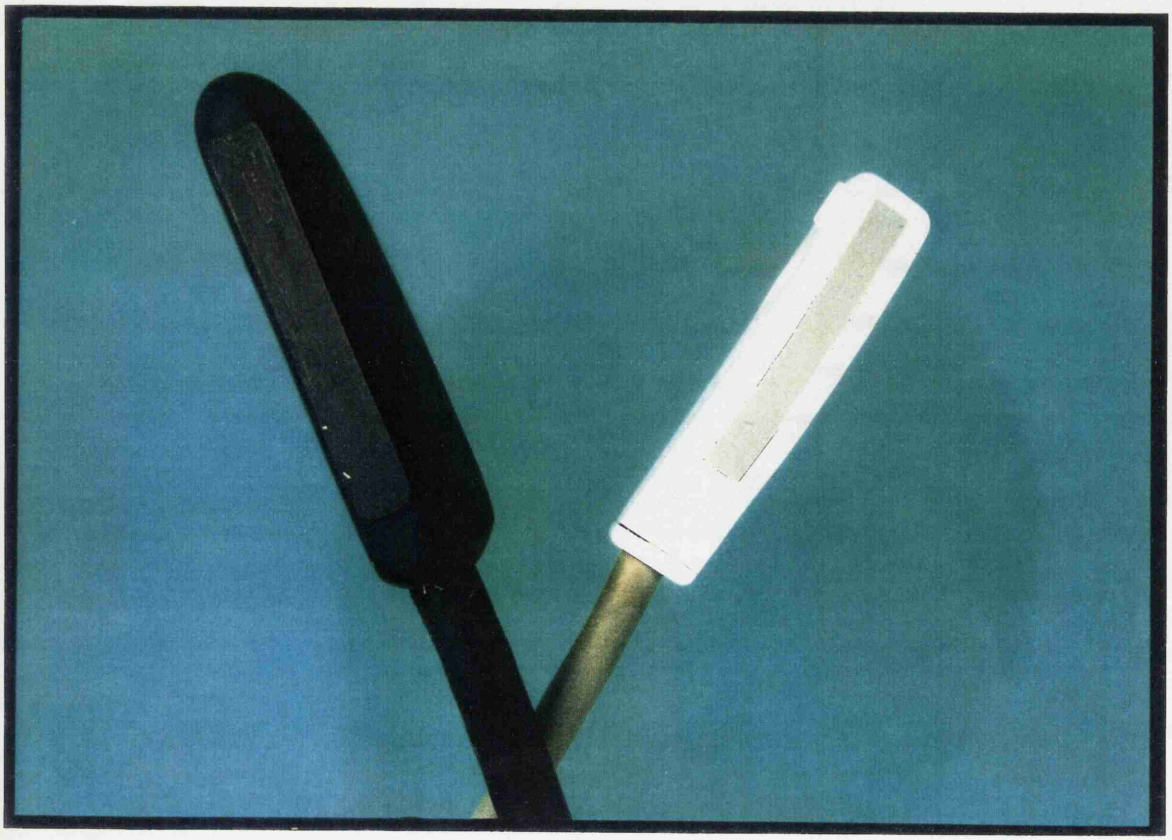
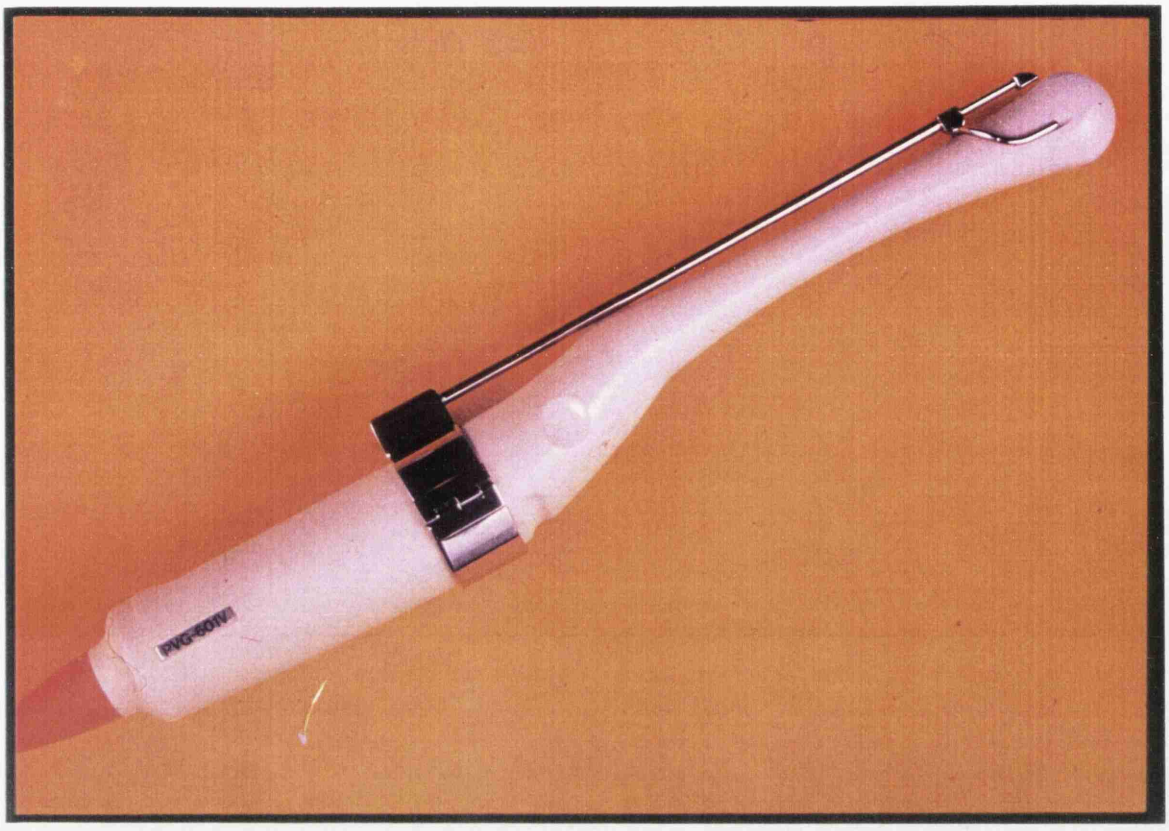


Figure 2. Equipment used for ultrasound guided follicular aspiration.
Upper: 6.0 MHz human transvaginal transducer fitted with the needle guide.

Lower: Foot pedal aspiration pump and a set of needles.
a: introducer with the stylet
b: 18g x 32cm
c: 18g x 45cm
d: Follicular fluid collection tube


PVC-501


Karivac 2
d
c
a
b

and inserted into the rectum. With gentle lateral movements of the probe, the right ovary first located and inspected, followed by the left ovary. The ovary was first examined for the presence of a corpus luteum. The probe was moved very slowly over the ovary from left to right changing the scanning angle, such that an image of optimal quality was obtained and the organ was thoroughly scanned. When measurements were made, the image was frozen and the structures of interest were measured using the electronic caliper facility.

2.2.2. Transvaginal scanning.

The Toshiba Capasee scanner fitted with a 6 MHz, 31cm long, human transvaginal transducer (model, 601v Toshiba) was used for transvaginal ultrasound scanning (TVS) (fig.2).

The procedure took place with the cow restrained in a crush, so that very little movement was possible during the session. To prepare the animal for TVS, 0.3 mg clenbuterol hydrochloride (Planipart, Boehringer Ingelheim, Berkshire, UK) was given intravenously for intestinal and uterine relaxation, followed after 10 minutes by intravenous injection of 0.7mg/100kg detomidine hydrochloride (Domosedan, SmithKline, Beecham Animal Health, Surrey, UK) to achieve mild sedation. Finally, an epidural anaesthetic of 5ml 2% lignocaine hydrochloride (Lignavet, C-Vet, Suffolk UK) was given to prevent abdominal straining. After such preparation of the cow, the tail was tied to one side.

The transducer was covered with a latex cover (Ultra-cover, Casmed, Surrey, UK) and to achieve optimal transmission of the ultrasound, coupling gel was applied in and outside of the cover. After emptying the rectum, the perineum and the vulva were cleaned thoroughly. The operator located the ovary with one hand, while the

other hand inserted the transducer in the vagina. The head of the transducer was located against the right vaginal wall, lateral to the cervical os. The right ovary was then positioned by manipulations per rectum so that it lay against the head of the transducer. The sonographic image of the ovary was improved by gentle rotations of the transducer and/or slight movements of the ovary. After the scanning of the right ovary was completed, the probe was moved to the left side of the vagina and the left ovary was examined. To prevent air invasion of the rectum, which by reason of the drug treatment would have been very difficult to remove, the operator's hand was not withdrawn from the rectum throughout the scanning session. Follicular structures were measured by an assistant upon instruction given by the operator.

2.2.3. Ultrasound guided follicular aspiration.

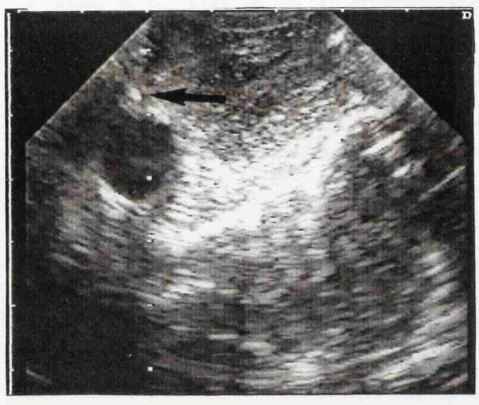
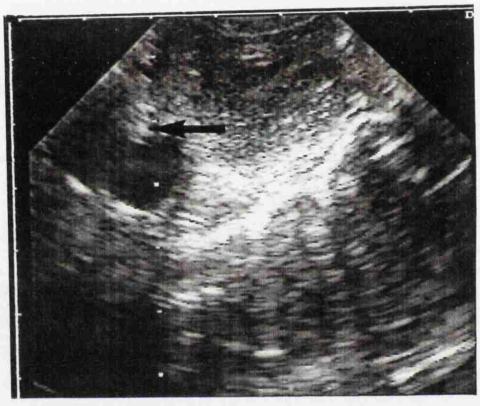
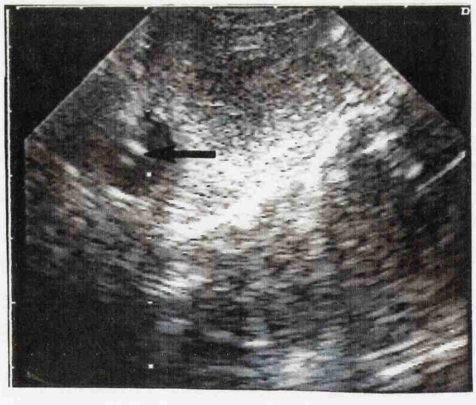
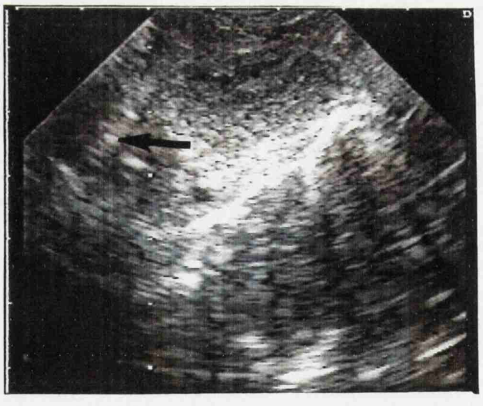
The technique was a modification of that developed and reported by our Research Group (Scott et al., 1994).

The same scanner as for the transvaginal scanning was used for the follicular aspirations. This scanner was equipped with a biopsy guideline, enabling the operator to predict the route of the needle on the monitor. The transducer was equipped with a clip-on needle guide (Toshiba, UK), which could accomodate needles of 15g or greater.

Three different types of needles were used. A 15 gauge, 29 cm needle bearing a central stylet was used as an introducer to penetrate the vaginal wall. The follicles were aspirated using 18 gauge, 32 or 45 cm needles with an echogenic tip (Casmed, Cheam, Surrey, UK) which could be passed through the introducer (fig. 2). The aspiration needle was connected by a 17g, 20cm long plastic extension to a

Figure 3 Ultrasound images of follicular aspiration
Four consecutive stages of follicular aspiration

Top left:	needle (arrow) in the ovarian tissue
Top right :	needle penetrating the follicle
Bottom left:	needle in the follicle
Bottom right:	the follicle is completely collapsed

25ml tube acting as collection reservoir. The collection reservoir was connected by 120cm long silastic tubing to the suction apparatus.

Two different suction units were used in the experiments. Initially an electric manually-activated peristaltic vacuum pump (Watson-Marlow 501) was used which was later replaced by a foot-pedal activated pump (Karri-vac 2, Rocket London UK). Both pumps were adjusted to aspirate 25 ml of water/minute (fig.2).

The pharmacological preparation of the animals was the same as for transvaginal scanning, except that the dose of detomidine hydrochloride was increased to 1.25mg/100kg. This drug treatment allowed approximately 20 minutes of sedation.

Before aspiration, both ovaries were rapidly scanned to estimate the follicular population and to locate the corpus luteum. Subsequently the transducer was placed ipsilateral to the ovary to be aspirated. An assistant introduced the 15g needle into the needle guide and punctured the vaginal wall. The stylet was then withdrawn and the aspiration needle was inserted. The operator manipulated the ovary per rectum so that the biopsy guideline on the monitor transected the follicle to be punctured. When the follicle was stable on the puncture line, the needle was advanced towards the follicle. As soon as the echogenic tip of the needle was seen in close proximity to the follicle the vacuum pump was activated, and suction was continued until the follicle appeared to have completely collapsed. The needle was gently rotated during aspiration in order to prevent blockage by a fold of the shrinking follicular wall (fig. 3). After the complete collapse of the follicle and depending on the experimental protocol, the needle was either withdrawn and flushed with phosphate buffer saline supplemented with foetal calf serum or was simply removed from the ovary until another follicle was positioned for aspiration. Having finished the aspiration of all scheduled follicles, the ovary was scanned again to verify that no follicle had escaped aspiration and the same procedure was

repeated on the other ovary. The aspirate from each follicle was collected, if required, into separate tubes containing a drop of heparinised saline.

2.3. Radioimmunoassays

To measure the plasma concentration of progesterone, oestradiol, LH and FSH double antibody radioimmunoassays were used.

2.3.1.Buffers.

General purpose buffer.

Phosphate buffer stock solution (PB 0.5M, pH 7.4), was prepared as followed and stored at 4°C.

Solution (a) 7.8 g sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$, MW 156.01) was dissolved in 100ml distilled water.

Solution (b) 14.02g disodium hydrogen orthophosphate anhydrous (Na_2HPO_4, MW 141.96) was dissolved in 200ml distilled water.

24ml of solution (a) was added to solution (b), and adjusted to pH 7.4. 0.1g thiomersal preservative (BDH Chemicals Ltd, England) was added.

To prepare PBS (0.05M) 1.75g Ethylenediamine tetracetic acid (EDTA) and 15.75g sodium chloride were dissolved in 175 ml of 0.5M PB and the solution was made up to 1.75 l with distilled water.

Assay Buffer

Fresh assay buffer was prepared every week and stored in the fridge, by dissolving 2g bovine serum albumin (BSA, FractionV, Sigma Chemical Co., St.Louis, U.S.A) into 800ml 0.05M PBS.

2.3.2. Assay protocols.

2.3.2.1 . General.

The gonadotrophin assays were carried out in disposable polystyrene tubes (LP3, Luckham Ltd, UK). For the steroid hormone assays the plasma was extracted in 13x100mm borosilicate glass tubes (Ciba-Corrning, Essex,UK). The ether extracts were dried and the assay was performed in 10.5x 7mm borosilicate assay tubes.

2.3.2.2 Luteinizing Hormone - Follicle Stimulating Hormone

2.3.2.2a Iodination

5μg of purified ovine LH (LER-1056C2)[a], or FSH (USDA-oFSH-19-SIAFP-1-2)[b] was reconstituted with 20μl of 0.05M PBS. Using a 100μl Hamilton syringe, 1mCi, of $Na^{125}I$ (Amersham, UK) was added to 20μl 0.05 M PBS and the radioactive solution was added to the hormone preparation. In this mixture 20μl of 0.5M PBS and 50μg of Cloramine T (BDH Chemicals Ltd) in 20μl of 0.05M PBS were added and the mixture was then incubated for 2 minutes in ice. The reaction was stopped by the addition of 100μg of sodium metasulphide in 20μl of 0.05M PBS. The solution was mixed gently and applied to the 7x30 sephadex G100 column (Pharmacia,Uppsala Sweden), and eluted with PBS 0.25% BSA. 30 fractions of 1ml were collected and 10μl fractions of each were counnted out in a gamma counter. The content of the peak tubes was diluted in PBS-BSA, pooled and stored at -20°C in 0.5 ml aliquots.

2.3.2.2.b Gonadotrophin standards

Purified ovine FSH (USDA-oFSH-19-SIAFP-RP-2)[b] and LH (NIH-oLH-S25)[c], were used for the standard curves. For LH, the range of the standards was 0, 1, 2, 4, 8, 16, 32, 64ng/ml, while an additional 128ng/ml standard was incorporated to the FSH set.

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a: kindly donated by Professor L.E. Reichert, Union University, Albany, NY, USA

b: kindly provided by USDA - ARS- Animal Hormone Program

c: kindly provided by the national hormone and pituitary Program, NIADDK Bethesda, MD, USA

2.3.2.2.c. Gonadotrophin antibodies

The first antibody (R151) for the LH was a rabbit antiserum raised against ovine LH (NIH-oLH-S25) was used at dilution of 1:20000, while the FSH antibody (A558/RIH) was a rabbit anti bovine purchased from UCB-Bioproducts, Belgium and used at a dilution of 1:10000.

The second antibody for both assays was a 1:20 anti-rabbit gamma globulin serum, freshly prepared in 1:200 normal rabbit serum (SAPU, Law Hospital, Carluke, Strathclyde, UK)

2.3.2.2.d. Gonadotrophins assay protocol

Both assays were set up to include two tubes for the total counts (TC), two tubes for the non specific binding (NSB) and two tubes for each standard and sample. Zero standard contained 100µl of assay buffer, while 100µl of either standard or sample was added to the other corresponding tubes. The sequence of the reagents addition was as follows:

I. Plasma samples 100µl or standards were added to the corresponding assay tubes.

II. The first antibody 200µl was added to all but the NSB tubes. In the latter PBS was added instead. The reagents were mixed and the tubes were incubated overnight at 4^0 C.

III. Iodinated hormone (12000-15000c.p.m. in 100µl PBS) was added to all the tubes which were again incubated overnight at 4^0 C.

IV. The second antibody was added to all tubes except the TC tubes and the tubes were incubated for a further night at 4^0 C for the LH assay and at room temperature for the FSH.

V. Separation of the bound and non bound phases was achieved by centrifugation of the tubes at 2000g for 15 minutes at 4^0 C using a MSE Mistral 6L centrifuge (Fisons Instruments, Sussex, UK). The supernatant was aspirated by a 18g needle connected to a pump attached to a running water tap and the tubes were subsequently counted for 1 min in an automatic Gamma counter (Minaxi Packard, Auto Gamma 5000series, United Technologies UK).

VI. The total binding of the assay was calculated in the tubes containing PBS instead of standard (the zero standard), while the non-specific-binding (NSB) was determined in tubes containing only PBS and second antibody. Calculations were made after the NSB value was subtracted from the value of each standard and sample.

2.3.2.3 Steroid Hormone Radioimmunoassay

The progesterone tracer[d] was prepared by iodination of 11α-hydroxyprogesterone glucuronide-tyramine using chloramine T and purified by solvent extraction and thin layer chromatography (Corrie et al., 1981).

The oestradiol tracer was prepared in our laboratory by a procedure that involves the iodination of histamine, the activation of oestradiol and the coupling of the activated oestradiol to the iodinated histamine, followed by separation in thin layer chromatography (Corrie et al, 1981).

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d: kindly provided by Dr C.E. Gray, The Royal Infirmary, Glasgow, UK

2.3.2.3.a. Oestradiol iodination.

The procedure was conducted as follows:

Iodination of histamine

In a 13 x100mm glàss tube the following reagents were mixed:

1. Histamine (10µl of 2.2µg in 5ml 0.5M Phosphate Buffer)
2. 1 mCi 125I Sodium Iodide(Amersham)
3. Chloramine T. (10µl of 50mg Chloramine T in 10 ml water).

The reagents were gently mixed and were left to react for 20 seconds.The reaction was stopped by the addition of sodium metabisulphide(10µl of 15 mg in 500µl water)

Activation of oestradiol

One mg of 17β-oestradiol-6-O-carboxymethyloxime, (Sigma, St Louis, USA) was disolved in 200µl dioxan and was stored in 50µl aliquots at -20^0 C.

In a fume cupboard two 10µl solutions each containing Tri-n-Butylamine/Dioxan and Isobutylchloroformate/Dioxan in a ratio 1:50, and 1:100 respectively were added to a 0.25 mg aliquot of the hormone. The mixture was mixed gently and incubated for 30 minutes at 10^{0}C in an ice water bath and was diluted with 300µl Dioxan precooled at 10^{0}C.

Following incubation, 50µl of this solution was transfered to the iodination mixture.

Coupling.

10µl 0.2M NaOH was added to the mixture of histamine/oestradiol which was incubated for one hour at 10^{0}C.

The solution was neutralised with 1ml 0.1M HCl and was subsequently extracted with 1ml Ethyl Acetate. Following a brief vortex the upper organic layer, which contained the activators was discarded and 1ml each of 0.1M NaOH and 0.5M Phosphate buffer pH 7 were added.

The mixture was then extracted twice with 1ml ethyl acetate and the pooled organic extract was reduced in volume by drying in 10ml glass tube over sodium sulphate.

Purification by thin layer cromatography (TLC).

The dry ethyl acetate extract was spotted on three TLC plates (PE SIL G/UV, Whatman Ltd, Kent, UK) which were subsequently developed for one hour in a mixture consisting of $CHCl_3$, MeOH, and acetic acid in the ratio 90: 10:1 respectively.

The plates were dried, wrapped in polythene film and scanned with a portable γ-counter. The hihgly radioactive bands were marked on the polythene and were cut out. The discs were put in 10ml glass tubes and were eluted with ethanol.

The different extracts were tested for specific and non-specific binding and displacement in a number of standard curves in order to select the best fraction.

2.3.2.3.b. Standards

Progesterone standards ranging from 0.31 to 20 ng/ml were made up by dissolving progesterone (Sigma, Chemical Co, Dorset, UK) in ethanol and than into assay buffer, and they were stored in 0.5ml aliquots at -20°C.

Oestradiol standards (Sigma UK) were prepared after an initially in of absolute alcohol and then by serial dilutions in assay buffer they finally ranged from 100 down to 0.8 pg/ml.

2.3.2.3.c. Antibodies

The first antibody for progesterone[e] was raised in sheep against 11α-hydroxyprogesterone hemisuccinate BSAand it was used at an initial dilution of 1:20000. The first antibody for oestradiol[f], was raised in sheep against 6 carboxymethyloxime-oestradiol-17β-BSA antibody. It was used at an initial dilution of $1:3x10^6$.

The second antibody for both assays was raised a 1:20 donkey anti-sheep:goat gamma globulin, prepared in a 1:200 normal goat serum (SAPU, Law Hospital, Carluke, Strathclyde, UK)

2.3.2.3.d. Progesterone and Oestradiol assay protocol

All samples and standards were extracted with ether prior to double antibody radioimmunoassay. Plasma and standards (0.2 and 0.4ml for progesterone and oestradiol assay respectively) were vortexed for 10 minutes with 3 ml di-ethyl ether in a multi-tube vortexer (SMI, Alpha Laboratories, UK). The aqueous phase was

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e: provided by B.Cook, The Royal Infirmary, Glasgow, UK

f: provided by Dr B.A.Morris, AFRC, APU, University of Surrey, UK

then frozen in methanol-dry ice bath and the ether phase containing the steroid was carefully transferred into clean 10.5x7mm borosilicate glass assay tubes. The ether was evaporated under air after the assay rack was placed in 37°C waterbath to accelerate evaporation

. The methodology after the stage of extraction and drying of the tubes was as follows:

I. Labelled hormone (10000-12000c.p.m.) was added to each tube, followed 15minutes later by the addition of 0.2ml of the first antibody in all but the NSB and TC tubes.

II. The progesterone and oestradiol assay were then incubated for 45 minutes at 37^0C and two hours at room temperature respectively.

III. The precipitating antibody (0.4ml) was added to all tubes, the reagents were mixed and the tubes were incubated overnight at 4^0C.

IV. Separation of bound and non-bound phases and the counting of the tubes was performed as described for LH and FSH.

2.4. Molecular Biology Techniques

2.4.1. **Introduction**: Polymerase Chain Reaction (PCR)

Polymerase chain reaction is a relatively new technique (Saiki et al., 1985) that allows more than a millionfold amplification of the DNA from a selected region of

the genome, provided that the nucleotide sequence of the of the particular region is at least partially known.

The principle of the PCR process involves the following steps:

a: Separation of the complementary strand of the selected DNA, achieved by heating the sample to temperatures around $94/95^0$ C.

b: The separated strands are then annealed with the addition of excess oligonucleotide primers, which match to the nucleotide sequence of the DNA to be amplified. The primers are usually 15-30 nucleotides in length and have different sequences, not complementary to each other. The primers hybridise specifically to their complementary sequences at the 3'ends of each strand of the target DNA. The possibility of reassociation of the two DNA strands is minimal since the concentration of the primers in the mixture is high, favouring the formation of the primer-template complex. The temperature and the time used for this step is dependent on the sequence to be amplified.

c: Extension of the primer-template complex by DNA polymerase. The extension occurs from the 3`end, where the pimer was initially coupled, towards the 5` end of the DNA strand. In early PCR protocols (Saiki et al., 1985; 1986; Noonan & Roninson 1988) the polymerase was derived from *Escherichia coli* and it was thermosensitive (Klenow fragment DNA polymerase I). This meant that fresh enzyme had to be added to the reaction mixture after the completion of each cycle. The isolation of the thermostable DNA polymerase from the bacterium *Thermus aquaticus* (*Taq* polymerase, see Saiki et al., 1988) superceded the cumbersome Klenow polymerase I. *Taq* polymerase is stable at 94°C and thus it can be added at the start of the reaction mixture and it remains active throughout many amplification cycles.

The set of three steps mentioned above is referred to as a cycle. The PCR product usually starts accumulating after the third cycle. After a number of cycles the PCR

product becomes the predominant template which is subsequently amplified. Effective DNA amplification usually requires 20-30 reaction cycles.

PCR is an extremely sensitive methodology since it enables the detection of a single DNA molecule in a sample, by the production of enormous numbers of copies of the specified DNA sequence. Furthermore, tiny amounts of RNA can be analysed. Complementary DNA (cDNA) is first synthesised after the catalytic action of the enzyme reverse transcriptase. This cDNA can be used as the template for the enzymatic amplification.

The PCR product can later be detected in agarose or polyacrylamide gels after it has been marked with a fluorescent dye. The DNA migrates through the gel at a rate that is dependent on its molecular size and the density of the gel.

2.4.1.a. **Procedures**: RNA extraction.

Granulosa cells were isolated from follicular fluid collected during follicular aspiration. The follicular fluid was centrifuged at 5000rpm for 10 minutes to allow the precipitation of the cells. The liquid phase was stored at -20°C for hormonal analysis and the small pellet, containing the granulosa cells, was frozen in liquid nitrogen.

300 µl of RNAzol (Biogenesis, Poole, UK) was added to the sample (granulosa or luteal cells) as soon as the latter was taken out from the liquid nitrogen container and the mixture was subjected to high frequency ultrasound treatment (Vibra cell, Sonics & Materials, Danbury, Connecticut, USA) for approximately 20 sec to break down the cellular membranes. Subsequently, chloroform (BDH, UK) was added at a rate of 10% of the total volume, the mixture was put in crushed ice for 5 minutes and centrifuged for 15 minutes at 14000rpm. The upper liquid phase was

then pipetted into a clean tube with an equal volume of isopropanol (Sigma, UK), kept in ice for 15 minutes and then centrifuged for 15 min at 14000rpm. A tiny sediment formed at the bottom of the tube, which was vortexed with 150µl of 80% ethanol (BDH AnalaR UK) and re-precipitated after a brief spin at 10000rpm. The liquid phase was discarded and any droplets were removed using a small piece of absorbing paper. The pellet was dissolved in 100µl of water (BDH, Biomedical, UK) and 10µl of sodium acetate pH 7 was added. 220µl of frozen ethanol (twice the volume of the mixture) was subsequently added and the sample, the solution was mixed and incubated at -20°C for 1 hour. The mRNA, in the form of a small pellet, was isolated after a further centrifugation and stored in liquid nitrogen after solubilisation in 10µl of water.

2.4.1.b. Reverse transcription

Complementary DNA was produced from the mRNA using the enzyme reverse transcriptase. The reaction mixture consisted of 6 mmol of $MgCl_2$, 1µl Buffer (250mM Tris-HCL pH 8.3 at room temperature, 375 mM KCl and 15mM $MgCl_2$), 10 mM dithothreitol, an antioxitant factor to protect the enzyme (DTT Gibco, BRL), 15µM deoxyribonucleoside triphosphate (dNTP), Random Hexamer, 30 units of reverse transcriptase (Superscripts^{TM}RT, Gibco UK), 6units of RNase inhibitor (rRNasin, Promega Madison, USA). The final volume was adjusted with water (BDH Biomedical, UK) to 10µl and the mixture was incubated for 1 hour at 37°C. The resulting cDNA was stored at -20°C.

2.4.1.c. PCR protocol.

The procedure was carried out in 0.5ml microcentrifuge tubes and the final reaction volume was 30µl. Water, 10 mM Tris HCl buffer (pH 8.8 at room temperature containing $(NH_4)_2SO_4$) and 2 mM $MgCl_2$ were added into the tube and the latter was exposed to ultraviolet (uv) light (Spectro-linker XL1500UV, New York, USA) at an energy level of 1 J/cm^2 to eliminate contamination. Subsequently 0.3µl of the two primers, 20µM of deoxynucleoside triphosphates (dNTP, Gibco BRL) and 5 units of the *taq* polymerase (AmpliTaq, Strategene Ltd, Cambridge, UK, or Finnzyme, Oy, Epsoo, Finland) were added. The mixture was capped with 30µl of mineral oil to prevent evaporation and internal condensation of the reagents and the tubes were placed in a PCR thermal reactor where 30-33 cycles were normally performed. The initial denaturation took place at 95°C for 2 minutes and each cycle consisted of the following three steps:

(I denaturation 94°C for 20 sec

(II) annealing 55°C for 30 sec

(III) extension 72°C for 1 min

After completion of the PCR amplification, the product was stained with loading buffer (Evans blue dye) and separated on 1% agarose gel (Amresco, Solon, Ohio) containing 5µl /100ml ethidium bromide (Sigma, UK) against a molecular marker (100bp ladder, Gibco). The gel electrophoresis was performed in TBE (Tris-borate EDTA) buffer at a constant voltage of 120-150 volts for 20 -30 minutes depending on the size of the gel. The gel was checked for any DNA bands under uv light and photographs were taken on either Polaroid film (Polaroid MP-4, Polaroid Corporation, Campridge, USA) or by a thermal printer (68B, Mitsubishi Electric Corporation, Tokyo, Japan).

2.4.2. Southern blotting

Southern blotting was developed by E.M. Southern (1975) and is the most widely used technique for analysing gene expression. To perform a Southern blot analysis, the DNA fragments are first separated by size on agarose gel. The gel is then coated with a nylon membrane to which the DNA fragments are transferred and bound by the flow of a appropriate buffer. Subsequently, a DNA probe labelled with a radioisotope hybridises with the complementary sequence of the DNA fragments on the membrane. The DNA fragments complementary to the probe can be detected by radiography of the membrane and the bands will indicate the size and the number of these fragments.

2.4.2.a. Preparation of the DNA probe

The technique was applied to study of the FSH receptor when very faint DNA bands were obtained on the agarose gel. The probe was prepared by PCR from a plasmid containing the rat FSH-receptor. The PCR product was carefully removed from the gel with a surgical blade and was put in a piece of dialysis tube (Visking, Medicell International Ltd. London, UK) with 1ml of 0.5xTris-acetate-EDTA (TAE) buffer. To remove the DNA from the bands, the tube was subjected to electrophoresis for 20min at 100 volts and the polarity was reversed for a few seconds before switching off. The fluid was treated with frozen alcohol for 1-2 hours, centrifuged at 14000rpm for 15minutes and the small sediment containing the DNA was dissolved in 15µl of water.

2.4.2.b. Labelling the DNA probe

The DNA probe (1µl) was labelled with 5µl of ^{32}P (Amersham UK) in the presence of 10µl of random hexamer, 1µl Exo-Klenow polymerase and 10µl primer buffer. After 10 minute incubation at 37°C the reaction was terminated by the addition of 2µl stop mixture and the radioladelled probe was kept in ice until further use. The mixture was then passed down a sephadex column and the purified solution was collected in 6 tubes. The contents of the three most radioactive tubes were stored in aliquots to be used later for the hybridisation.

2.4.2.c. Southern blotting protocol

The agarose gel underwent denaturation for 30 minutes in 0.5M NaOH and 1.5M NaCl followed by neutralisation for further 30 minutes in 0.5 Tris HCl and 1.5M NaCl. Prior to denaturation the gel was photographed with a ruler on the side, to mark the size of the bands. To transfer the DNA bands from the gel to the nylon membrane, an improvised devised was used consisting of a plastic box wrapped with absorbent paper. This acted as wick on top of which the gel was laid with the other side covered with the nylon membrane (Biotrans Nylon membrane, ICN Biomedicals, Aurora, Ohio) and the system was placed in a container where 150-200ml of 20x SSC RNA-free solution was poured. A pile of paper towels was then placed on top of the nylon membrane and pressed down by a 250-500g weight.

2.4.2.d Hybridisation

The nylon membrane was prepared for hybridisation after incubation for 1 hour at 63°C with the pre-hybridisation solution, which contained 8.16 ml water, 120µl herring sperm DNA(Gibco,BRL), 120µl of 10% sodium dodecyl sulphate (SDS), 3ml 20x SSC and 600µl of 100x Denhardt's solution. The incubation was carried out in an oven (micro oven, Hybaid Ltd, UK) equipped with a rotating tube holder. Before the addition of the radiolabelled probe the latter was heated at 98°C for 5 minutes and then cooled immediately on ice for 10 minutes. The mixture was then incubated overnight at 65°C. The next morning the unbound probe was washed out with two solutions of 2x and 1x SSC, each one applied for 30 minutes. The membrane was covered with X-ray film (Hyper film-MP Amersham) and kept at -70°C for 12 hours for development of the autoradiogram on which the position of the DNA fragments, complementary to the probe, appeared as black bands.

2.4.2.e. Primer sequences

P450scc

41. 5` GCC ATC TCG TAC AAG TGC CAT TGC

40. 5` GCC GTC TAC AAG ATG ATG TTC CAC AC

P450arom.3. 5` GAG CAT GTT AGA GGT GTC CAG CAT

5. 5`GGA TTG GAA GTG CCT GCA ACT ACT

FSH rec.

16. 5` CCC TTA AGC CTT AAA ATA GAC TTG TTG CA

64. 5`CCT CTG CCA GGA GAG CAA GGT GAC

GAPDH

50. 5` GTG AAG GTG GGA GTC AAC G

51. 5`` GGT GAA GAC GCC AGT GGA CTC

$P450_{17\alpha}$

42. 5` CTT GTC GGA CCA AGG AAA AGG CGT

7. 5` CAA CCA CGG GAA TAT GTC CAC CAG

2.5 Statistical Analysis

To investigate the effects of repeated multiple aspiration on the secretion of ovarian steroids and pituitary gonadotrophins between aspirated and control cows, mean pre-aspiration hormone concentration was subtracted from each post-aspiration hormone concentration and the balance was tested using analysis of variance.

In the dominant follicle experiment pre- and post- aspiration values were tested by analysis of variance. Mean post-aspiration values were compared to the pre-aspiration means using two-tailed Tuckey's procedure for pairwise comparison of means, followed by analysis of variance for a single factor experiments with repeated measurements.

Chapter 3

RESULTS

3.1 Radioimmunoassays

3.1.1 FSH and LH radioimmunoassay

3.1.1.1 FSH and LH Iodination

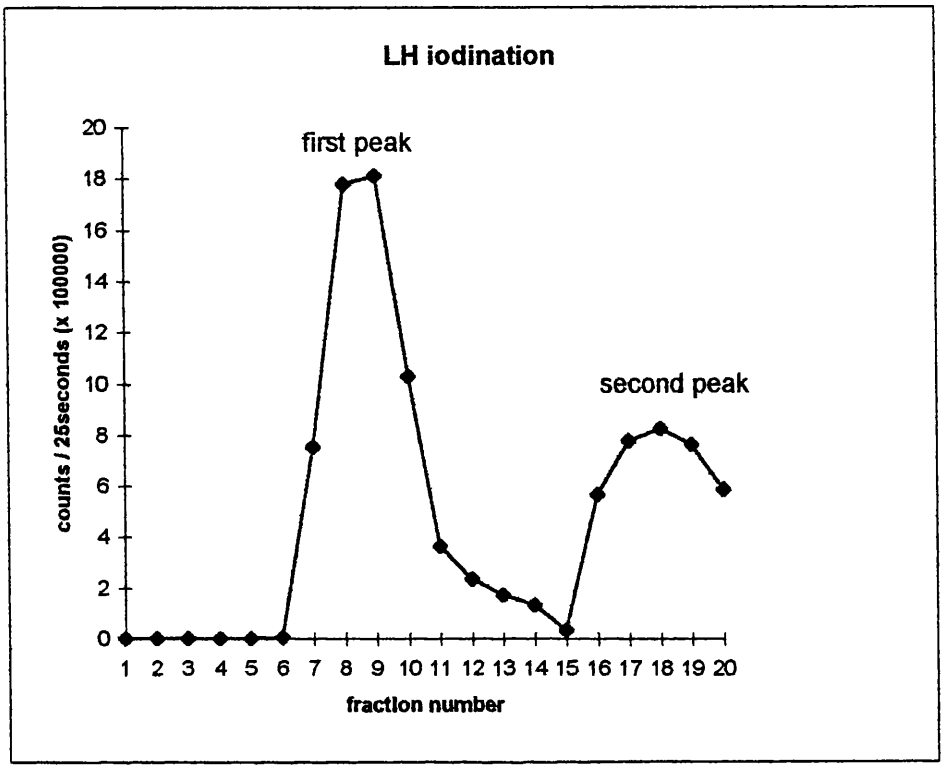
Two peaks of radioactivity were eluted from the Sephadex column, as shown in fig. 4. Consecutive fractions from each peak were tested for antibody binding and displacement after coincubation with either 1 or 8 ng/ml LH or FSH. The radioactivity of 10µl fractions was counted for 25sec and the total binding and the percentage of non specific binding for a typical iodination of FSH and LH is shown in tables 1 and 2 respectively.

Fractions 8-11 usually gave the highest binding and the lowest NSB. Fractions from the second peak possessed acceptable antibody binding but they gave high NSB and very little displacement in the presence of unlabelled LH and FSH.

Table 1. Radioactivity and immunoactivity of different fractions following FSH iodination

Fraction	CPM	%NSB	%TC	1ng/ml	8ng/ml
6	549850				
7	1848250	2.7	15.3	100.5	64.9
8	2994150	3.4	20.6	97.2	60.1
9	**3285850**	**2.8**	**22.8**	**93.7**	**62.4**
10	324028	4.1	20.1	88.6	65.5
11	2700300	5.3	19.8	91.1	88.9
16	783950	4.5	17.4	98.3	94.6
17	976550	6.8	24.1	105.3	91.5
18	1156280	7.1	21.4	98.9	94.2

Figure 4. Typical elution pattern of iodination of ^{125}I - LH (upper panel) and ^{125}I - FSH (lower panel).


LH iodination
first peak
second peak
counts / 25seconds (x 100000)
fraction number
0
2
4
6
8
10
12
14
16
18
20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

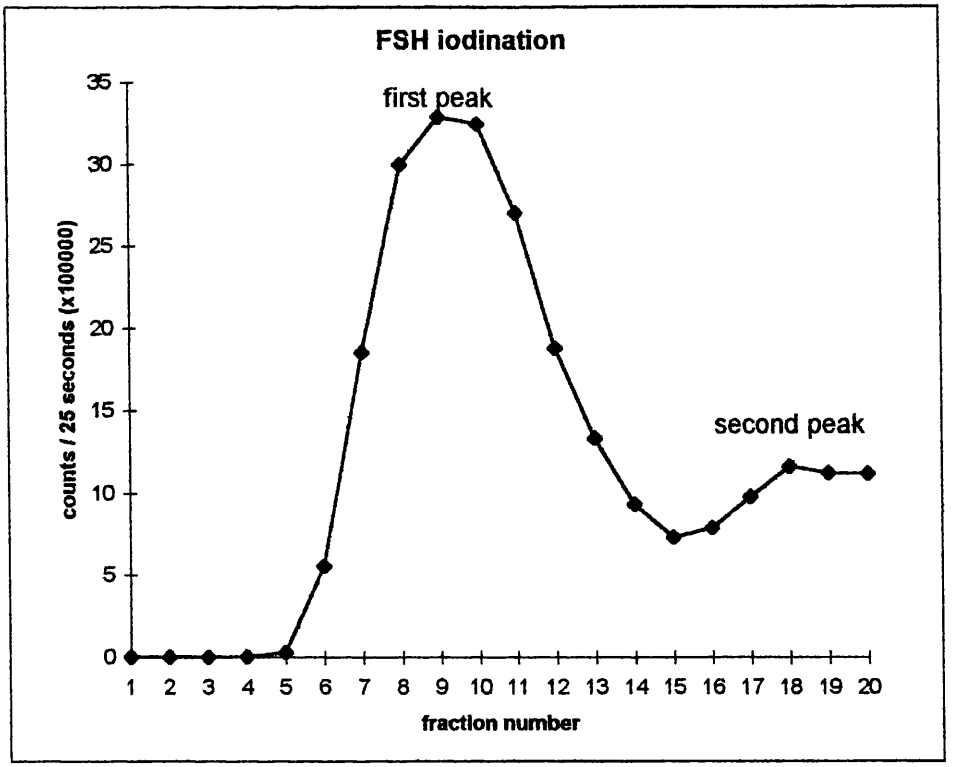

FSH iodination
first peak
second peak
counts / 25 seconds (x100000)
fraction number
0
5
10
15
20
25
30
35
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Table 2. Radioactivity and immunoactivity of different fractions following LH iodination

Fraction	CPM	%NSB	%TC	1ng/ml	8ng/ml
6	5472				
7	752160	3.4	26.8	96.8	52.7
8	**1777950**	**3.3**	**43.8**	**94.2**	**44.6**
9	1810250	3.8	44.6	88.4	49.7
10	1028900	5.5	39.8	89.8	55.6
11	363884	5.1	45.7	97.2	79.9
16	566050	6.5	55.1	104.2	88.1
17	778300	5.2	51.2	99.8	103.7
18	736296	5.1	56.1	101	95.4

3.1.1.2 FSH and LH assay validation

The mean standard curves derived from 13 LH and 11 FSH assays are shown in figures 5a and 5b respectively. In both cases, increasing the concentration of the unlabelled hormone enabled good progressive displacement of the labelled hormone.

Sensitivity

The sensitivity of the assay, defined as twice the standard deviation of the zero standard was 0.21 ± 0.01ng/ml and 0.3 ± 0.07ng/ml in the LH and FSH assays respectively (n=7 assays).

Specificity

Specificity of LH and FSH antibodies in terms of their crossreaction with other pituitary hormones have been found to be as follows:

LH	oGH (NIH-oGH-13)	1.3%
	oFSH (NIH-oFSH-16)	<0.1%
	oTSH (NIH-oTSH-9)	<0.1%
	oPRL (NIH-oPRL-1-2)	<0.1%
FSH	bLH	0.2%
	bTSH	0.1%
	bGH	<0.01%
	bPRL	<0.01%

Precision

The intra- and inter- assay coefficient of variation for two plasma pools containing high and low concentrations were

for LH: 4.3 and 8.8% and 3.8 and 5.7% respectively (n=14).

for FSH: 5.4 and 9.1% and 4.6 and 7.3% (n=12)

3.1.2 Steroid hormone radioimmunoassay

3.1.2.1 Oestradiol iodination

The various radioactive bands obtained after TLC (see Materials & Methods) were checked for total binding, %NSB and % displacement from zero, (either as single or pooled solutions) after the radioactivity of 10µl fractions from each band was assessed for 25 sec.

3.1.2.2 Standard curves

The recovery of different concentrations of progesterone and oestradiol when added to assay buffer varied from 91 - 98% and 85-103% for progesterone(n=3) and oestradiol (n=5) respectively (Fig. 6a, b)

Mean standard curves obtained from 17 oestradiol and eight progesterone assays are shown in fig. 7a and 7b respectively.

3.1.2.3 Assay validation

Specificity

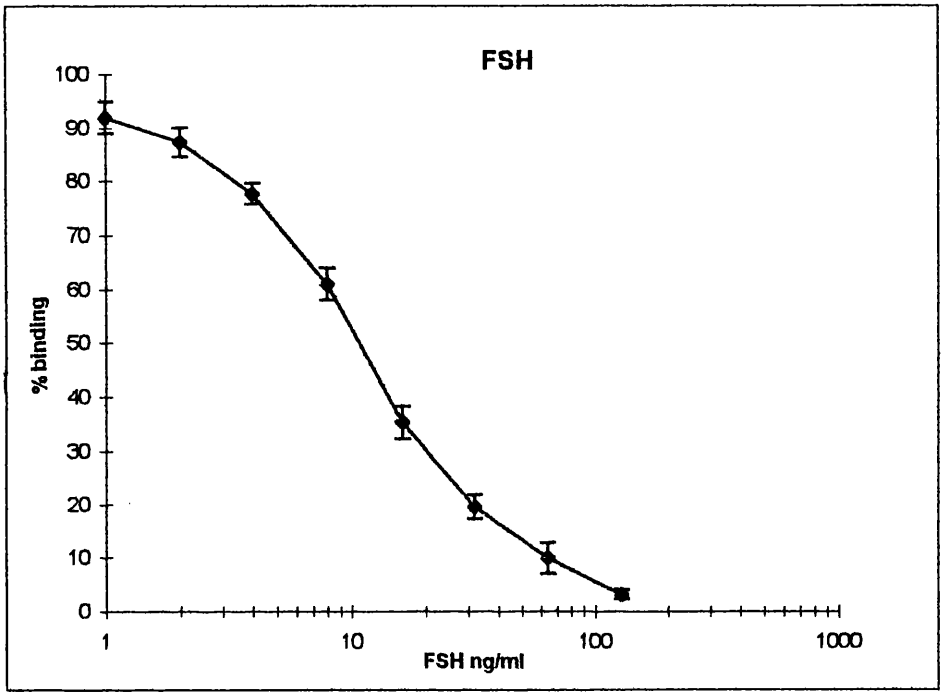
Crossreaction of progesterone antibody with various steroids was as follows

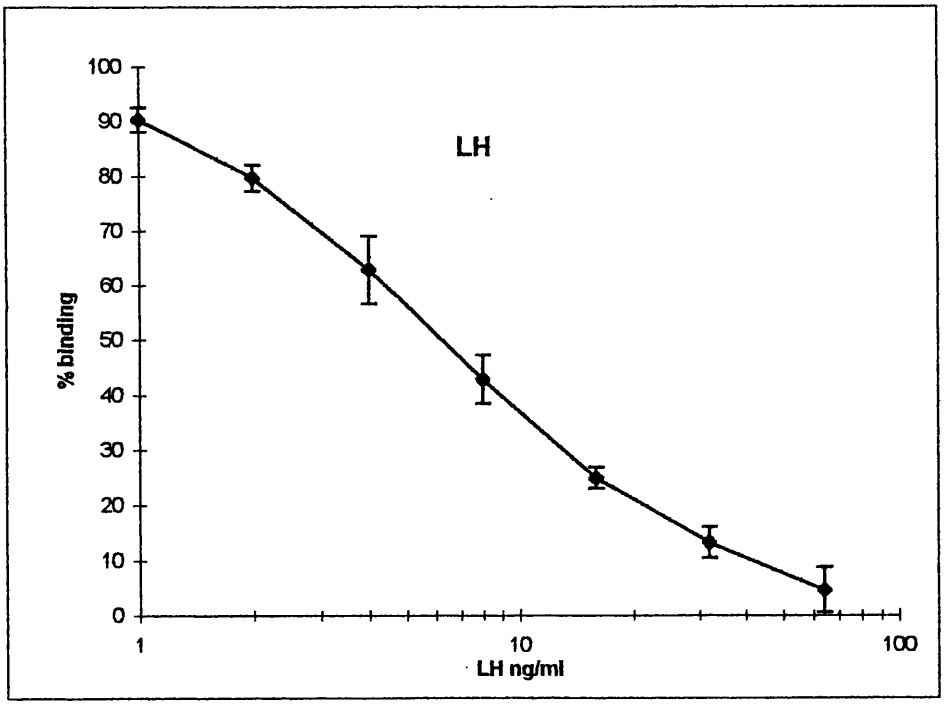
11α-hydroxyprogesterone	61%
11-deoxycorticosterone	4%
17α-hydroxyprogesterone	1.5%
20α-hydroxypregn-4-en-3-one	1%
other steroids	<1%

Oestradiol antibody crossreactivity is given bellow

oestrone	12%
oestriol	1.3%
androgens	<1%
progesterone	<1%
corticosterone	<1%

Figure 5. Composite standard curves for FSH (n=11) and LH (n=13) Vertical bars denote ± S.D.


FSH
100
90
80
70
60
50
40
30
20
10
0
% binding
1
10
100
1000
FSH ng/ml


LH
100
90
80
70
60
50
40
30
20
10
0
% binding
1
10
100
LH ng/ml

Precision

Coefficients of variation for two plasma pools containing low and high plasma concentration are given in the table that follows:

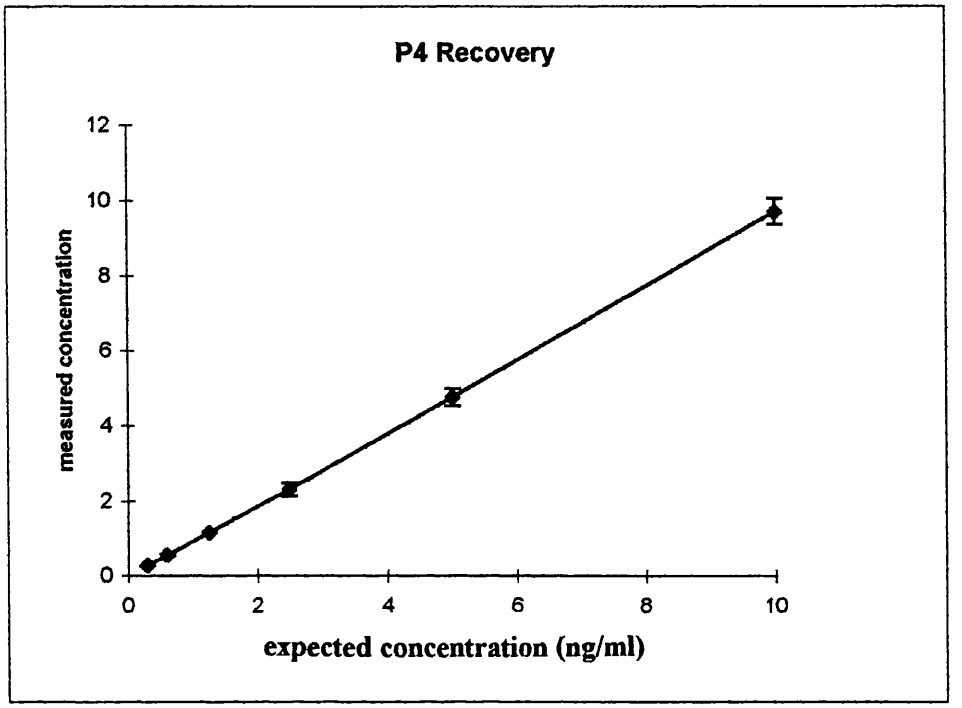
Table 3. Inter and intra-assay coefficients of variation for progesterone and oestradiol radioimmunoassay in sample pools containing low and high concentrations of hormone

		Progesterone(n=8)	**Oestradiol** (n=12)
Inter-	High	8.5%	11%
	Low	13%	8.9%
Intra-	High	6%	6.7%
	Low	5%	7%

Sensitivity

Assay sensitivity (2 X SD of zero standard) was 0.2 ± 0.1ng/ml for the progesterone assay(n=8) and 1.0 ± 0.5pg/ml for the oestradiol assay (n=12).

Figure 6. Mean recovery after extraction of progesterone P_4 (n=3) and oestradiol (n=5)
Vertical bars denote ± S.D.


P4 Recovery
measured concentration
0
2
4
6
8
10
12
0
2
4
6
8
10
expected concentration (ng/ml)

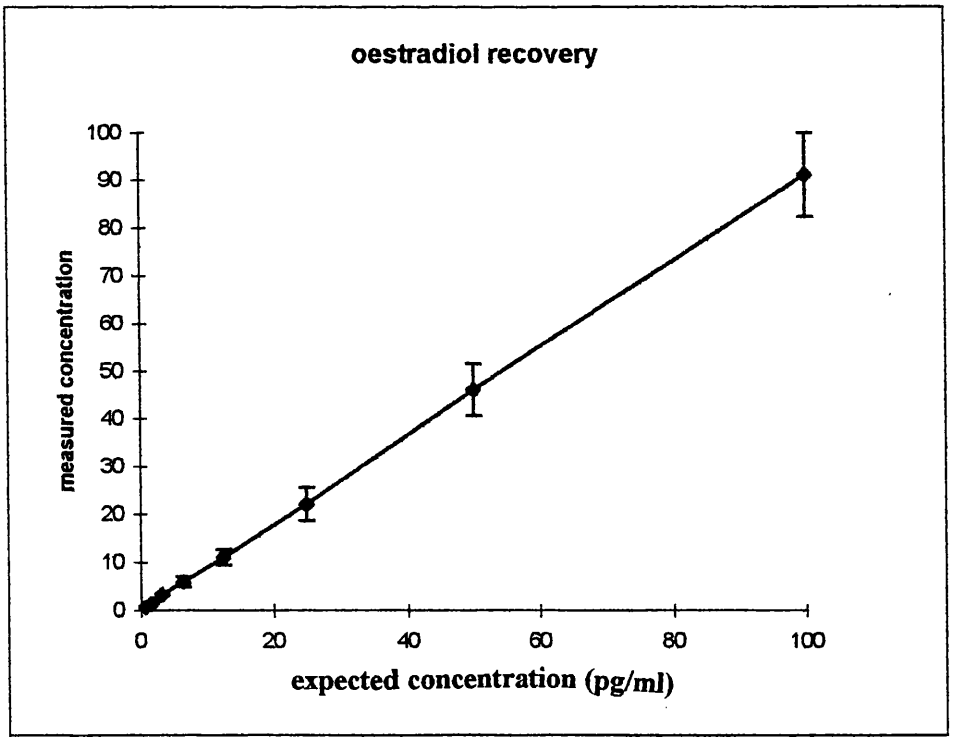
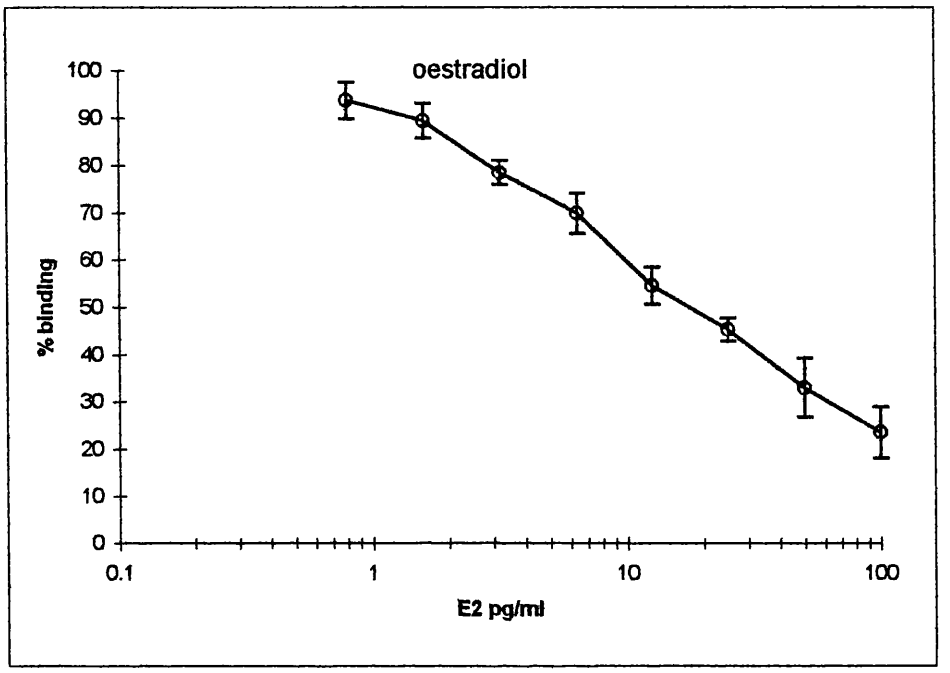
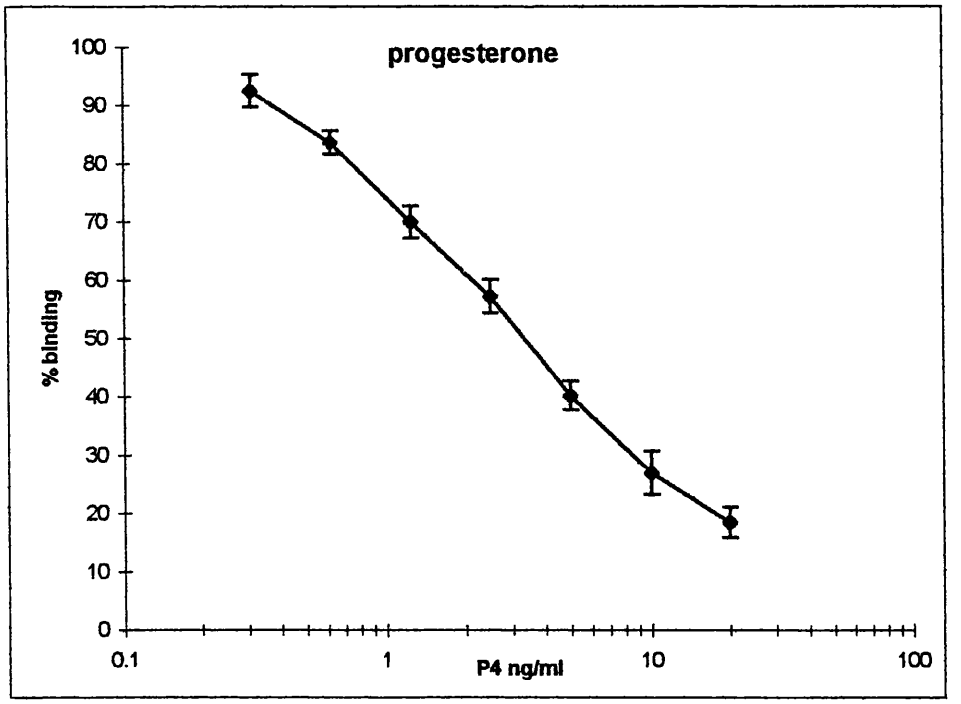

oestradiol recovery
measured concentration
0
10
20
30
40
50
60
70
80
90
100
0
20
40
60
80
100
expected concentration (pg/ml)

Figure 7 Composite standard curves for oestradiol (n=17) and progesterone (n=8)
Vertical bars denote ± S.D.


oestradiol
% binding
100
90
80
70
60
50
40
30
20
10
0
0.1
1
10
100
E2 pg/ml


progesterone
% binding
100
90
80
70
60
50
40
30
20
10
0
0.1
1
10
100
P4 ng/ml

3.2 Ultrasound guided ovum pick-up

3.2.1 Introduction

The aims of this preliminary study were

a) to develop a technique for transvaginal ultrasound guided follicular aspiration which could be performed repeatedly on the same donor cow with minimal disruption to her oestrous cycle and

b) to grade the recovered oocytes for their suitability for *in vitro* fertilisation

Two trials were carried out using four cows in the first and three cows in the second. It was attempted to aspirate all follicles >3mm at approximately one week intervals, during the early, mid and late stages of the luteal phase i.e approximately days 3-4, 9-12 and 14-17 of the oestrous cycle, over 3-4 cycles.

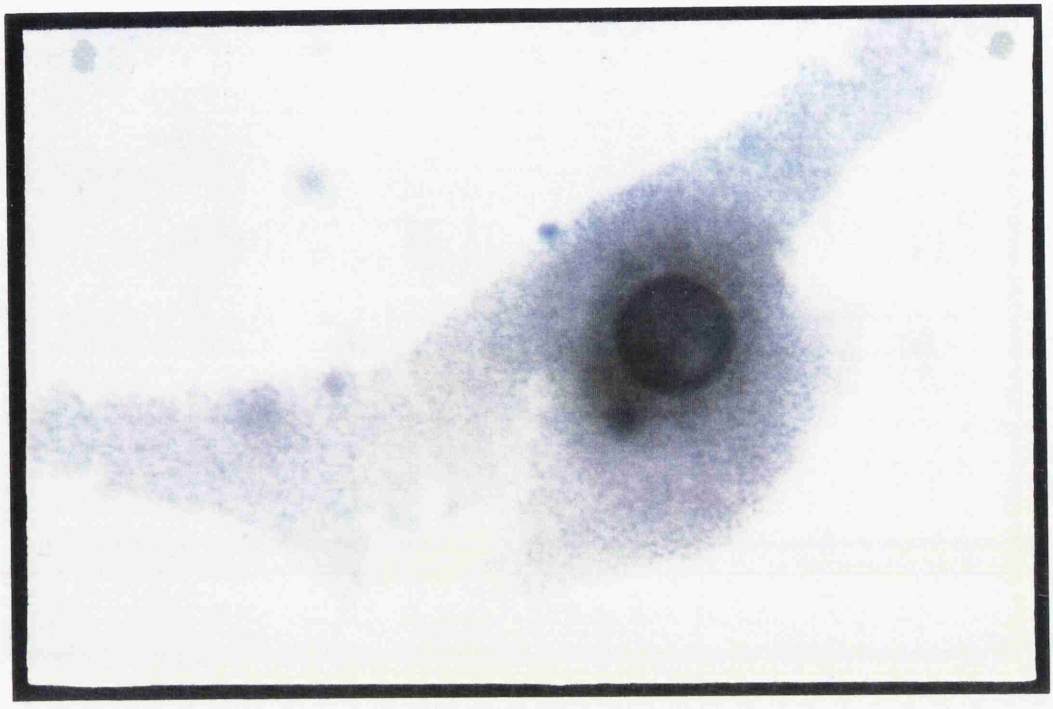
Oocyte quality

Oocyte quality was determined as described by de Loos et al. (1989) as follows:

Grade 1	Compact multilayered cumulus; homogenous ooplasm; cumulus-oocyte complex (COC) light and transparent
Grade 2	Compact multilayered cumulus; homogenous ooplasm but with some rough appearance; oocyte with some dark peripheral zones; COC less transparent.
Grade 3	Irregular ooplasm with dark clusters; COC dark; loose cumulus.
Grade 4	Expanded cumulus with scattered cells in dark clusters; irregular COC and ooplasm, and denuded oocytes (fig. 8, 9).

Figure 8 **Upper:** Grade one oocyte (x40) surrounded by compact multilayer of cumulus cells.

Lower: Grade two oocyte (x100) some dark clusters are noted in the ooplasm (Arrows)



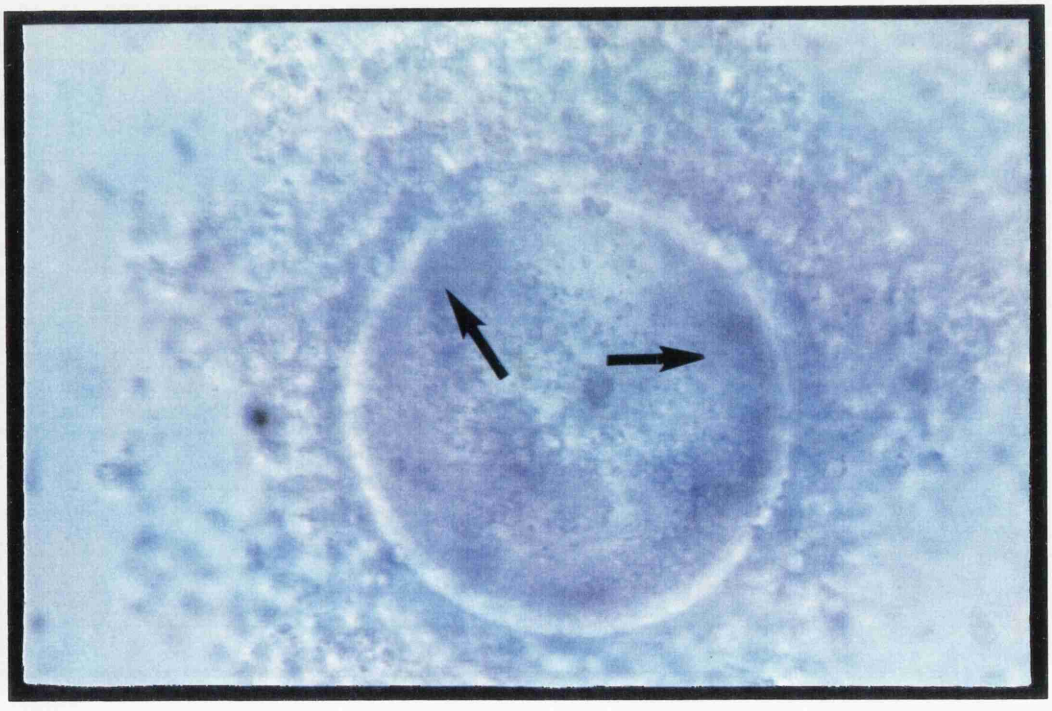
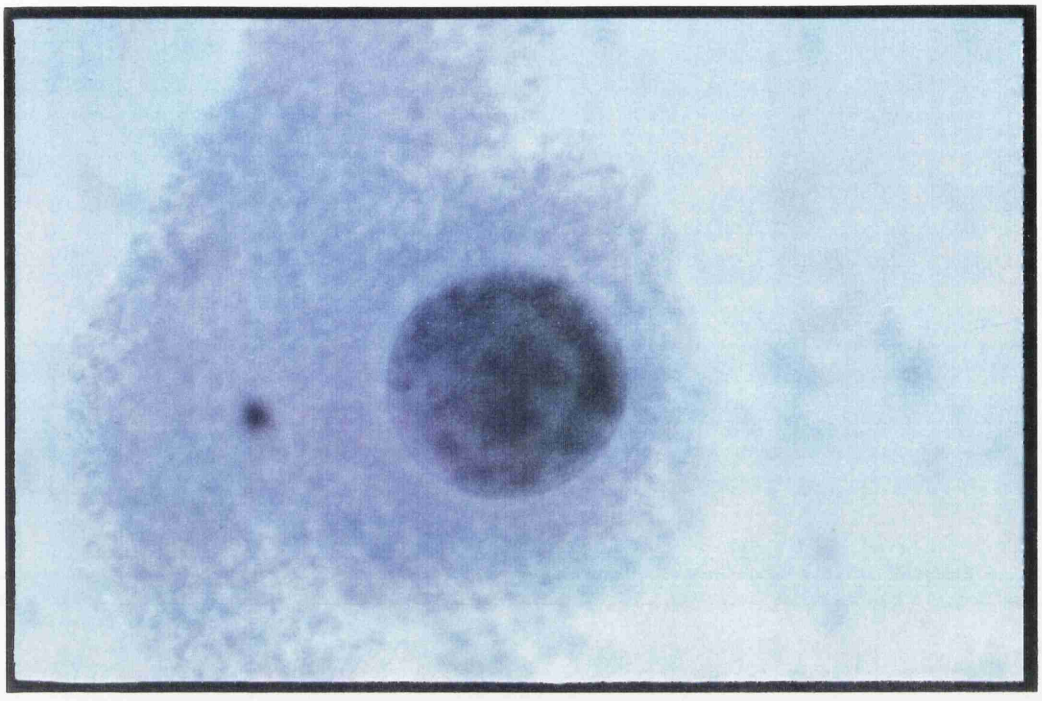
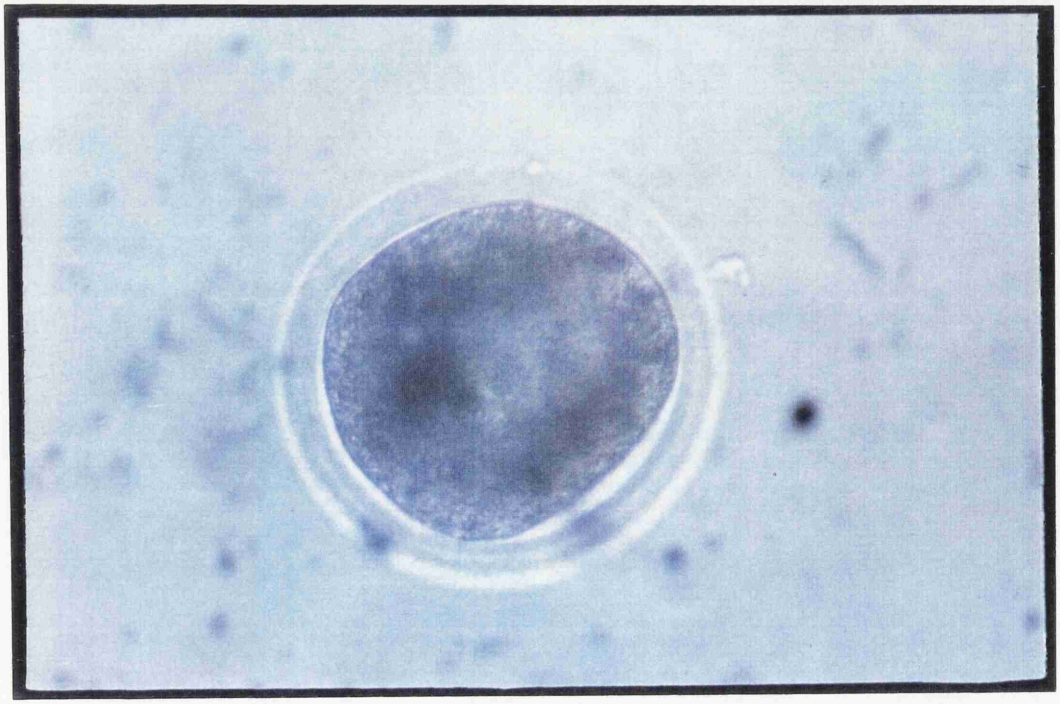


Figure 9 **Upper**: Grade three oocyte (dark irregular ooplasm)

Lower: Denuded oocyte -grade four.





3.2.2 Oocyte recovery

In the course of oocyte recovery during the early, mid and late stages of the luteal phase from seven cows, 189 follicular punctures were attempted and 177 follicles were aspirated as detailed in table 4. The size range of the aspirated follicles according to the stage of the luteal phase is given in table 5.

Table 4. Oocyte recovery rate during the three stages of the luteal phase

	Stage of the luteal phase			
	Early	Mid	Late	Total
Attempts	45	77	67	189
Aspirations	42	70	65	177
% Success	95.5	91	96.5	93.7
No of oocytes	15	23	16	54
% recovery	35.7	32.8	24.6	30.5

Table 5. Classification of follicles aspirated during the three luteal stages according to their size

	Follicular diameter (mm)				
Stage of cycle	≤4	4-6	7-9	10-12	≥13
Early	1	22	8	6	5
Mid	-	43	11	8	8
Late	-	40	10	6	9
Total	1	105	29	20	22
% aspir.	0.56	59.3	16.3	11.2	12.5

In total 54 oocytes were collected from 177 aspirated follicles (recovery rate 30.5%).

Oocyte recovery rate was initially below 10% but performance improved and overall recovery rate reached 30.5% after 13 follicular aspiration sessions as shown in table 6. The highest yield of oocytes was achieved during the mid luteal phase (42.6%, $P<0.1$), while during the early and late luteal phases the recovery rate was 27.8 and 29.6% respectively (table 9).

Table 6. Summary of oocyte collection rates from 13 aspiration sessions

session	stage of cycle	number of cows	number of aspirated follicles	number of oocytes collected	oocyte recovery rate %
1	E	4	11	1	9.1
2	M	4	12	1	8.3
3	L	4	23	4	17.4
4	E	4	12	5	41.6
5	M	4	26	9	34.6
6	L	4	25	8	32
7	E	4	11	5	45.4
8	M	4	16	6	37.5
9	L	2	9	3	33.3
10	E	2	4	1	25
11	M	3	13	4	30.7
12	L	3	9	4	57.1
13	E	1	6	3	50
TOTAL			177	54	30.5

There was no significant difference between the number of follicles aspirated from the right and left ovaries. However, more oocytes were collected from the right ovary (recovery rates for right and left ovary were 37.2 and 24.1% respectively) as summarised in table 7.

Table 7. Variation in oocyte collection rates from each ovary at different stages of the luteal phase

	Right ovary		Left ovary	
stage of cycle	folls aspirated	No of oocytes	folls aspirated	No of oocytes
Early	20	11	22	4
Mid	38	12	32	11
Late	35	9	30	7
TOTAL	93	32	84	22

In the course of ten oestrous cycles a mean of 18.5 ± 3.75 follicles were punctured per cycle. No difference was observed in the number of aspirated follicles between the mid and late stages of the luteal phase (7.9 ± 1.3 and 5.6 ± 1.4 for mid and late-stages respectively) while during the early luteal phase the mean number of aspirated follicles was 4.7 ± 1.5.

The number of follicles available for aspiration in each stage of the cycle as well as the oocyte recovery rate varied between idividual cows. The mean number of oocytes harvested from each cow per oestrous cycle is given in table 8.

Table 8. Mean number of oocytes collected from individual cows

Cow ID	Number of collections	Follicles aspirated	Oocytes obtained	% Recovery
47	n=3	17.7±1.2	5	28.2
5	n=2	14±3.5	6	41.7
18	n=2	12.5±2.1	2.5	20.4
99	n=2	9.5±2.12	1.5	15.6
96	n=2	11.5±2.1	2.5	21.7
9	n=1	14	7	50
156	n=1	9	4	44.4

3.2.3. Oocyte quality

The majority (74.1%) of the harvested oocytes were of either grade three or four. Eight oocytes were of grade one (14.8%) and six of grade two (11.1%). Table 9 details the classification of the oocytes according to their quality and cycle stage.

Table 9. Variations in oocyte quality at different stages of the luteal phase.

GRADE	EARLY		MID		LATE	
	No of oocytes	% recovered	No of oocytes	% recovered	No of oocytes	% recovered
1	3	20	4	17.4	1	6.2
2	1	6.7	2	8.7	3	18.8
3	4	26.7	10	43.5	4	25
4	7	46.7	7	30.4	8	50
TOTAL	15	27.8	23	42.6	16	29.6

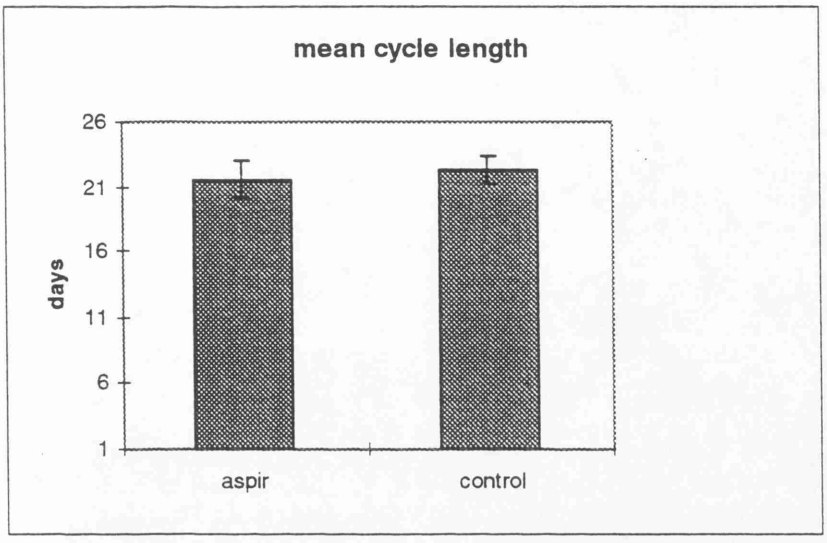
3.3.1. Effect of repeated multiple follicular aspiration on interoestrous interval

The oestrous cycles of ten cows were closely monitored over two to three consecutive cycles. Six cows were subjected to weekly transvaginal ultrasound guided follicular aspiration between days 3-5, 9-12 and 14-17 and four cows were scanned transvaginally without aspiration (mock aspiration).

Of 22 interoestrous intervals in the aspirated group, 20 fell in the range 18-26 days (21.62 ± 1.45). For two cows there was a delay in return to oestrus in one cycle: the first (cow 4) returning to oestrus naturally after 34 days and the second (cow 156) returning after 61 days but only after prostaglandin injection to cause regression of a persistant corpus luteum. The data from the latter cycle are not included in the results. In control animals, the average cycle length (n= 8 cycles) was 22.37 ± 1.06 days (range 21-26).

When aspirations were performed during the luteal phase, there was no significant difference in the mean interoestrus interval of the aspirated and control cows as shown in text fig. 1.

Text fig. 1 Mean oestrous cycle length of aspirated and control cows



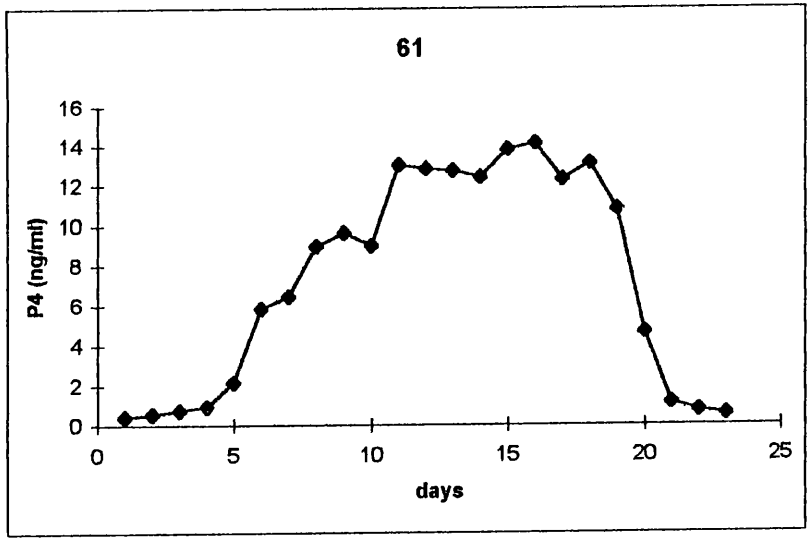
3.3.2. Effect of follicle aspiration upon ovarian and pituitary hormone production

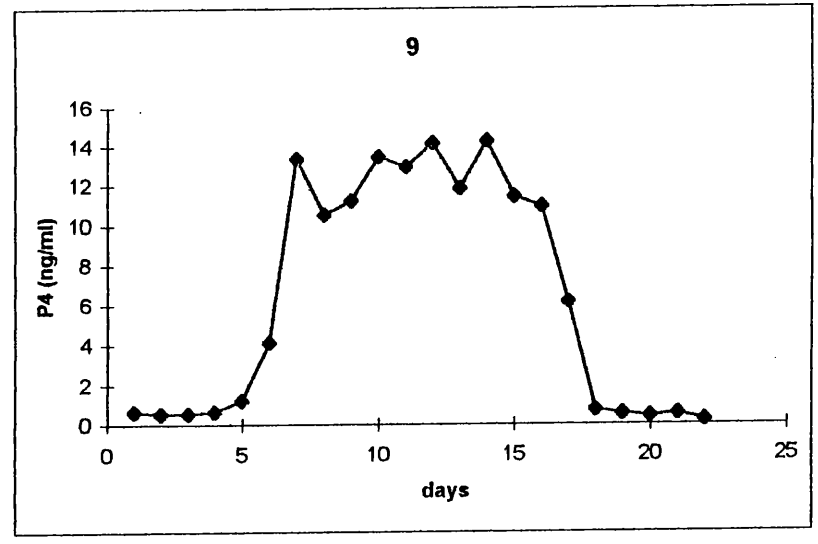
The eefect of multiple follicle aspiration was tested by looking at plasma progesterone concentration in daily blood samples and plasma LH and oestradiol in blood samples collected at hourly intervals from 4 hours before to 45 hours after aspiration. The results are given below.

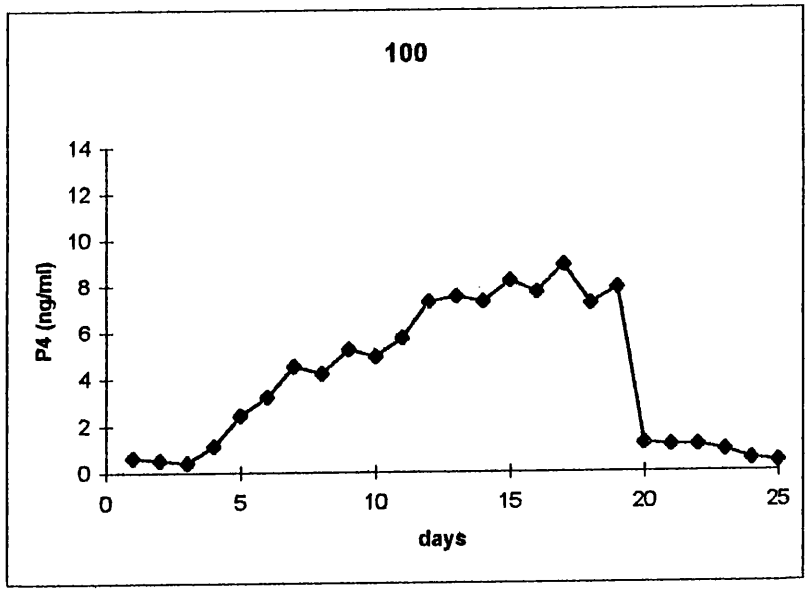
3.3.2.1 Plasma progesterone after multiple follicle aspiration

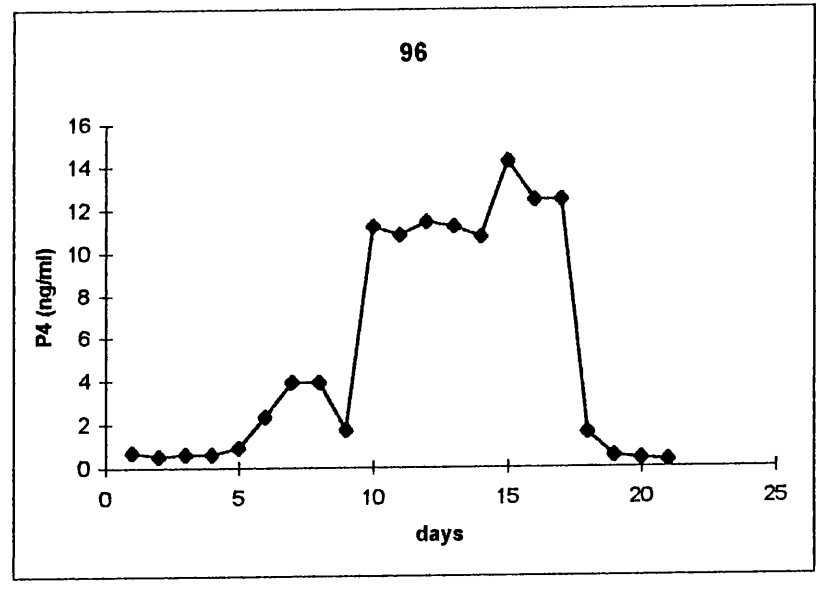
Plasma progesterone concentrations from samples collected throughout the cycle from representative aspirated and control animals were used to construct progesterone profiles for each cycle as shown in figures 10 and 11. Progesterone concentration increased from day 4 to near maximal levels around day12 of the cycle and remained maximal until day 16 to 18. In both groups the maximum

Figure 10. Progesterone concentration in daily in four cows subjected to repeated multiple follicular aspiration..


61
P4 (ng/ml)
0
2
4
6
8
10
12
14
16
0
5
10
15
20
25
days


9
P4 (ng/ml)
0
2
4
6
8
10
12
14
16
0
5
10
15
20
25
days


100
P4 (ng/ml)
0
2
4
6
8
10
12
14
0
5
10
15
20
25
days


96
P4 (ng/ml)
0
2
4
6
8
10
12
14
16
0
5
10
15
20
25
days

progesterone concentration was between 12 and 14ng/ml. Only in one cow from the each group was plasma progesterone found to be below 10ng/ml (cows 100 and 158). There was no significant difference in mean plasma progesterone concentration at any stage of the cycle between the aspirated and control cows (table 10)

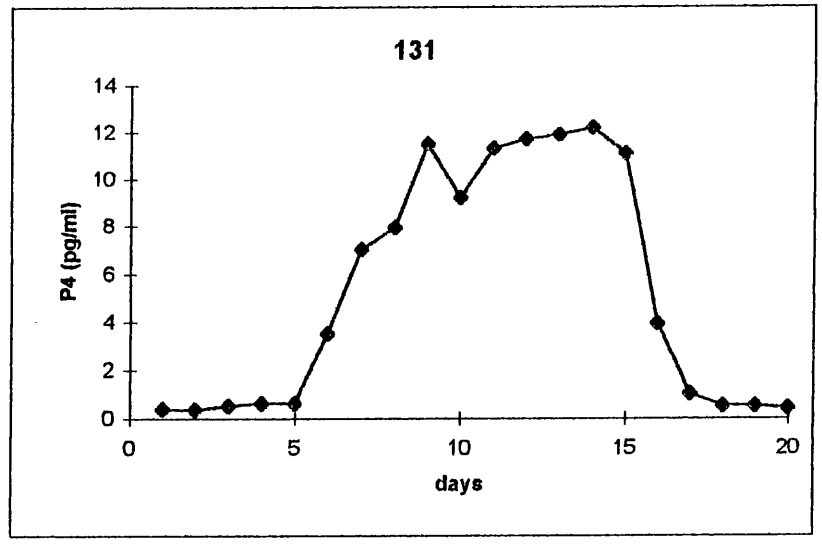
Table 10. Mean plasma progesterone concentration in aspirated and control cows at three stages of the luteal phase

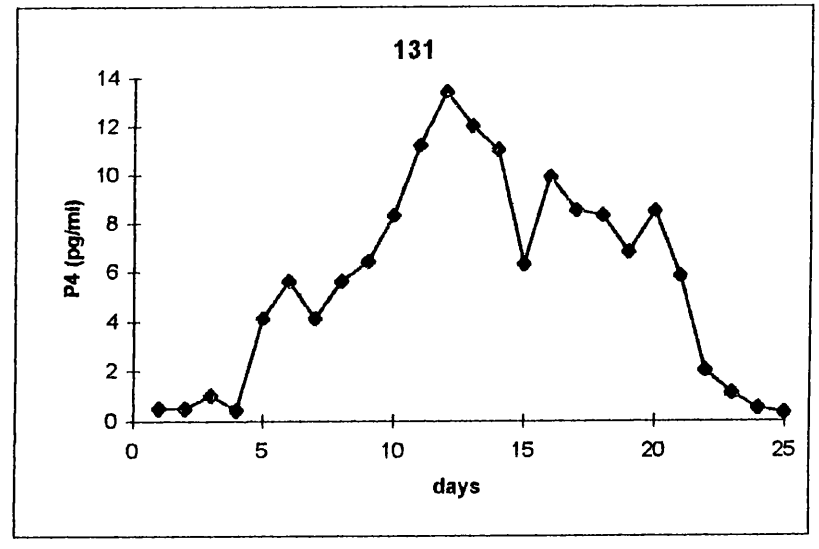
	Early luteal	Mid luteal	Late luteal
Aspirated	0.71 ± 0.56	7.15 ± 2.26	5.88 ± 3.04
Control	0.54 ± 0.17	9.36 ± 3.42	4.1 ± 2.8
Significance	P>0.05	P>0.05	P>0.05

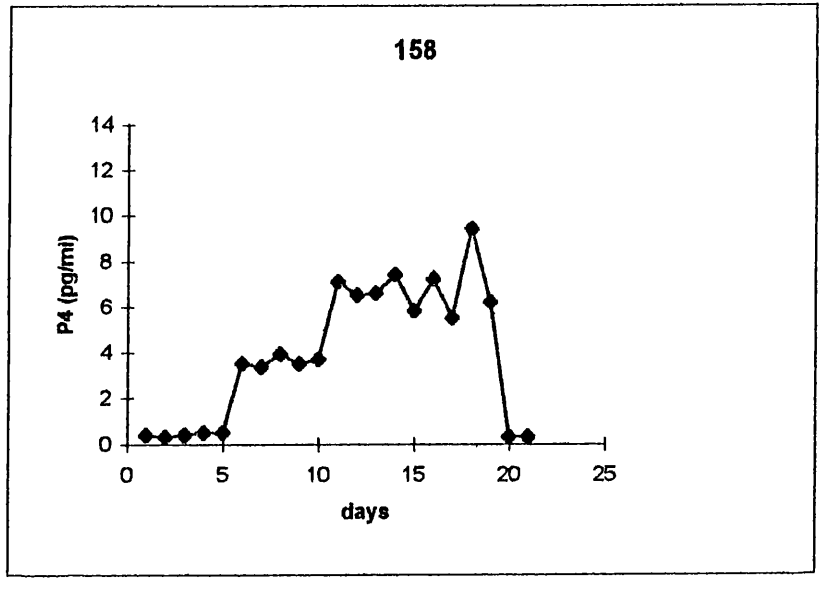
3.3.2.2 Plasma oestradiol after multiple follicle aspiration

Plasma oestradiol concentration in samples collected at hourly intervals from four hours before until 45 hours after aspiration or mock aspiration is summarised in table 11.

Figure 11 Progesterone concentration in daily samples of two consecutive cycles of two control cows


131
P4 (pg/ml)
0
2
4
6
8
10
12
14
0
5
10
15
20
days


131
P4 (pg/ml)
0
2
4
6
8
10
12
14
0
5
10
15
20
25
days


158
P4 (pg/ml)
0
2
4
6
8
10
12
14
0
5
10
15
20
25
days

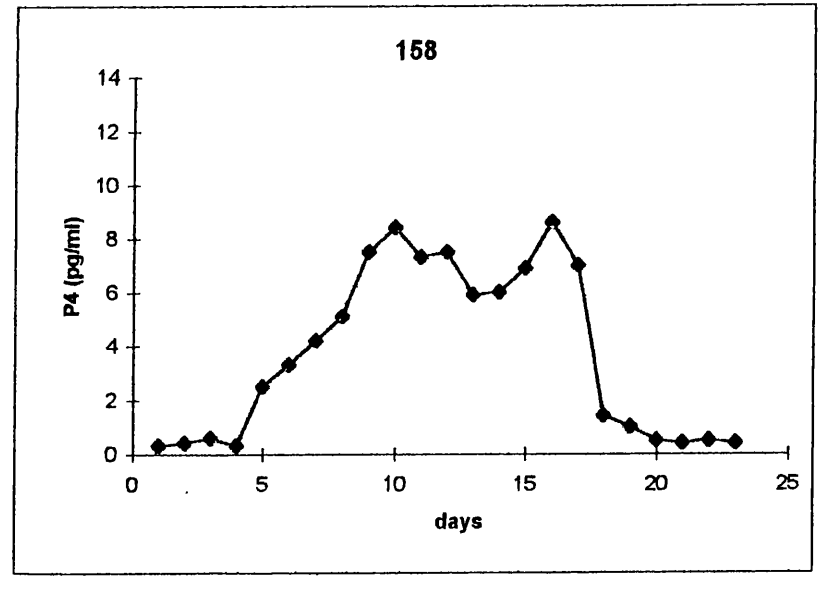
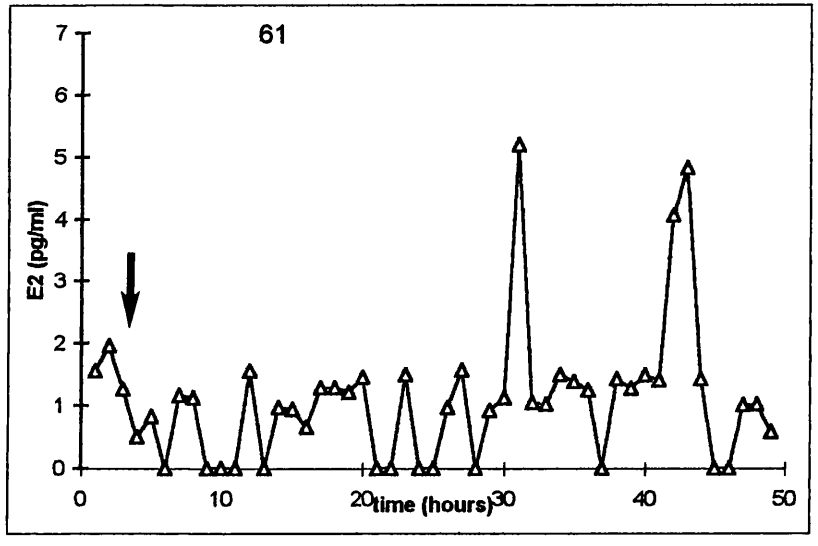
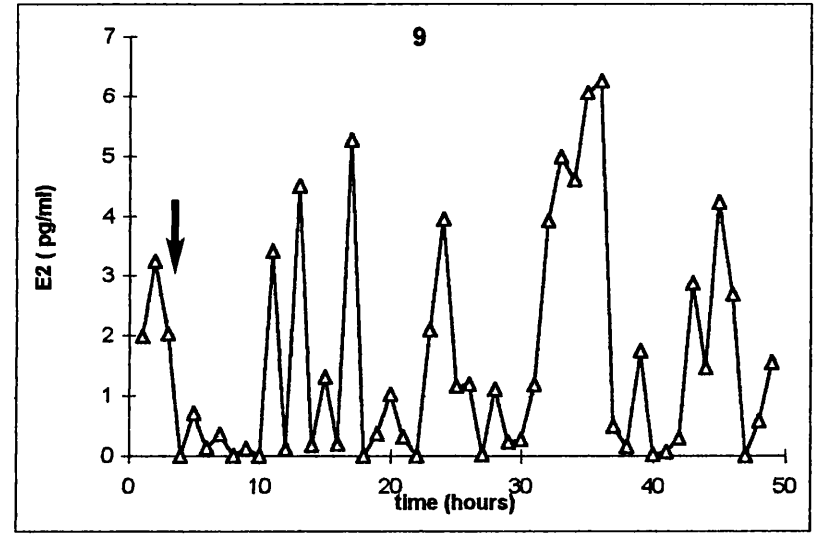
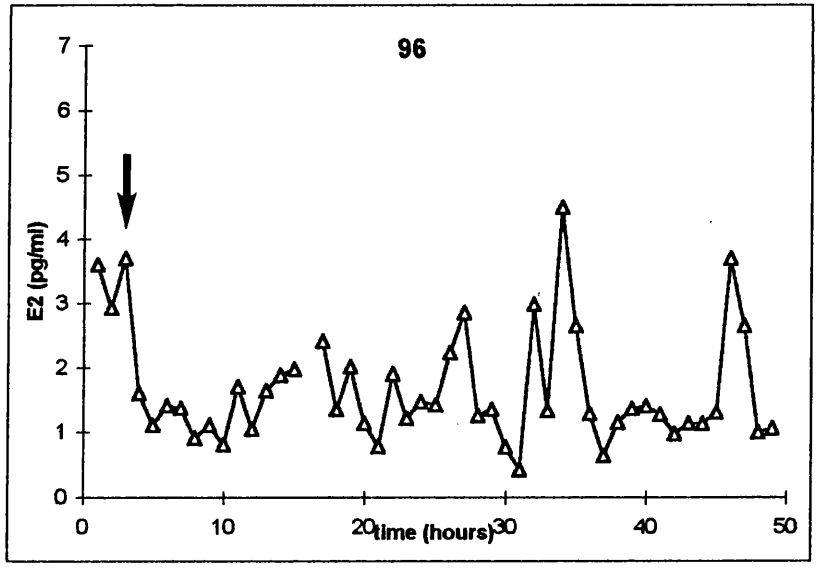

158
P4 (pg/ml)
0
2
4
6
8
10
12
14
0
5
10
15
20
25
days

Figure 12 Plasma oestradiol concentration in hourly samples from four representative cows subjected to multiple follicular aspiration during the **early** luteal phase. The arrow indicates the time of aspiration.


61
E2 (pg/ml)
time (hours)
0
1
2
3
4
5
6
7
0
10
20
30
40
50


9
E2 (pg/ml)
time (hours)
0
1
2
3
4
5
6
7
0
10
20
30
40
50


96
E2 (pg/ml)
time (hours)
0
1
2
3
4
5
6
7
0
10
20
30
40
50

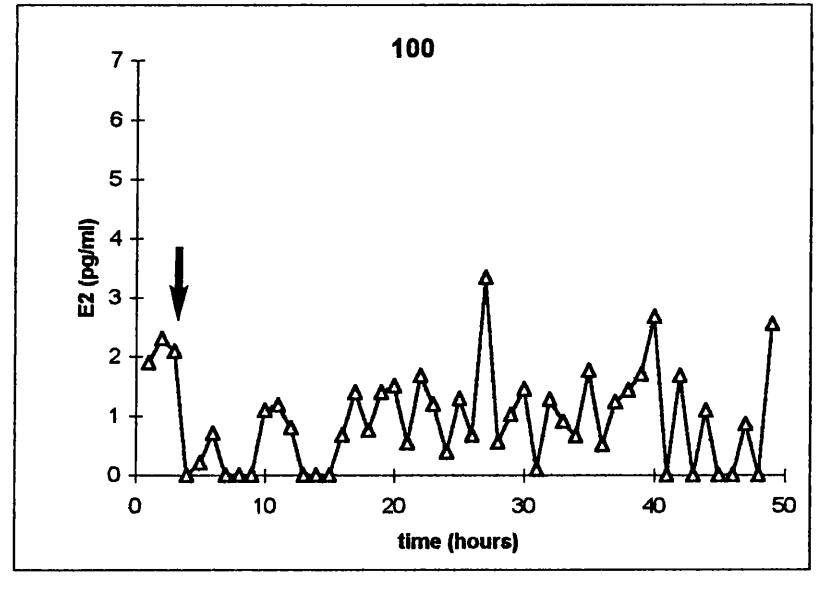
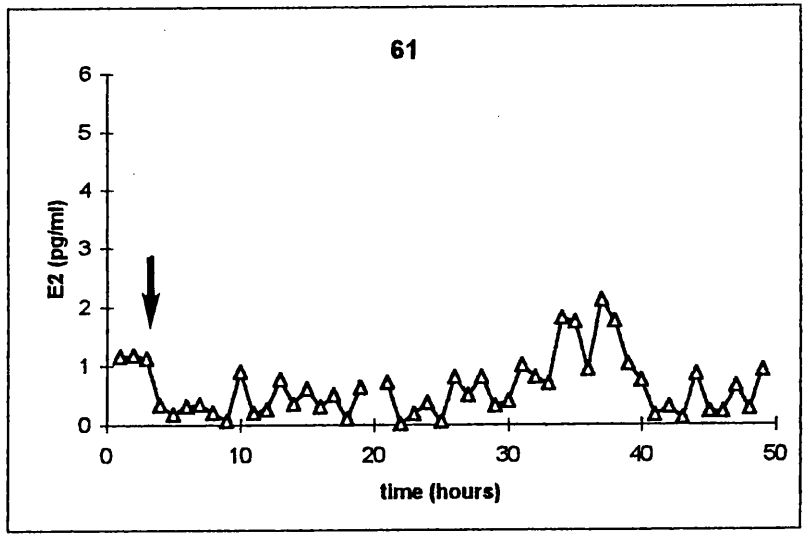
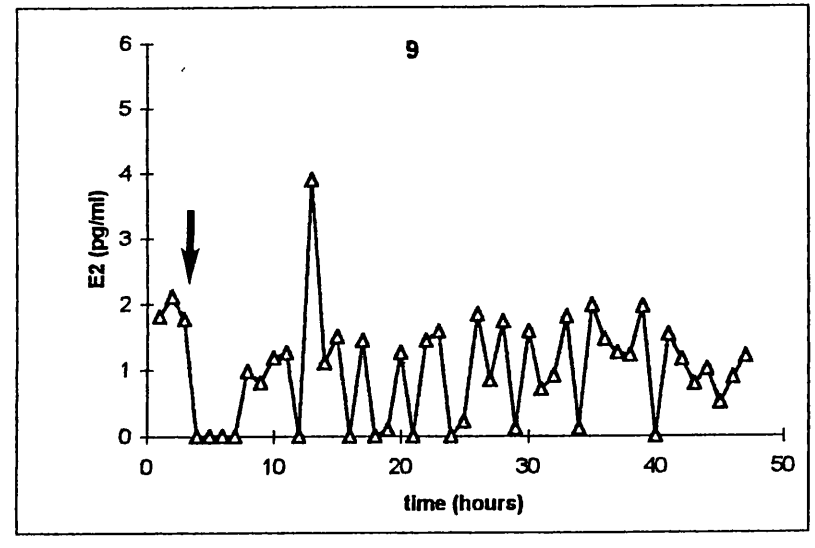
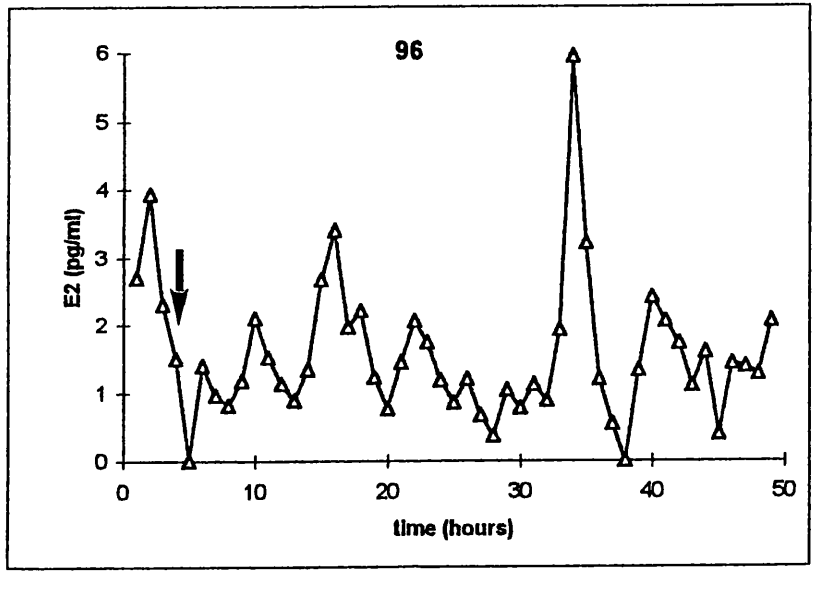

100
E2 (pg/ml)
time (hours)
0
1
2
3
4
5
6
7
0
10
20
30
40
50

Figure 13 Plasma oestradiol concentration in hourly samples from four representative cows subjected to multiple follicular aspiration during the **mid** luteal phase. The arrow indicates the time of aspiration.


61
E2 (pg/ml)
time (hours)
0
1
2
3
4
5
6
0
10
20
30
40
50


9
E2 (pg/ml)
time (hours)
0
1
2
3
4
5
6
0
10
20
30
40
50


96
E2 (pg/ml)
time (hours)
0
1
2
3
4
5
6
0
10
20
30
40
50

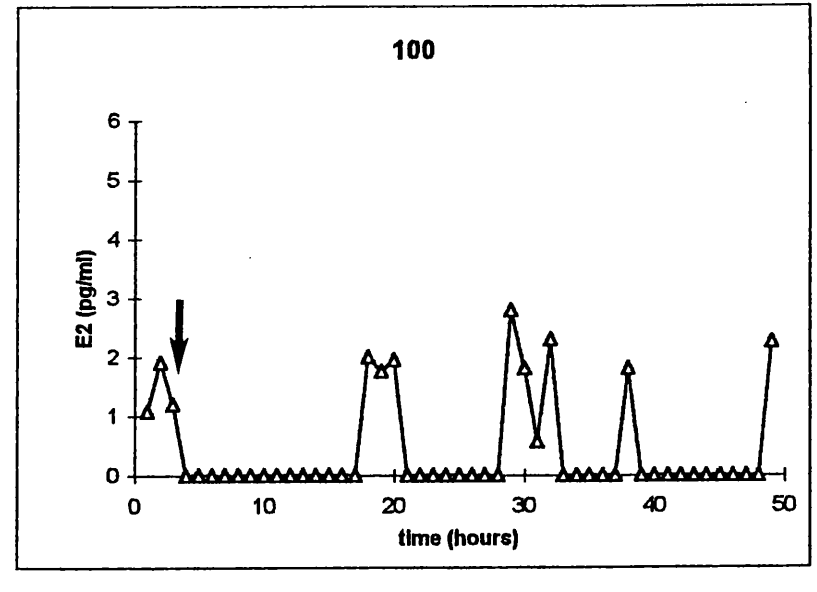

100
E2 (pg/ml)
time (hours)
0
1
2
3
4
5
6
0
10
20
30
40
50

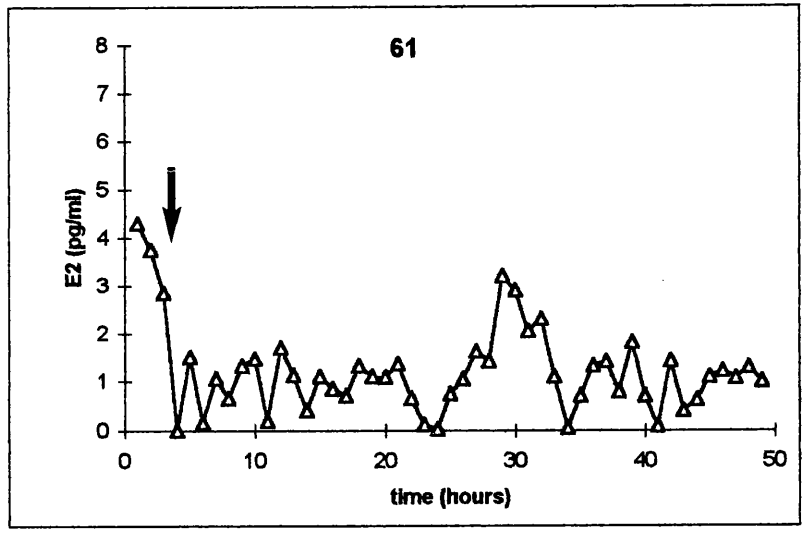
Table 11. Plasma oestradiol concentration (Mean ± SD) during the pre-aspiration / sham period and for each subsequent six hour period.

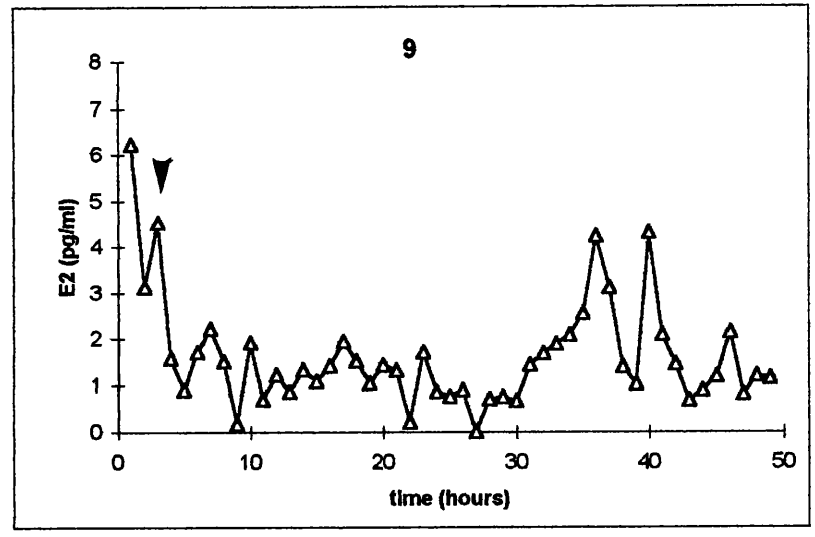
Time(h)	Early luteal phase		Mid luteal phase		Late luteal phase	
	Aspirated	Control	Aspirated	Control	Aspirated	Control
-4 - 0.	1.9±0.9	1.85±1.9	1.2±1	1.48±1.07	2.86±1.7	3.86±1.6
1-6	0.4±0.3	1.64±1.37	0.67±0.5	1.34±1.2	1.2±1.05	3.9±2.5
7-12	1.04±0.9	1.81±1.76	0.95±0.9	2.04±1.46	1.17±0.9	4.2±2.7
13-18	1.5±1	2.15±1.5	1±0.85	1.77±1.2	1.13±0.6	3.71±3.5
19-24	1.4±1.1	2.77±2.14	0.78±0.6	1.86±1.32	1.25±1	5.6±2.89
25-30	1.8±1.5	2.07±.1	1±0.77	1.34±0.86	1.84±1.75	6.25±2.7
31-36	2.2±1.8	2.71±1.4	1.36±1.2	1.55±1.31	1.71±1.47	6.9±2.9
37-42	1.47±1.2	2.39±1.2	0.83±0.7	1.71±1.05	1.22±1.13	7.6±2.3

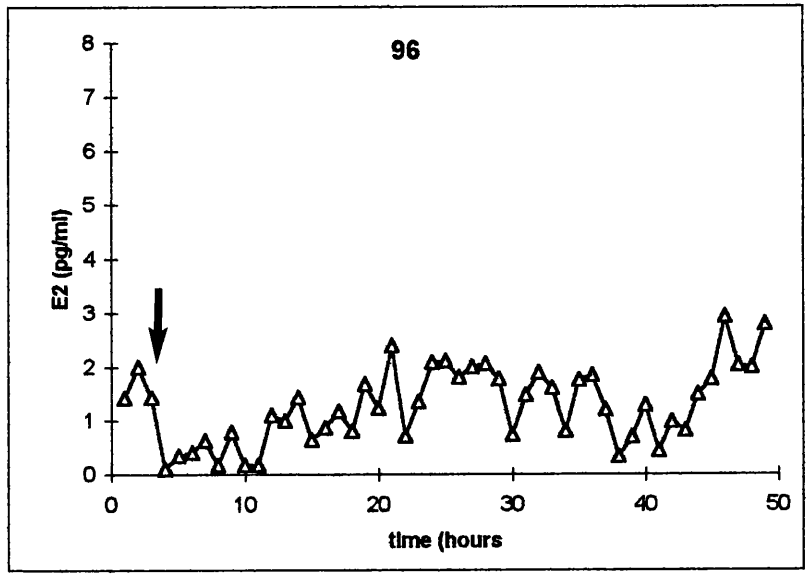
At all stages of the luteal phase, post aspiration values were significantly lower when compared to those of the pre-aspiration period, while no significant effects of mock aspiration on oestradiol were observed in the control cows. Oestradiol values fluctuated considerably in each cow and differed between cows, and with stage of the luteal phase (figs. 12, 13, 14 and 15, 16).

During the early stage of the luteal phase mean pre -aspiration plasma oestradiol in the aspirated animals was 1.9 ± 0.9pg/ml ranging from 1.59 ± 0.34 to 3.4 ± 0.42 pg/ml while in the same period control cows had a mean concentration of 1.85 ± 1.9pg/ml ranging from 0.36 ± 0.1 to 4.3 ± 2.7 pg/ml. Following aspiration mean plasma concentration was 1.25 ± 1 and 2.25 ± 1.6pg/ml in the aspirated and the control animals respectively.

Figure 14 Plasma oestradiol concentration in hourly samples from four representative cows subjected to multiple follicular aspiration during the **late** luteal phase. The arrow indicates the time of aspiration.


61
E2 (pg/ml)
time (hours)


9
E2 (pg/ml)
time (hours)


96
E2 (pg/ml)
time (hours

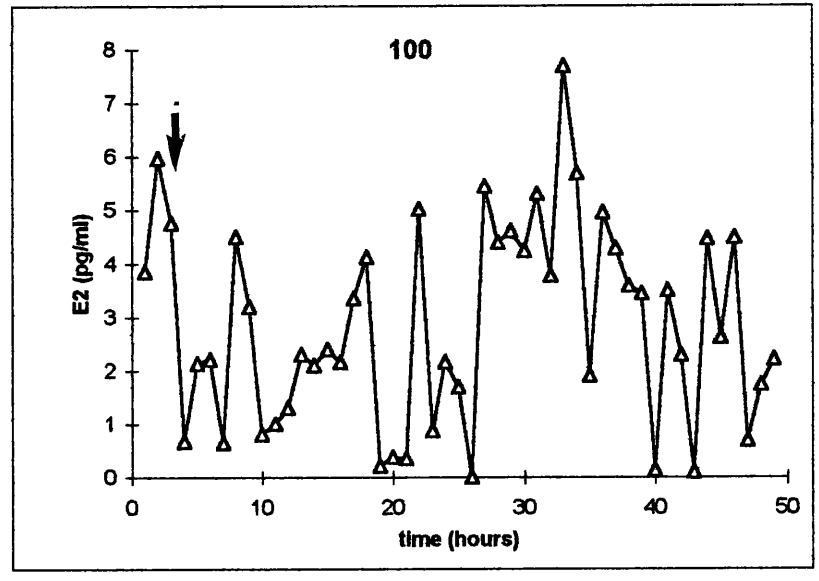
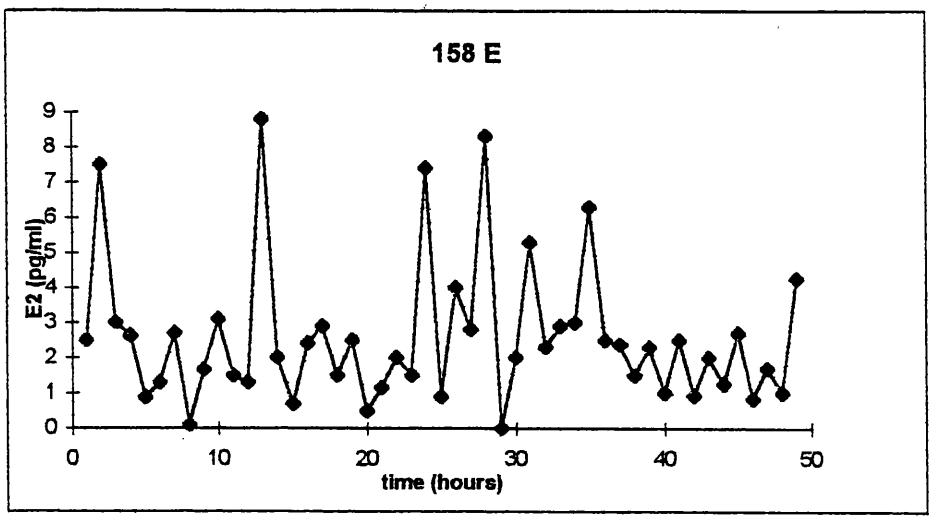
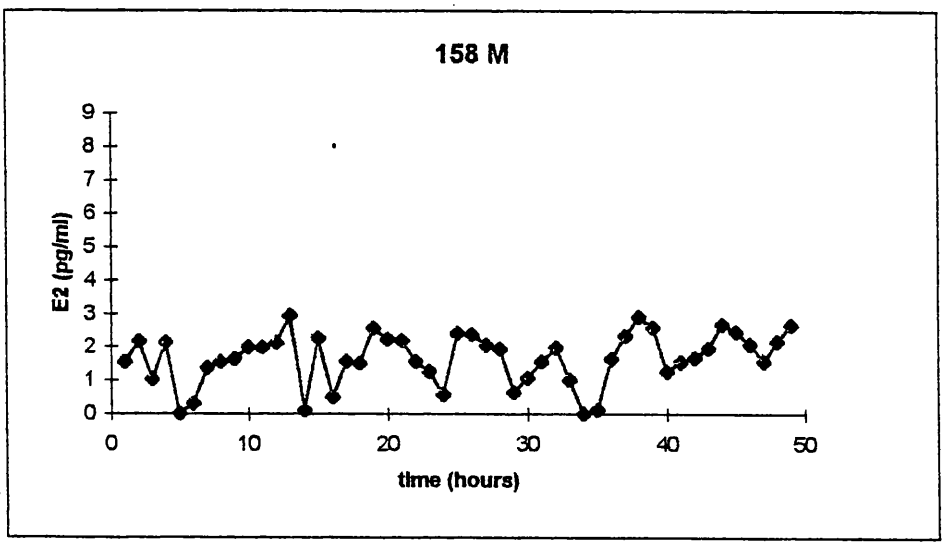
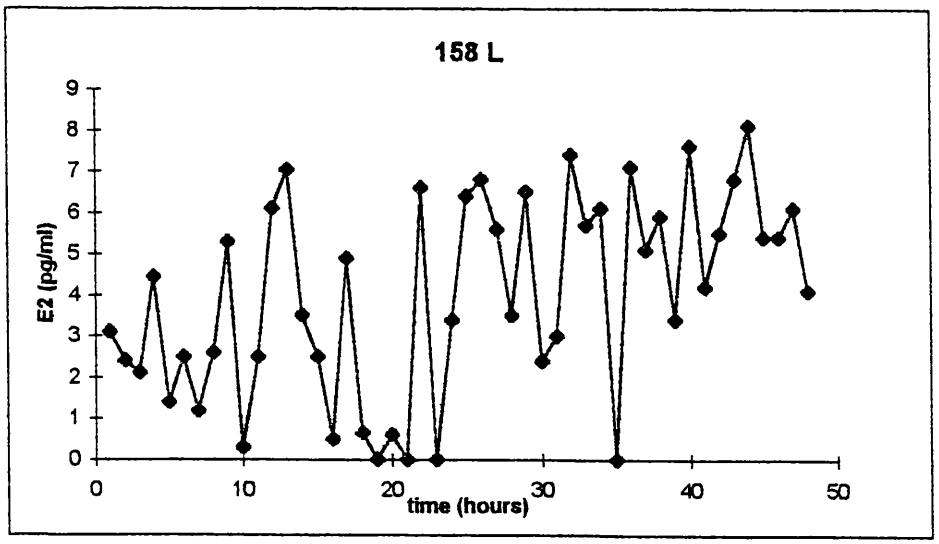

100
E2 (pg/ml)
time (hours)

Figure 15 Plasma oestradiol concentration in hourly samples from a control cow (158) during the early, (E), mid (M) and late (M) luteal phase.


158 E
E2 (pg/ml)
time (hours)
0
1
2
3
4
5
6
7
8
9
0
10
20
30
40
50


158 M
E2 (pg/ml)
time (hours)
0
1
2
3
4
5
6
7
8
9
0
10
20
30
40
50


158 L
E2 (pg/ml)
time (hours)
0
1
2
3
4
5
6
7
8
9
0
10
20
30
40
50

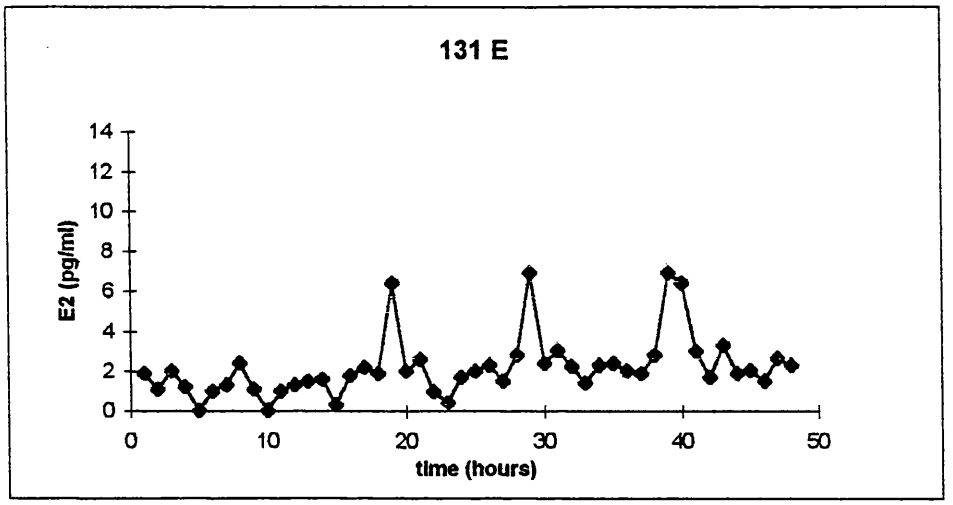
In the mid stage of the luteal phase mean pre-aspiration plasma oestradiol concentrations were 1.2 ± 1 and 1.48 ± 1.07pg/ml in the aspirated and the control cows ranging from 0.9 ± 0.19 to 3.55 ± 1.3pg/ml and 1 ± 1.2 to 1.66 ± 0.49pg/ml respectively. After aspiration mean oestradiol was 1.9 ± 1.2pg/ml in the control cows while and reduced to 0.89 ± 0.8pg/ml in the aspirated cows.

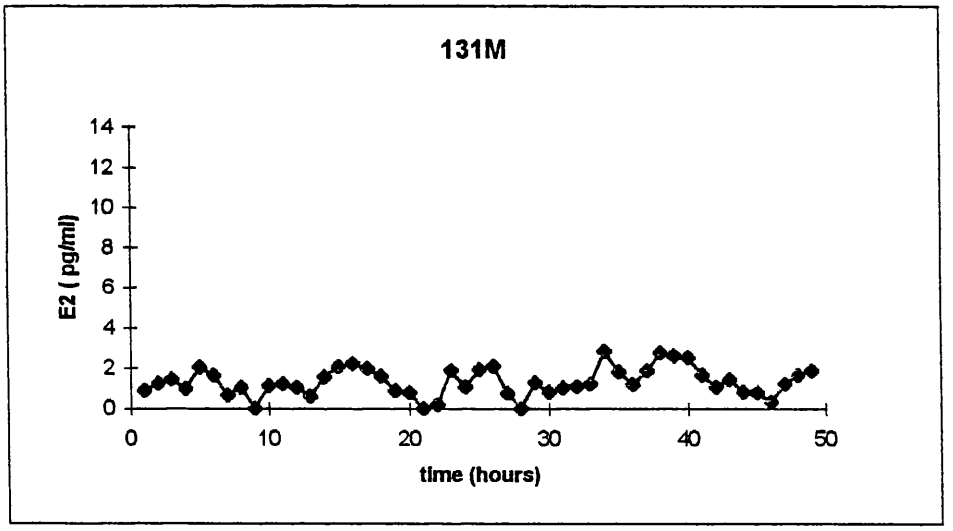
Similarly, during the late stage of the luteal phase mean pre-aspiration plasma oestradiol was 2.86 ± 1.7pg/ml ranging from 4.62 ± 1.54 to 1.61 ± 0.32pg/ml in the aspirated group and 3.86 ± 1.6pg/ml ranging from 3.86 ± 1.6 to 2.5 ± 0.5pg/ml in the controls. After aspiration mean oestradiol concentration declined to 1.86 ± 1.2pg/ml in the aspirated group, while in the control cows it slightly increased to 4.26 ± 3.2pg/ml.

To investigate the effect of the multiple follicle aspiration on the mode of oestradiol and LH secretion the mean pre-aspiration values were subtracted from the mean post aspiration values, in both groups (fig. 17, 18) and the balances were tested using analysis of variance. For the entire bleeding period, mean plasma oestradiol was found to be significantly lower after aspiration at any stage of the luteal phase ($P<0.05$). Analysis of variance revealed that following aspiration mean oestradiol concentrations were significantly lower in the aspirated cows when compared to the controls during the mid and late luteal phases ($P<0.05$), while in the early luteal phase the difference was not significant ($P=0.9$). However, from observation of the data presented in figure 17 it would appear that there was a clear decrease in plasma oestradiol concentrations after aspiration regardless of cycle stage.

In both groups, mean pre-aspiration oestradiol concentrations were highest during the late and lowest during the mid luteal phase (table 11).

Figure 16. Plasma oestradiol concentration in hourly samples from a control cow (131) during the early, (E), mid (M) and late (M) luteal phase.


131 E
E2 (pg/ml)
14
12
10
8
6
4
2
0
0
10
20
30
40
50
time (hours)


131M
E2 (pg/ml)
14
12
10
8
6
4
2
0
0
10
20
30
40
50
time (hours)

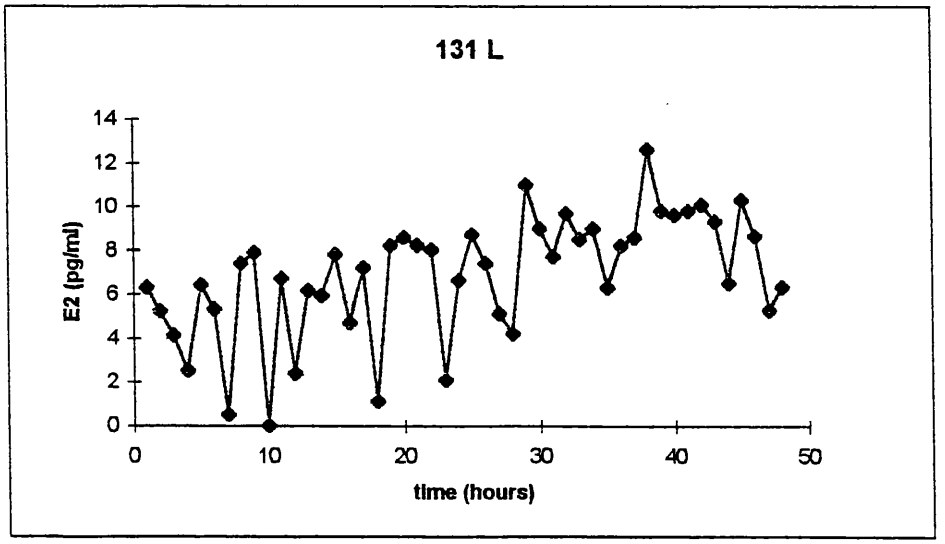
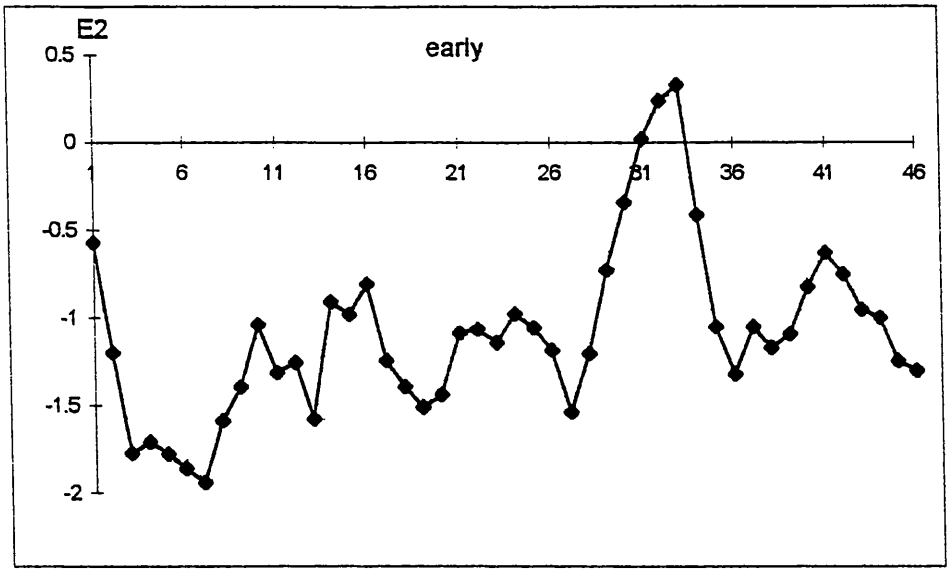
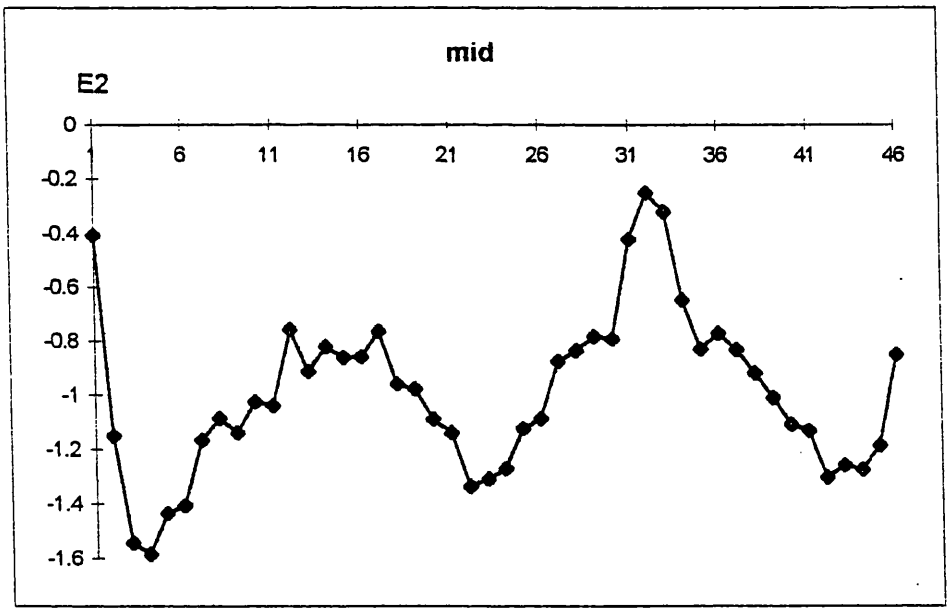

131 L
E2 (pg/ml)
14
12
10
8
6
4
2
0
0
10
20
30
40
50
time (hours)

Figure 17 Oestradiol plasma concentration post-aspiration during the early, mid and late stages of the luteal phase. Each point is the mean of the aspirated cows and is balanced by subtraction of the pre-aspiration mean value.


E2
early
0.5
0
-0.5
-1
-1.5
-2
1
6
11
16
21
26
31
36
41
46


mid
E2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
-1.4
-1.6
1
6
11
16
21
26
31
36
41
46

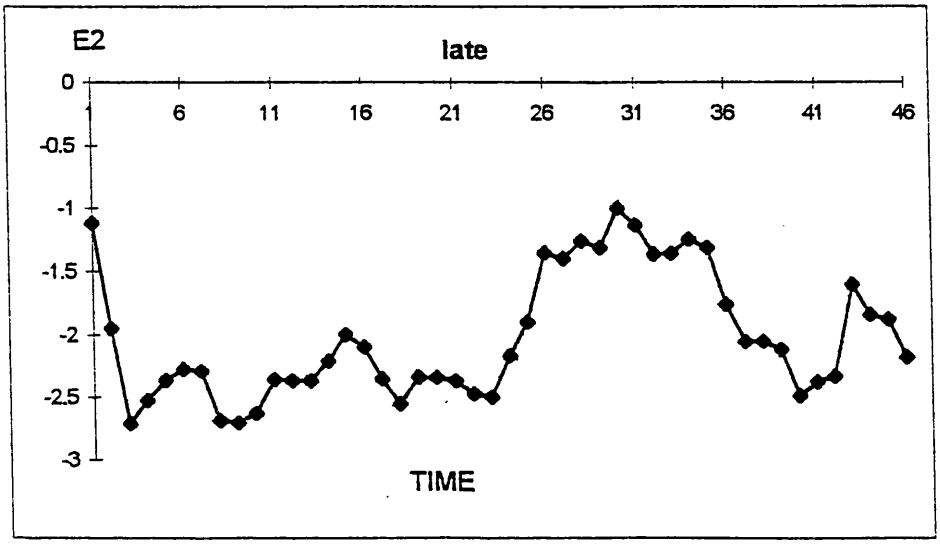
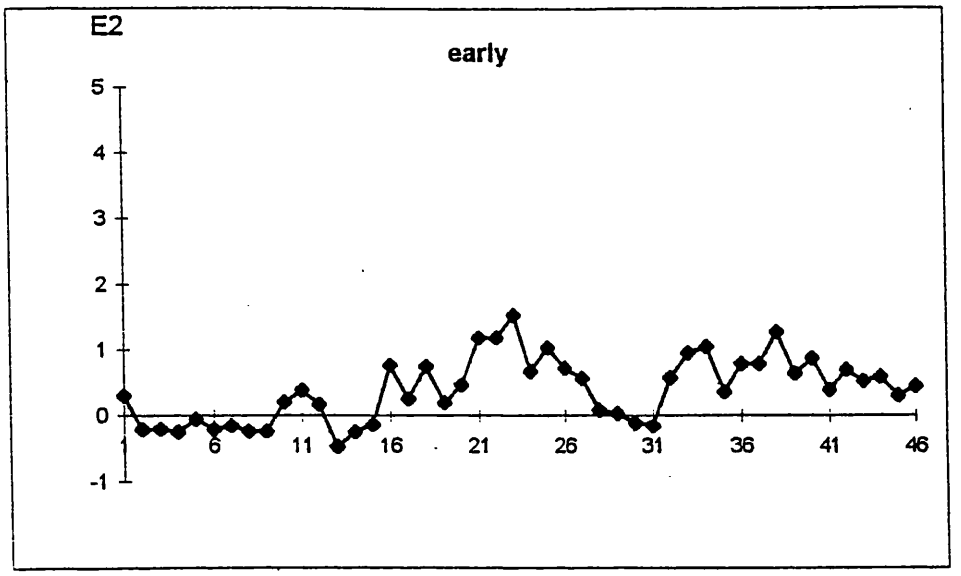
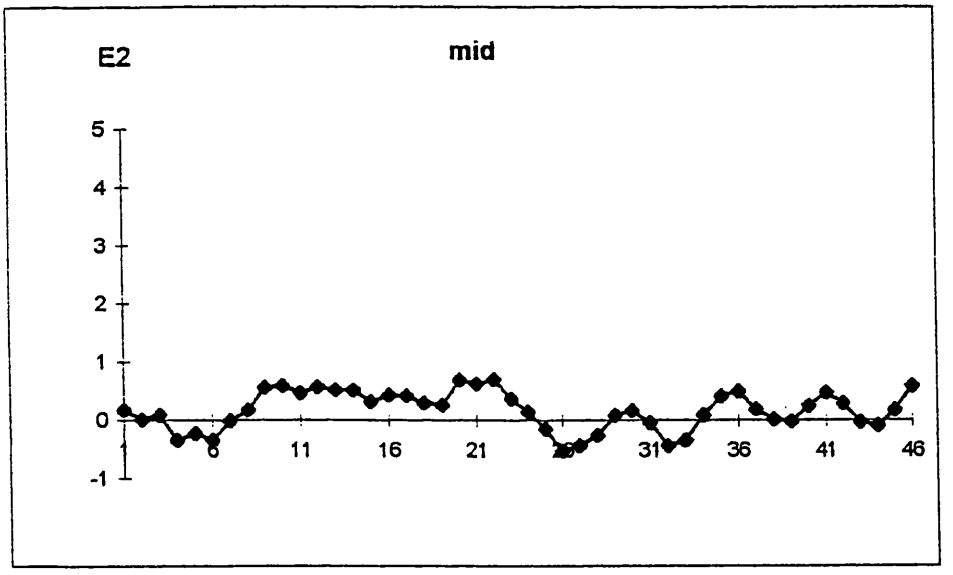

E2
late
0
-0.5
-1
-1.5
-2
-2.5
-3
1
6
11
16
21
26
31
36
41
46
TIME

Figure 18 Oestradiol plasma concentration in the mock-aspirated control cows during the early, mid and late stages of the luteal phase. Each point is the mean of the aspirated cows and is balanced by subtraction of the pre-mock-aspiration mean value.


E2
early
5
4
3
2
1
0
-1
1
6
11
16
21
26
31
36
41
46


E2
mid
5
4
3
2
1
0
-1
1
6
11
16
21
31
36
41
46

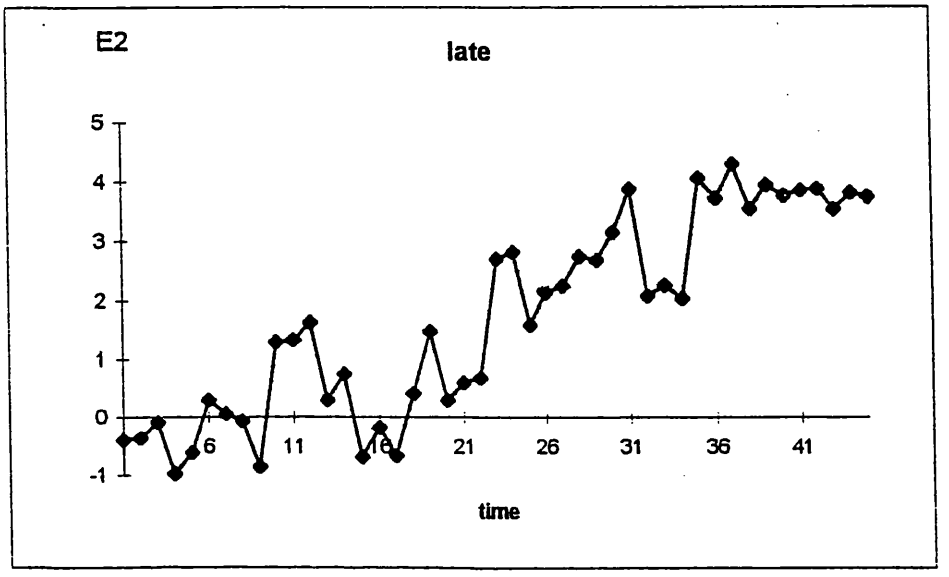
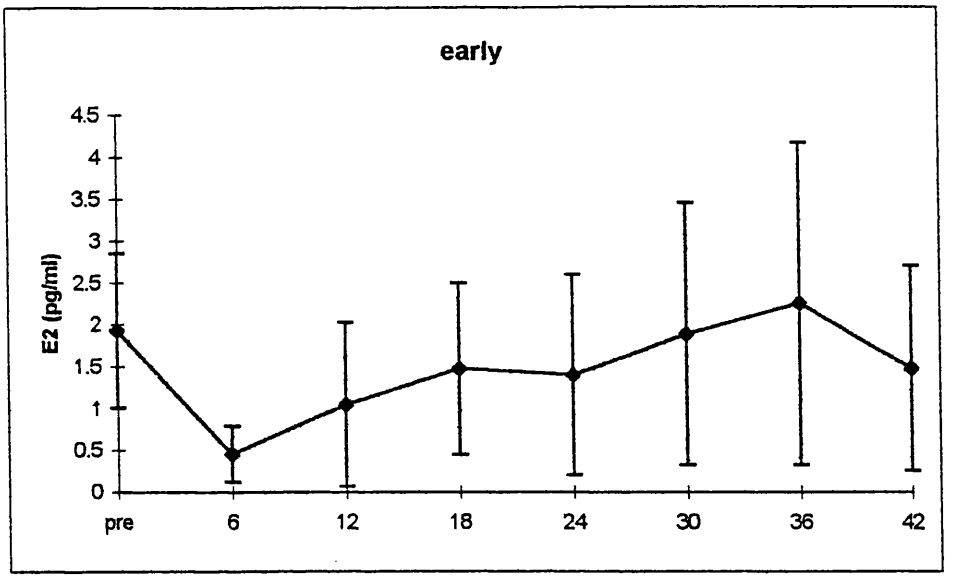
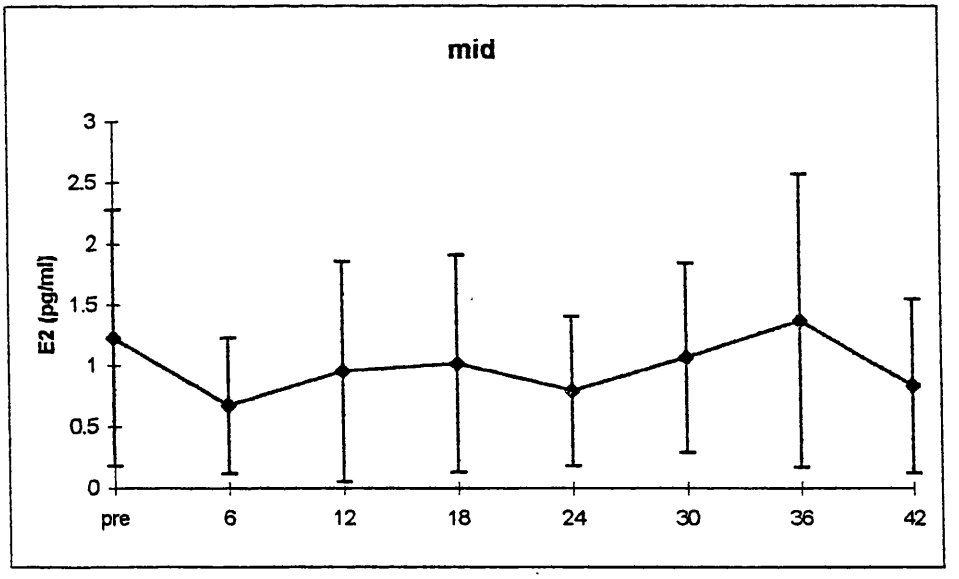
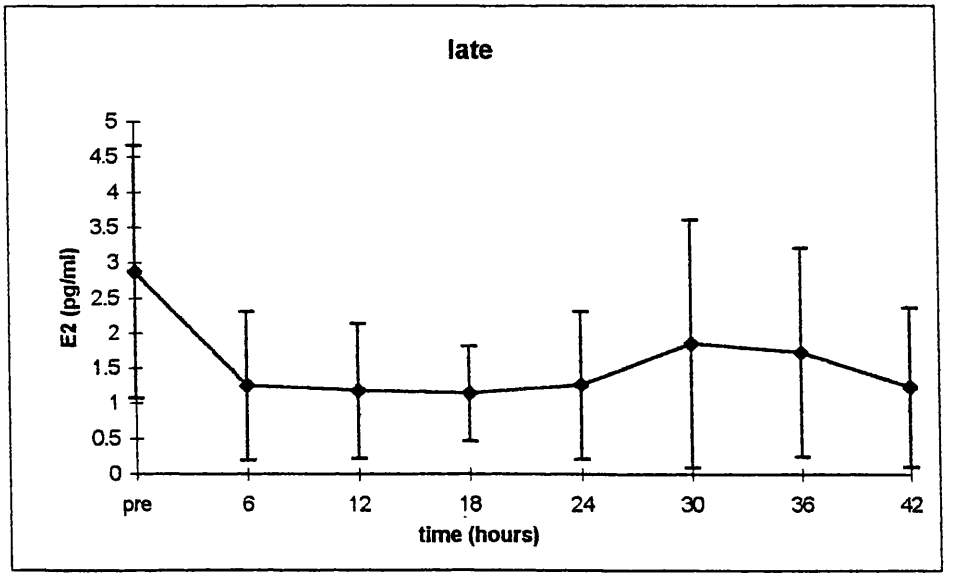

E2
late
5
4
3
2
1
0
-1
6
11
21
26
31
36
41
time

Figure 19 Oestradiol plasma concentration pre- and post-aspiration (mean ± S.D) during the early, mid and late stages of the luteal phase. Each post aspiration value is the mean for a 6 hour interval.


early
E2 (pg/ml)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
pre
6
12
18
24
30
36
42


mid
E2 (pg/ml)
3
2.5
2
1.5
1
0.5
0
pre
6
12
18
24
30
36
42


late
E2 (pg/ml)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
pre
6
12
18
24
30
36
42
time (hours)

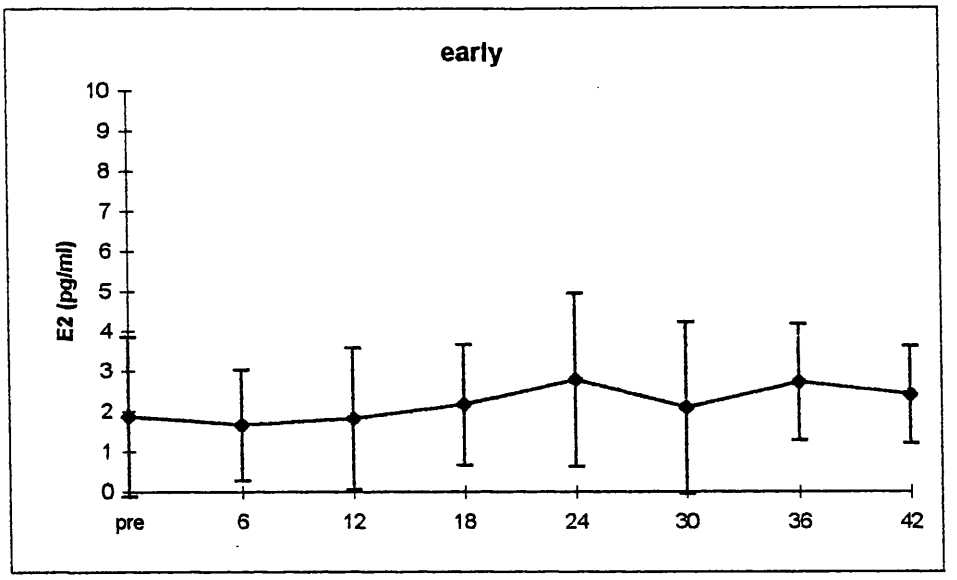
From the dramatic decrease in plasma oestradiol concentration observed shortly after aspiration plasma oestradiol then tended to increase after the sixth hour post-aspiration in the early and mid stages of the luteal phase but not until after 18 hours in the late stage (fig. 12, 13, 14 and 17). The most profound drop in plasma oestradiol was observed in cow 100 during the mid luteal phase, when the hormone concentration in sequential post aspiration samples was below the detection limit of the assay.

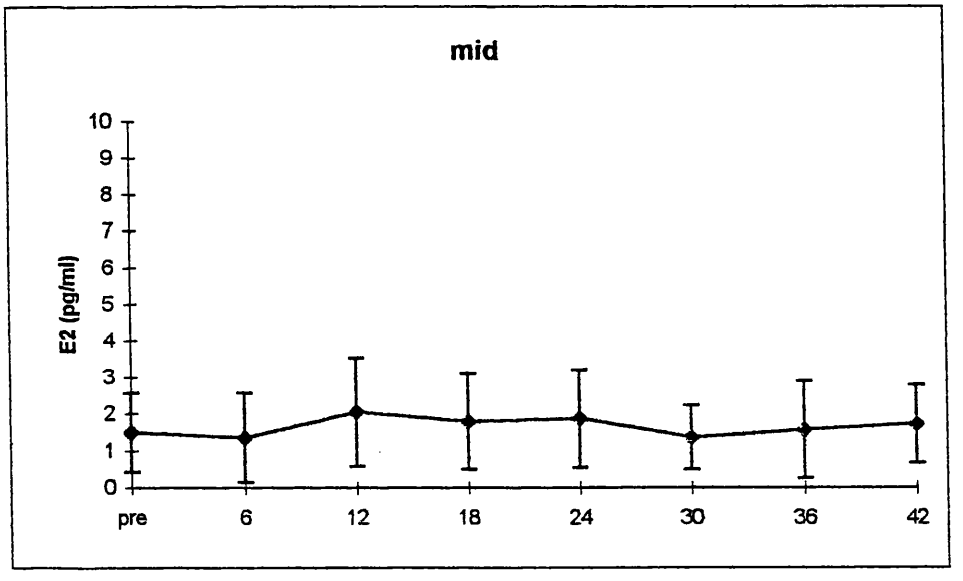
Mean oestradiol concentration in six hours periods after aspiration is shown in table 11. Between 31 to 36 hours after multiple follicle aspiration during the early and late stages of the luteal phase, mean plasma oestradiol was significantly higher ($P<0.05$) when compared to the earlier periods (fig. 19). In the mid luteal phase a period of increased oestradiol concentration was detected 25-30 hours post-aspiration but it was not significantly different from the other six hourly periods ($P>0.05$). However, by 30 hours after aspiration during the early luteal stage, mean plasma oestradiol had increased to levels comparable to that of the pre-aspiration period (fig. 17, 19)

A gradual increase in mean plasma oestradiol concentration was detected in the control group, 12 and 18 hours after the sham aspiration during the early and late luteal phase, respectively while during the mid luteal phase, there was a tendency for oestradiol to increase six hours after the sham aspiration and to remain higher for about 12 hours (fig. 18, 20)

A hormone pulse in plasma was defined as any increase in hormone concentration that exceeded the inter assay coefficient of variation by at least three times and was followed by a decrease to basal levels.

Figure 20 Oestradiol plasma concentration pre- and post-mock-aspiration (control cows) during the early, mid and late stages of the luteal phase. Each post aspiration value is the mean for a 6 hour interval.


early
E2 (pg/ml)
0
1
2
3
4
5
6
7
8
9
10
pre
6
12
18
24
30
36
42


mid
E2 (pg/ml)
0
1
2
3
4
5
6
7
8
9
10
pre
6
12
18
24
30
36
42

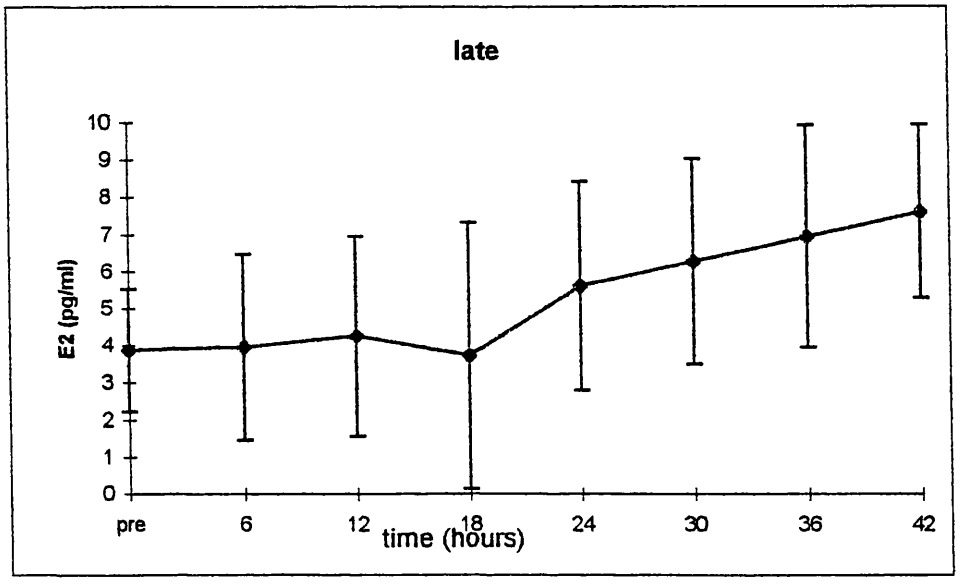
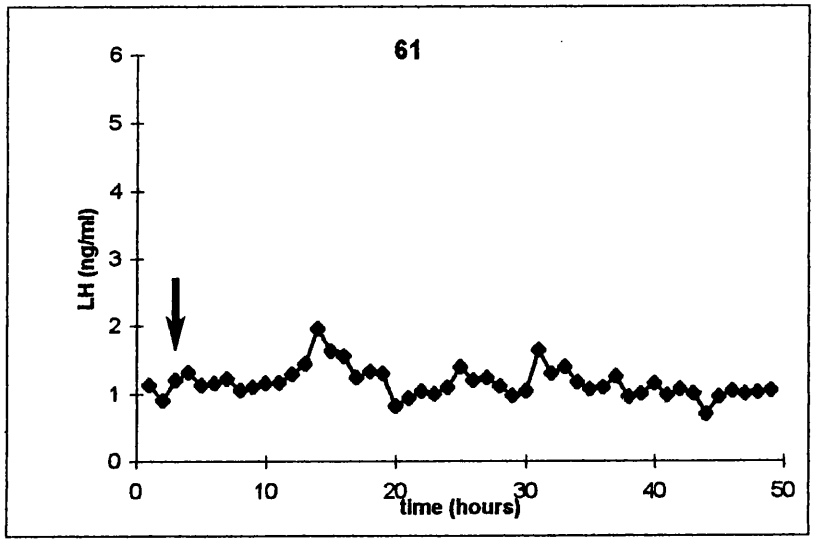
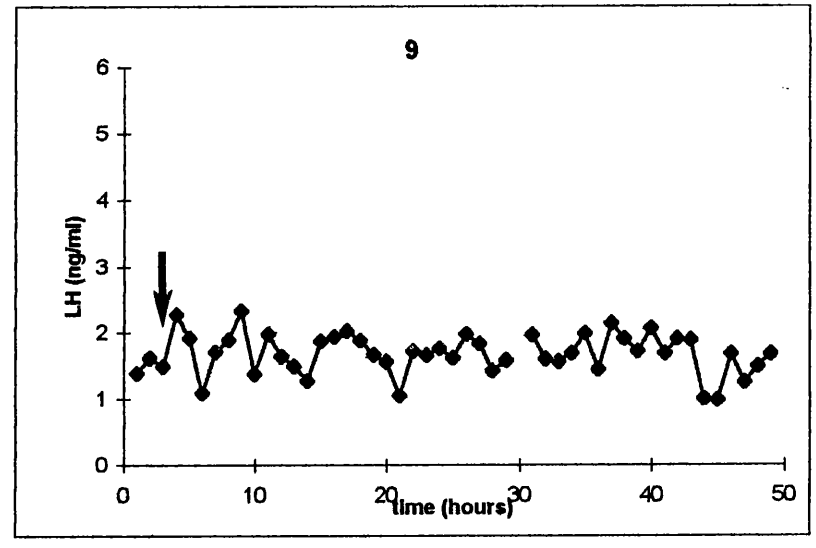
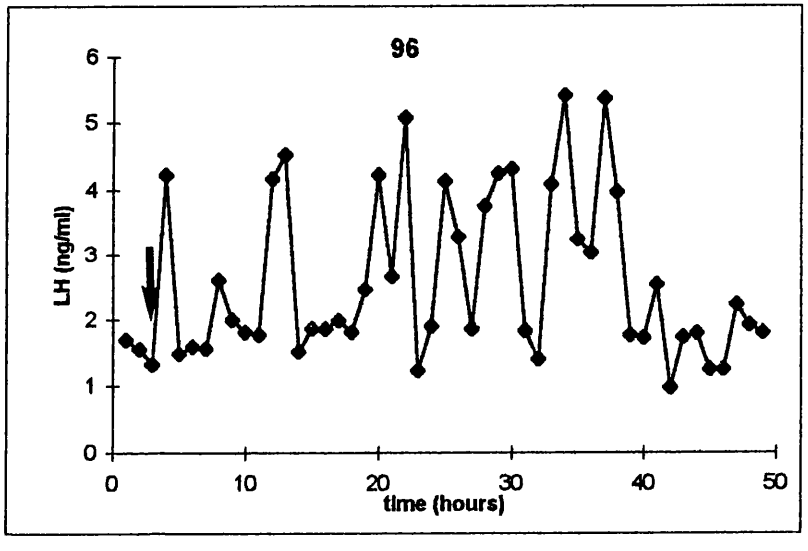

late
E2 (pg/ml)
0
1
2
3
4
5
6
7
8
9
10
pre
6
12
18
24
30
36
42
time (hours)

Figure 21 Plasma LH concentration in hourly samples from four representative cows aspirated during the **early** luteal phase. The arrow indicates the time of aspiration


61
LH (ng/ml)
time (hours)
0
1
2
3
4
5
6
0
10
20
30
40
50


9
LH (ng/ml)
time (hours)
0
1
2
3
4
5
6
0
10
20
30
40
50


96
LH (ng/ml)
time (hours)
0
1
2
3
4
5
6
0
10
20
30
40
50

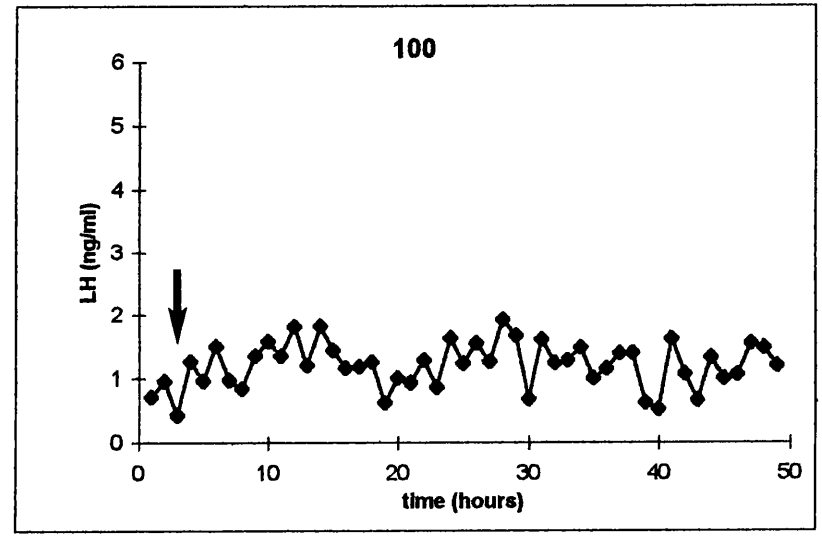
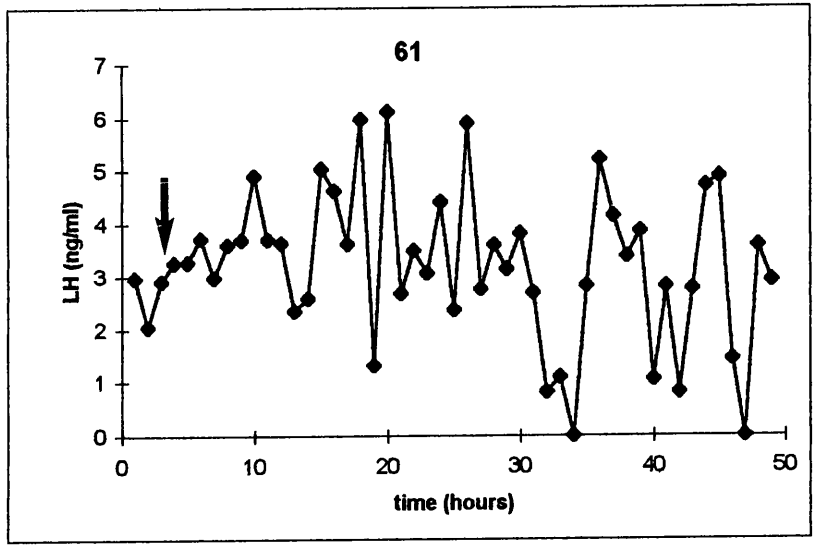
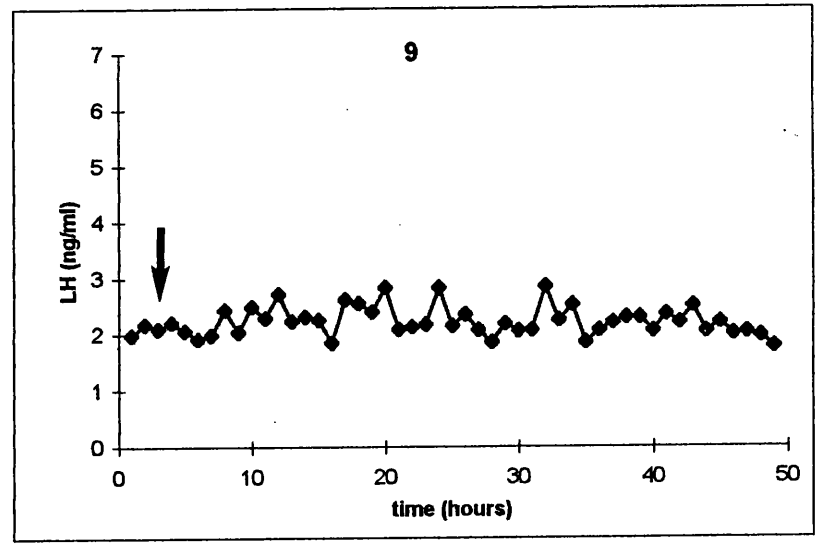
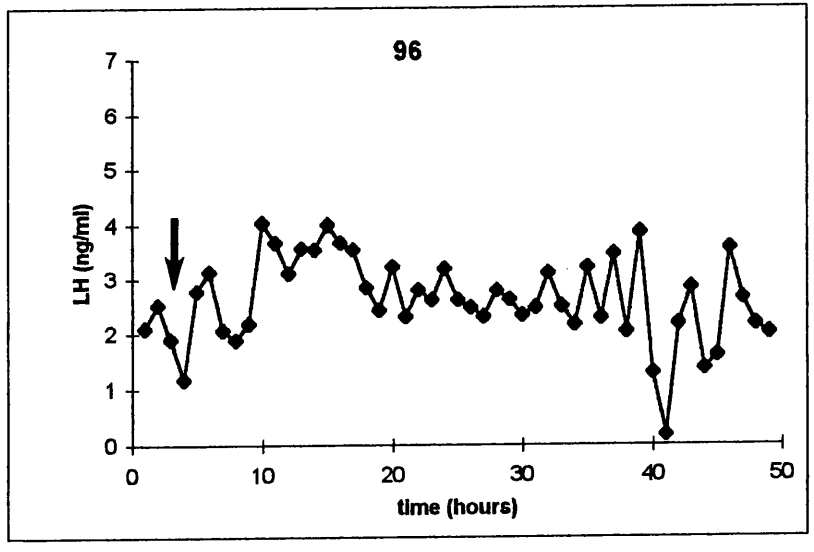
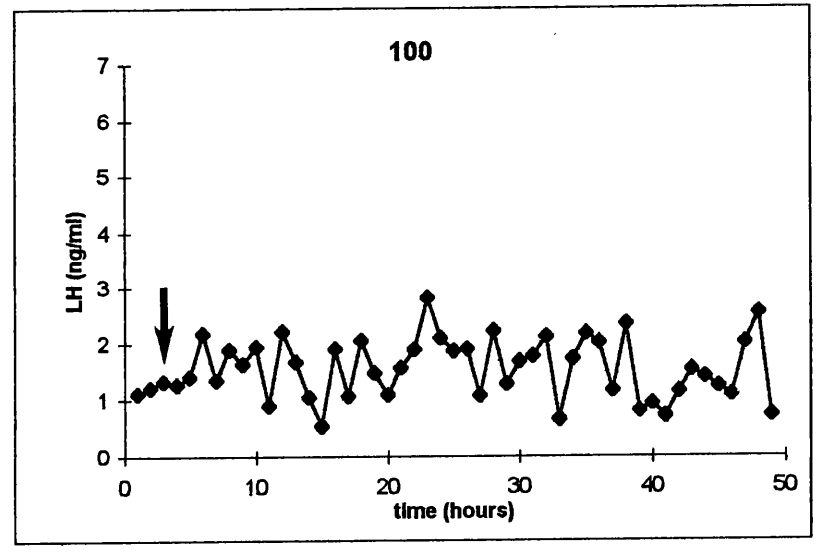

100
LH (ng/ml)
time (hours)
0
1
2
3
4
5
6
0
10
20
30
40
50

Figure 22 Plasma LH concentration in hourly samples from four representative cows aspirated during the **mid** luteal phase. The arrow indicates the time of aspiration


61
LH (ng/ml)
0
1
2
3
4
5
6
7
0
10
20
30
40
50
time (hours)


9
LH (ng/ml)
0
1
2
3
4
5
6
7
0
10
20
30
40
50
time (hours)


96
LH (ng/ml)
0
1
2
3
4
5
6
7
0
10
20
30
40
50
time (hours)


100
LH (ng/ml)
0
1
2
3
4
5
6
7
0
10
20
30
40
50
time (hours)

Oestradiol pulse frequency was higher in the aspirated animals during the early and mid luteal phase and lower in the late luteal phase when compared to the controls, but these differences were not significant (P>0.05). No significant effect of cycle stage was detected in the aspirated cows, while in the control cows, oestradiol pulse frequency was significantly higher in the late luteal stage than in the mid luteal phase (table 13)

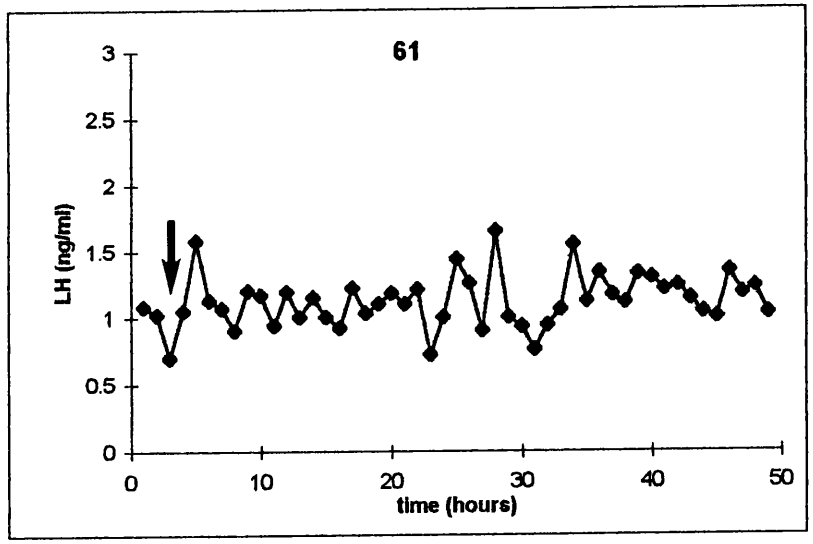
3.3.2.3. Plasma LH after multiple follicle aspiration

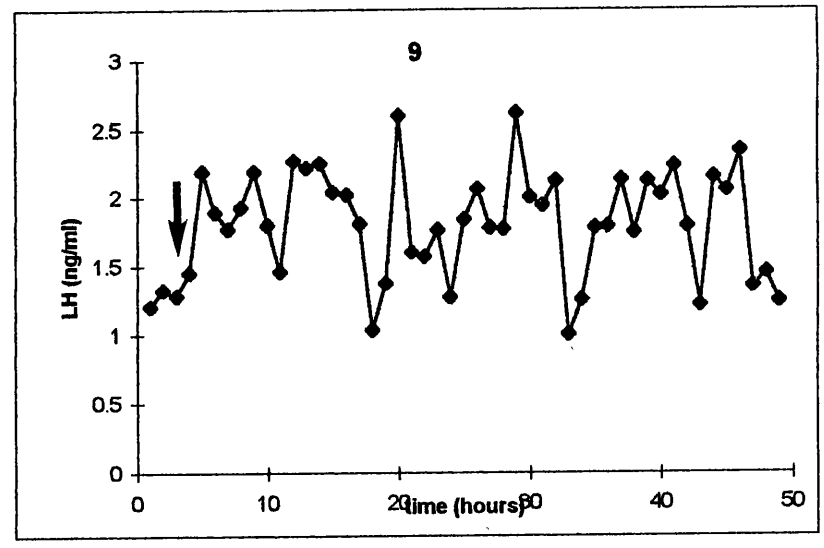
Plasma LH concentrations in samples collected at hourly intervals from four hours before until 45 hours after multiple follicle aspiration or mock aspiration are shown in table 12. In both groups, pre-aspiration LH levels were highest in the mid- and lowest during the late stages of the luteal phase. After follicular aspiration, a rise in plasma LH concentration was observed in all animals regardless of the stage of the cycle. However, the magnitude of this rise varied considerably between individual cows (fig. 21, 22, 23).

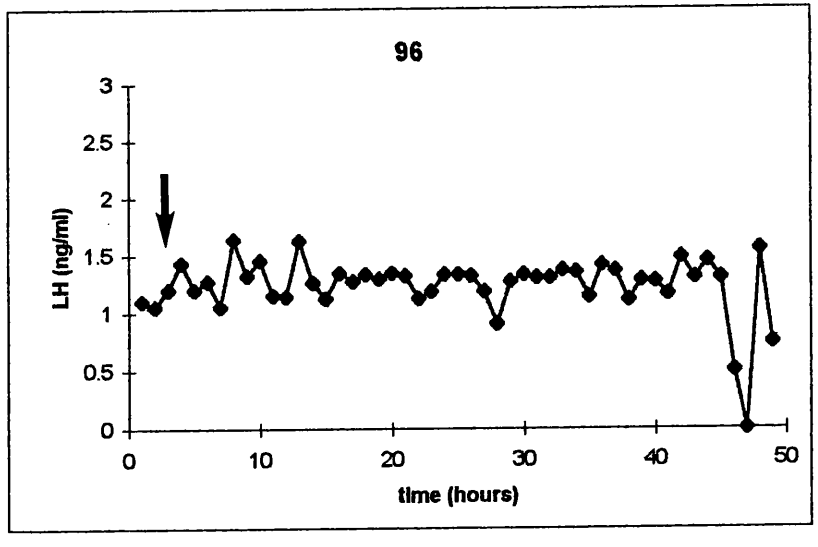
After multiple follicle aspiration in any of the three stages of the luteal phase, there was a clear tendency for an increase in mean plasma LH concentration, but this rise in the post-aspiration LH levels was not statistically significantly different from those of the pre-aspiration period.

When pre-aspiration LH values were subtracted from those of the post aspiration period as depicted in figure 24, mean plasma LH concentration steadily increased for more than 30 hours after aspiration in the early and mid luteal phases. During the early luteal phase, there was a clear tendency for mean LH concentration to drop after the 36th hour, while in the mid luteal phase, two periods of decreased mean LH values were detected, one starting about 28 hours and a second 37 hours after aspiration. In the late luteal phase, the mean LH concentration throughout

Figure 23 Plasma LH concentration in hourly samples from four representative cows aspirated during the **late** luteal phase. The arrow indicates the time of aspiration


61
LH (ng/ml)
time (hours)
0
0.5
1
1.5
2
2.5
3
0
10
20
30
40
50


9
LH (ng/ml)
time (hours)
0
0.5
1
1.5
2
2.5
3
0
10
20
30
40
50


96
LH (ng/ml)
time (hours)
0
0.5
1
1.5
2
2.5
3
0
10
20
30
40
50

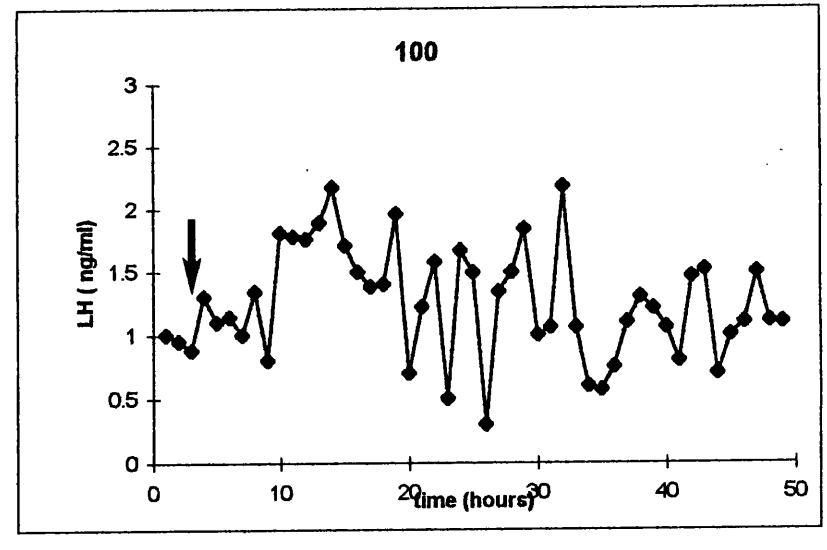
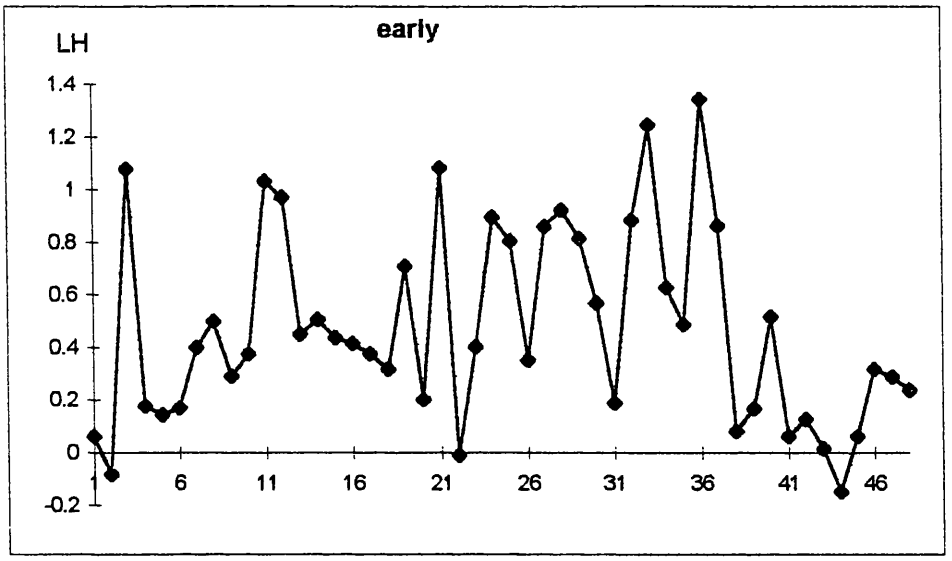
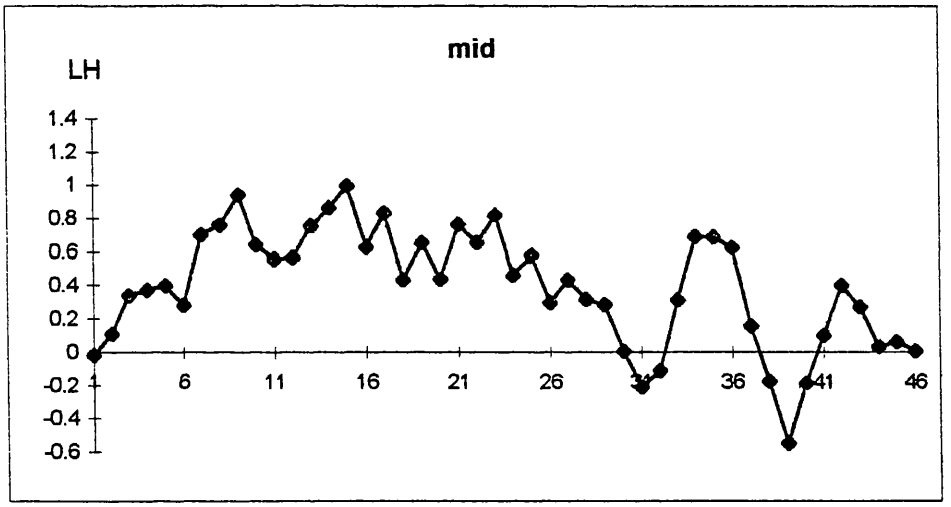

100
LH (ng/ml)
time (hours)
0
0.5
1
1.5
2
2.5
3
0
10
20
30
40
50

Figure 24 Plasma LH concentration post-aspiration during the early, mid and late stages of the luteal phase. Each point is the mean of the aspirated cows and is balanced by subtraction of the pre-aspiration mean value.


early
LH
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
1
6
11
16
21
26
31
36
41
46


mid
LH
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
6
11
16
21
26
36
41
46

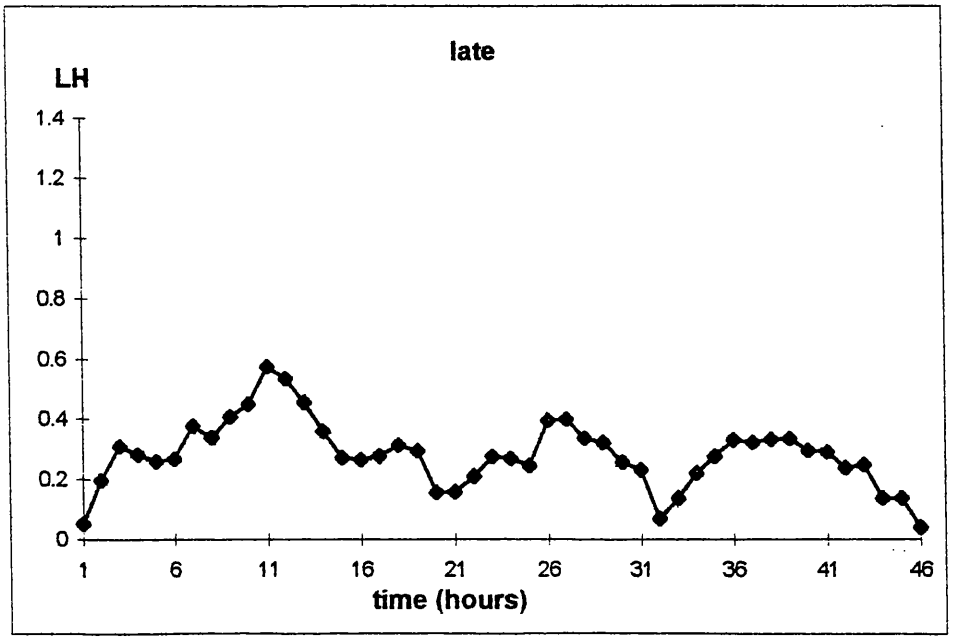
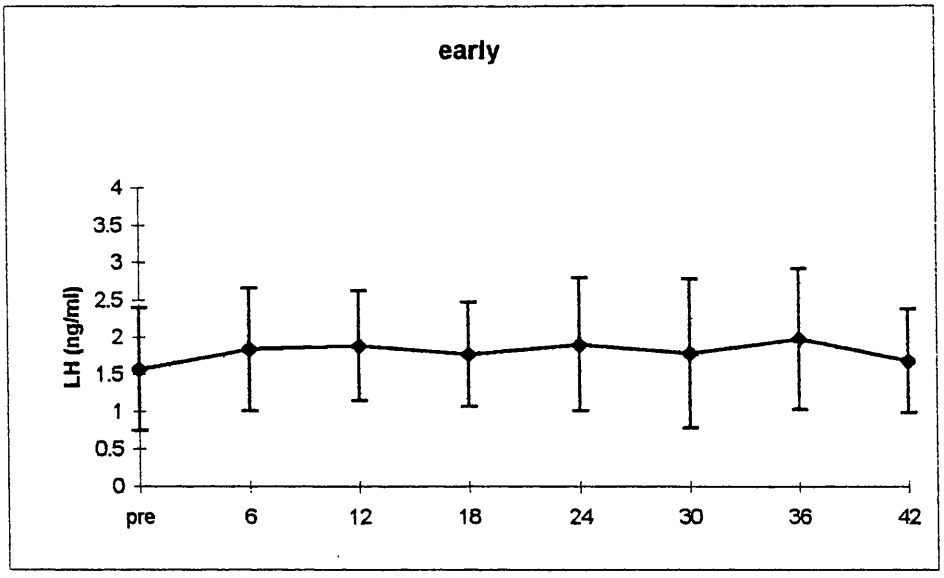
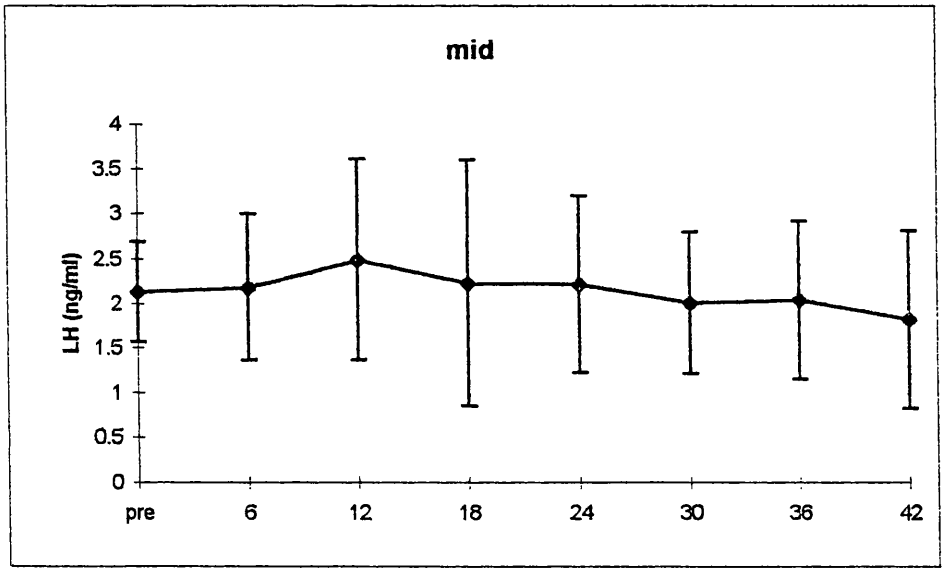
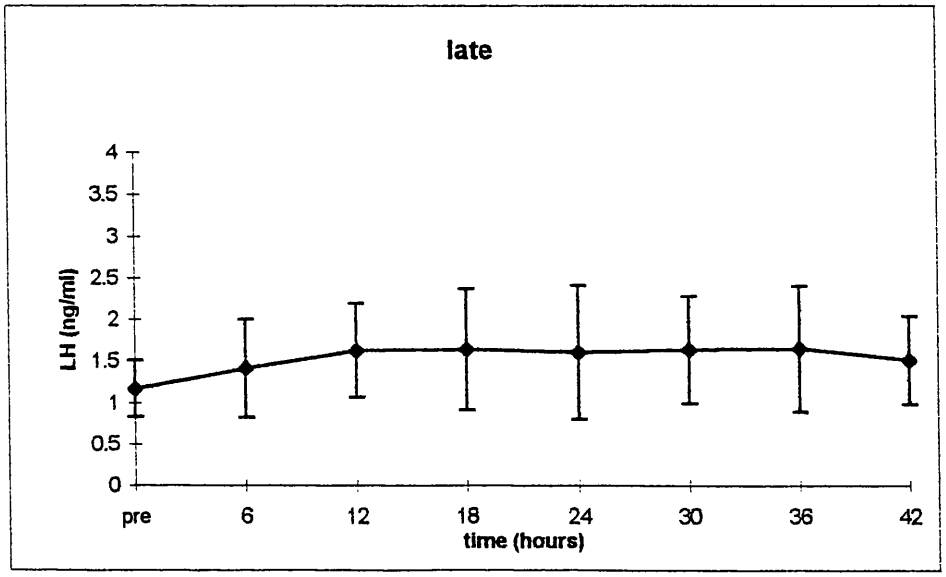

late
LH
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
6
11
16
21
26
31
36
41
46
time (hours)

Figure 25 LH plasma concentration pre- and post-aspiration (mean ± S.D) during the early, mid and late stages of the luteal phase. Each post aspiration value is the mean for a 6 hour interval.


early
LH (ng/ml)
4
3.5
3
2.5
2
1.5
1
0.5
0
pre
6
12
18
24
30
36
42


mid
LH (ng/ml)
4
3.5
3
2.5
2
1.5
1
0.5
0
pre
6
12
18
24
30
36
42


late
LH (ng/ml)
4
3.5
3
2.5
2
1.5
1
0.5
0
pre
6
12
18
24
30
36
42
time (hours)

the post aspiration bleeding period was steadily higher than the pre-aspiration values (table 3, fig. 24, 25)

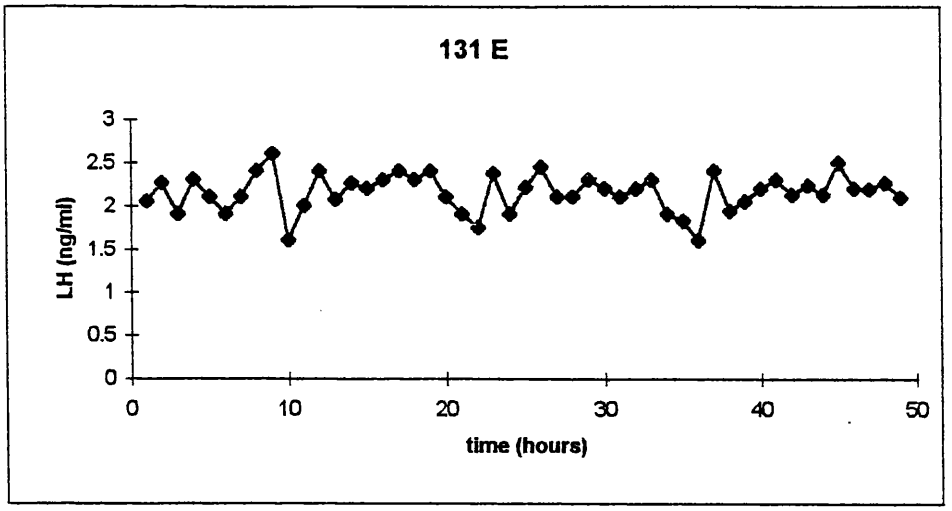
In the control group, plasma LH concentration remained basal throughout the bleeding period with minor fluctuations (fig. 26, 27, 28). During the late luteal phase, an increase in mean plasma LH was detected 16 hours post sham-aspiration, but this was not significantly higher than the preceding values.

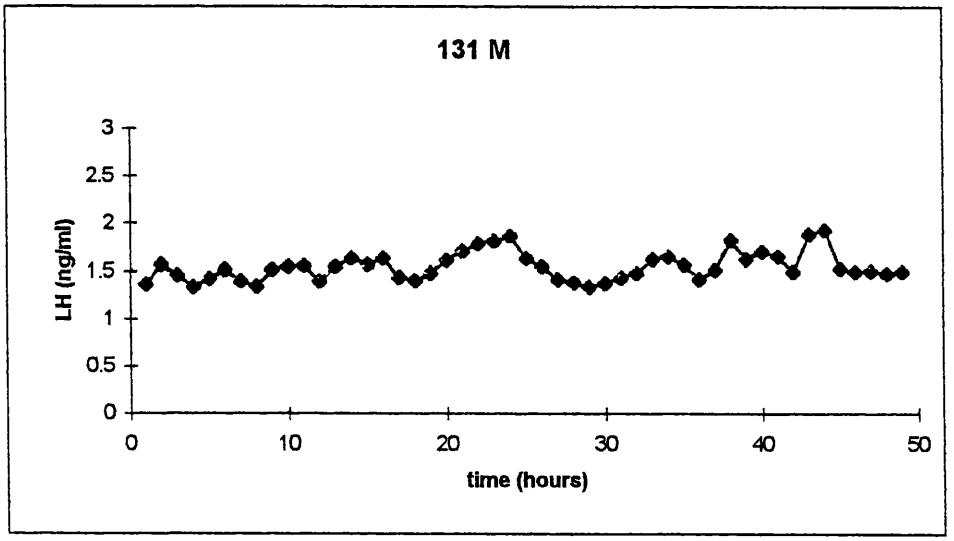
In the control cows, mean LH pulse frequency varied at different stages of the cycle being significantly lower during the mid luteal phase compared to the early and late stages of the luteal phase. However, LH pulse frequency was higher in the aspirated animals when compared to the controls ($P<0.05$ for the early and late luteal phase and $P<0.001$ for the mid luteal phase).

Table 12. Plasma LH concentration (Mean ± SD) during the pre- aspiration / sham period and for each subsequent six hour period

Time (h)	Early luteal		Mid luteal		Late luteal	
	Aspirated	Controls	Aspirated	Controls	Aspirated	Controls
-4 - 0.	1.56±0.8	1.81±0.46	2.12±0.56	1.88±0.45	1.16±0.3	1.53±0.4
1-6	1.83±0.8	1.85±0.4	2.17±0.8	1.94±0.47	1.4±0.5	1.59±0.56
7-12	1.89±0.71	1.52±0.38	2.45±1.1	1.8±0.32	1.62±0.56	1.66±0.47
13-18	1.76±0.73	1.89±0.44	2.24±1.3	1.82±0.31	1.8±0.8	1.57±0.6
19-24	1.81±0.93	1.82±0.45	2.2±0.8	1.87±0.32	1.63±0.64	1.7±0.4
25-30	1.77±0.75	1.85±0.52	1.85±0.9	1.8±0.36	1.63±0.72	1.7±1.345
31-36	1.97±0.94	1.82±0.3	2±0.72	1.82±0.33	1.5±0.52	1.61±0.3
37-42	1.67±0.64	1.79±0.4.	1.95v0.91	1.85±0.35	1.68±0.74	1.71±0.34

Figure 26 Plasma LH concentration in hourly samples from a control cow (131)during the early, (E), mid (M) and late (M) luteal phase.


131 E
LH (ng/ml)
3
2.5
2
1.5
1
0.5
0
0
10
20
30
40
50
time (hours)


131 M
LH (ng/ml)
3
2.5
2
1.5
1
0.5
0
0
10
20
30
40
50
time (hours)

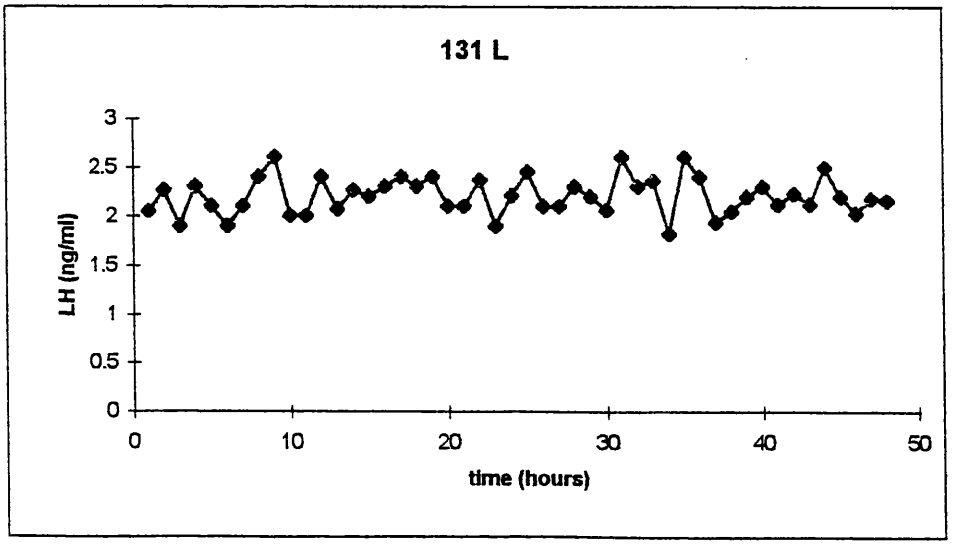
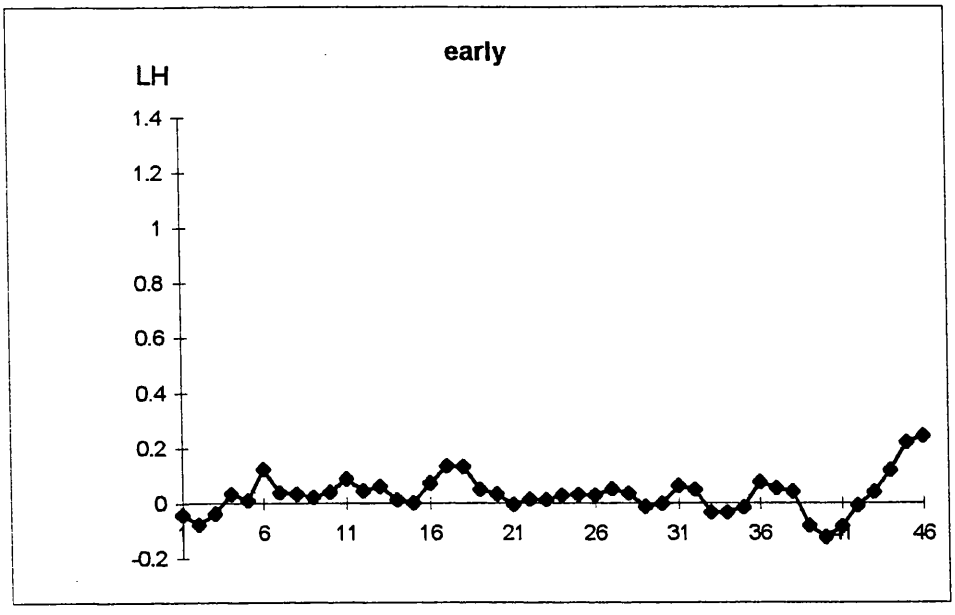
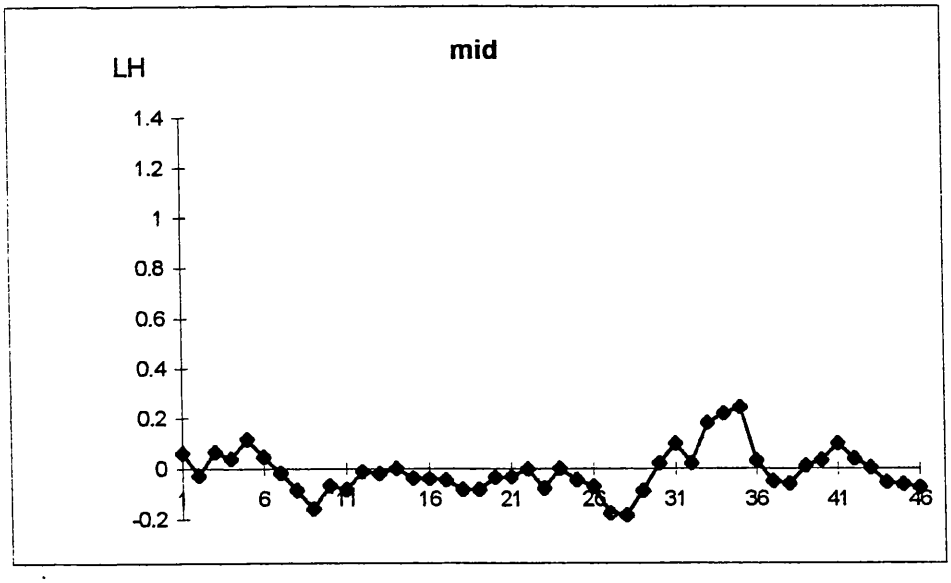

131 L
LH (ng/ml)
3
2.5
2
1.5
1
0.5
0
0
10
20
30
40
50
time (hours)

Figure 27 LH plasma concentration in the mock-aspirated control cows during the early, mid and late stages of the luteal phase. Each point is the mean of the aspirated cows and is balanced by subtraction of the pre-mock-aspiration mean value.


early
LH
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
1
6
11
16
21
26
31
36
41
46


mid
LH
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
1
6
11
16
21
26
31
36
41
46

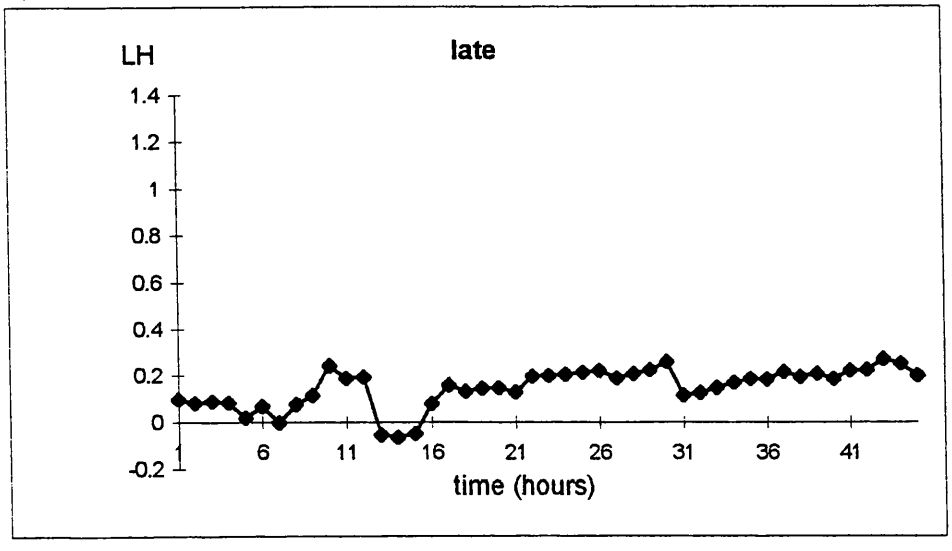
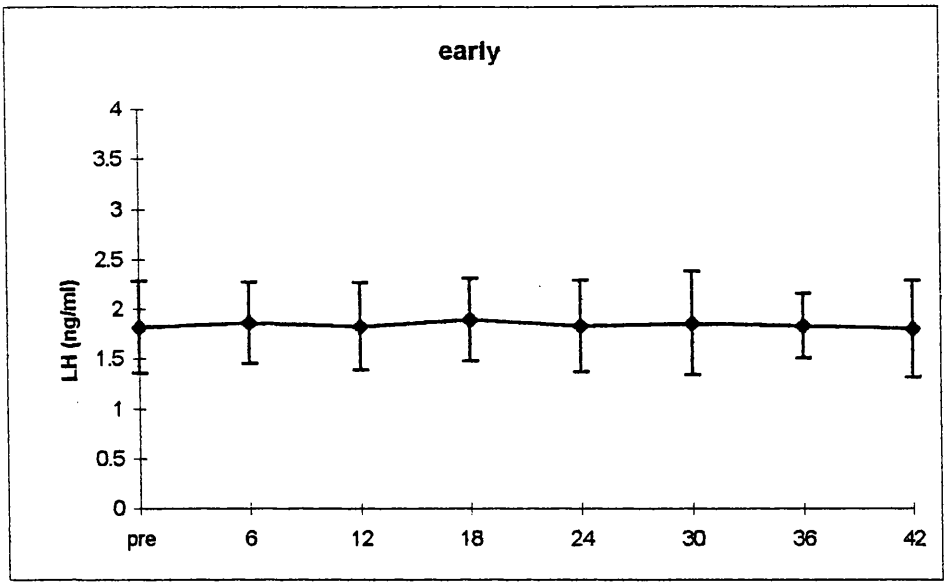
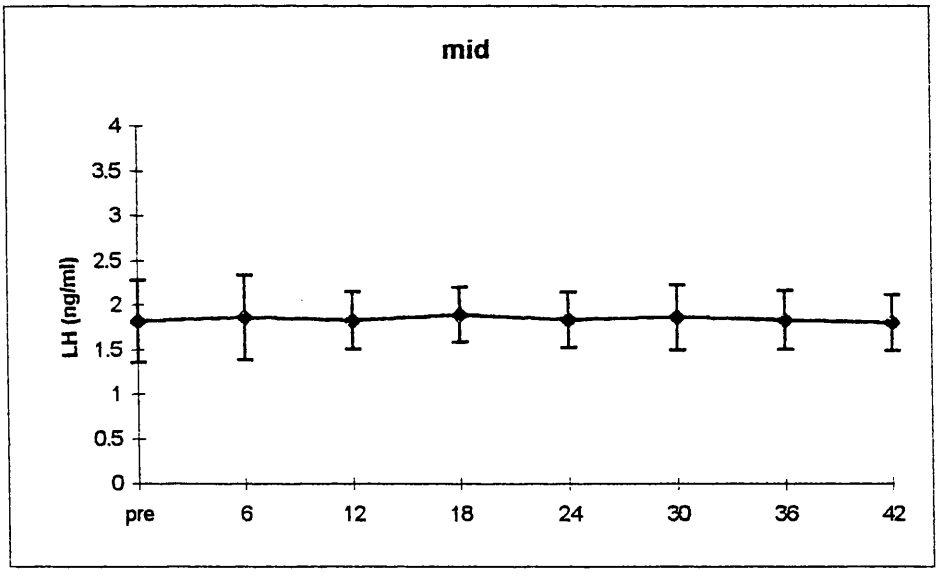

late
LH
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
1
6
11
16
21
26
31
36
41
time (hours)

Figure 28 LH plasma concentration pre- and post-mock-aspiration (control cows) during the early, mid and late stages of the luteal phase. Each post aspiration value is the mean for a 6 hour interval.


early
LH (ng/ml)
4
3.5
3
2.5
2
1.5
1
0.5
0
pre
6
12
18
24
30
36
42


mid
LH (ng/ml)
4
3.5
3
2.5
2
1.5
1
0.5
0
pre
6
12
18
24
30
36
42

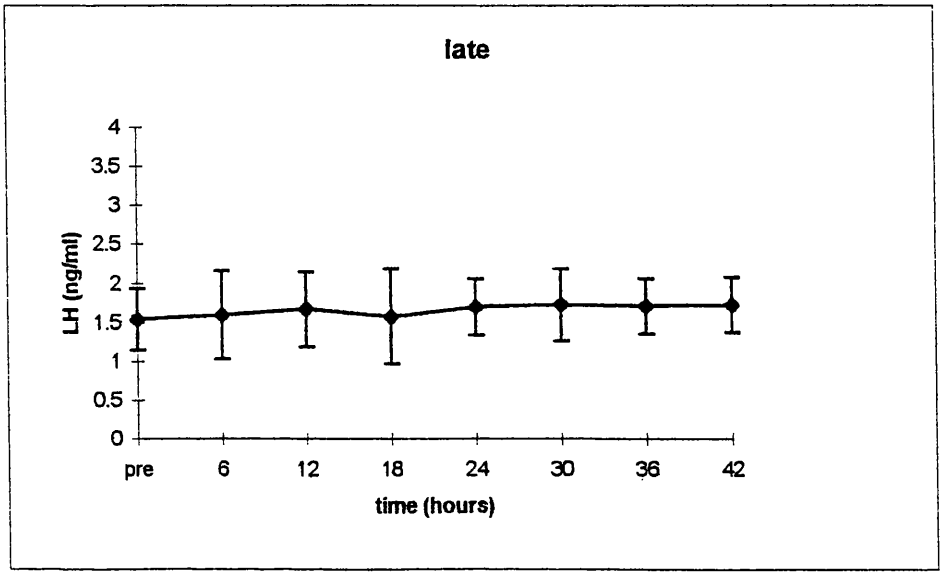

late
LH (ng/ml)
4
3.5
3
2.5
2
1.5
1
0.5
0
pre
6
12
18
24
30
36
42
time (hours)

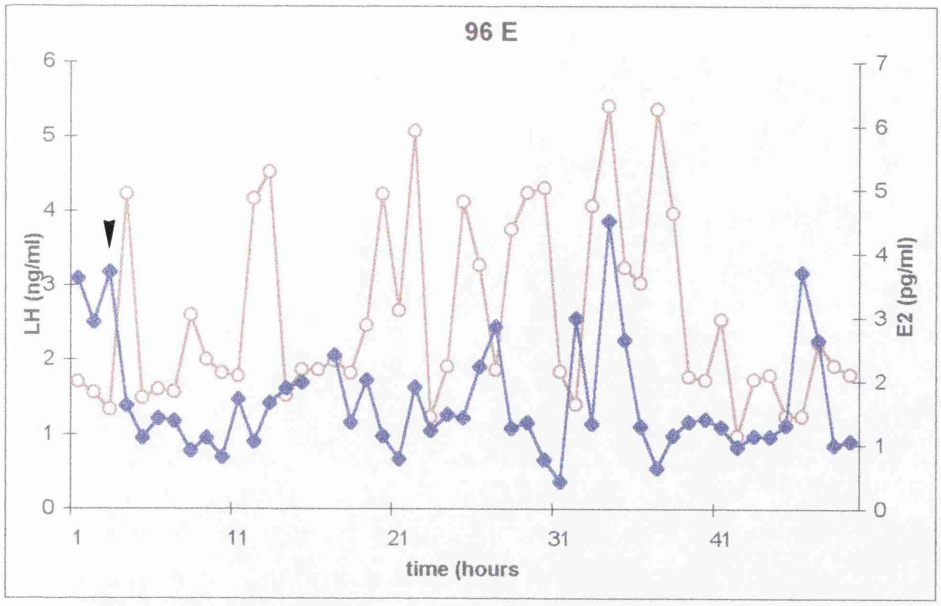
Table 13. Mean ±SD of oestradiol and LH pulse frequency and pulse maxima of the two hormones during the three stages of the luteal phase.

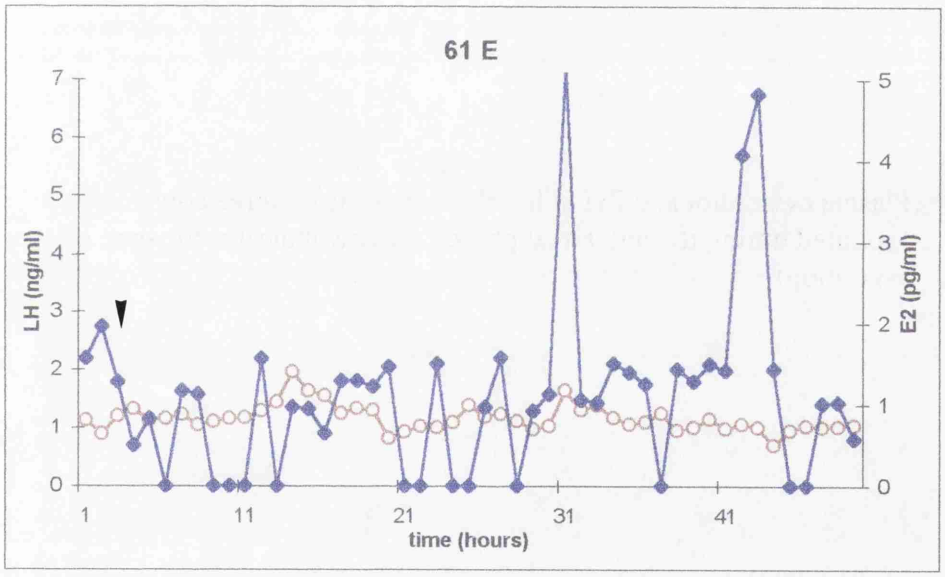
	Early luteal		mid luteal		late luteal	
Pulses/42h	Aspirated	Controls	Aspirated	Controls	Aspirated	Controls
Oestradiol	9±1.4	8.3±0.49	8±1.09	6.7±0.43	8.2±0.73	9.3±1
LH	7.33±1.7	4.75±0.8	5.8±0.68	2.5±0.86	7.1±1.07	5.7±1.09
Pulse maxima						
Oestradiol	4.2	3.9	5.9	6.4	5.4	12.6
LH	4.07	2.4	6.4	3.7	4.13	2.6

3.3.3 Effects of multiple follicular aspiration on oestrus and endocrine parameters after prostaglandin-induced luteolysis

A luteolytic dose of prostaglandin $F_{2\alpha}$ (PG) was injected during the mid luteal phase (day 8) in two cows to be subjected to aspiration and one control cow. Follicular aspiration was performed four hours later and hourly blood samples were collected from the time of PG injection until ovulation was confirmed by transrectal ultrasonography. Oestrus detection was performed every six hours. Standing heat was detected 96 and 108 hours after PG injection in the aspirated animals and 72 hours afterwards in the control. The preovulatory LH surge occured 86 and 92 hours after PG injection in the aspirated and 65 hours in the control cow. Peaks in plasma oestradiol were detected 85 and 88 hours after the

Figure 29 Plasma oestradiol and LH in hourly collected samples from three cows aspirated during the early luteal phase. (Arrow indicates the time of aspiration)


96 E
LH (ng/ml)
E2 (pg/ml)
time (hours


61 E
LH (ng/ml)
E2 (pg/ml)
time (hours)

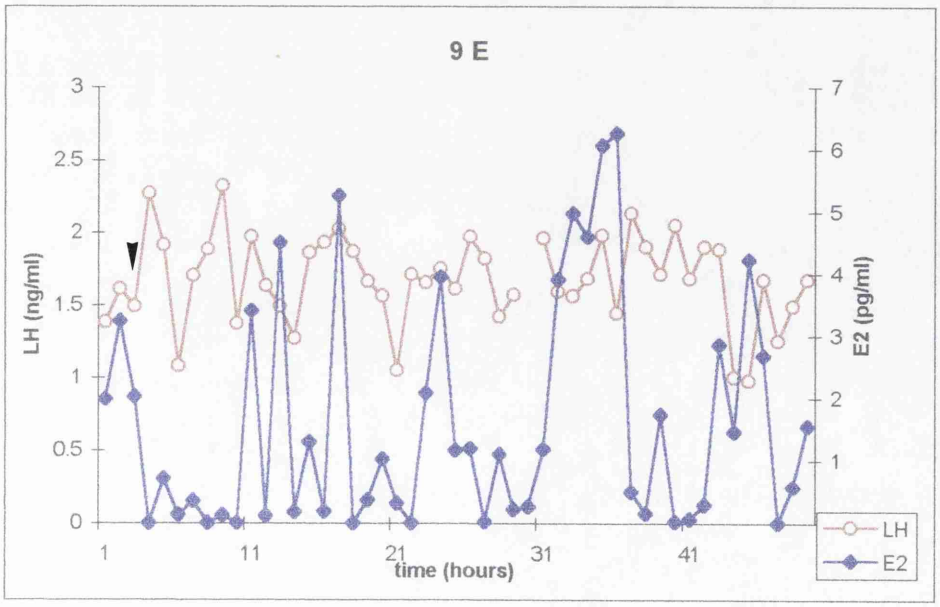
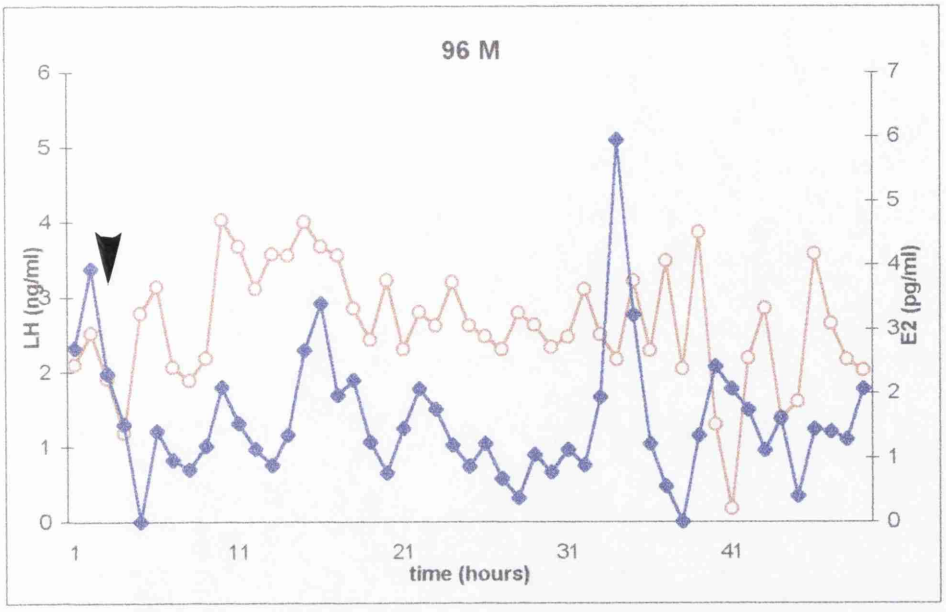
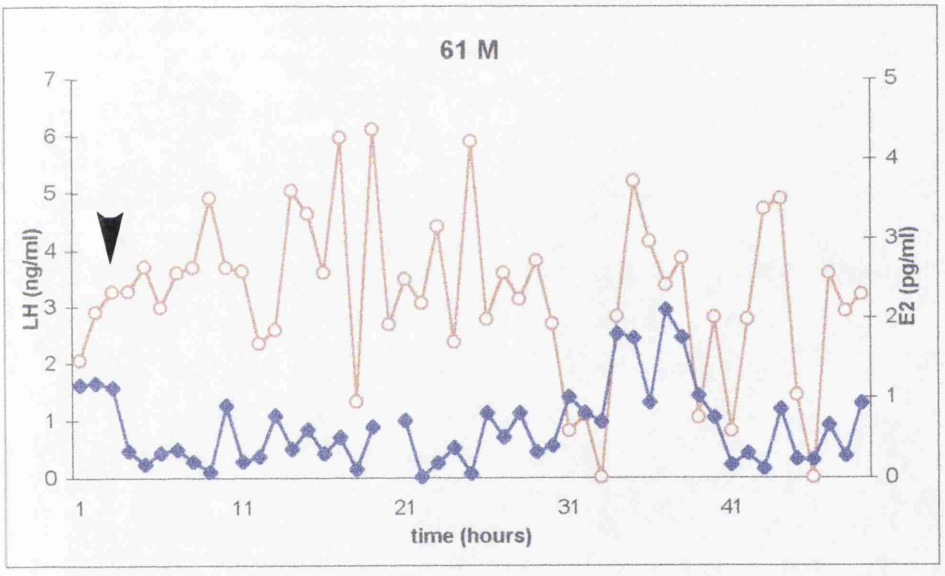

9 E
LH (ng/ml)
E2 (pg/ml)
time (hours)
LH
E2

Figure 30 Plasma oestradiol and LH in hourly samples from three cows aspirated during the mid luteal phase. (Arrow indicates the time of aspiration)


96 M
LH (ng/ml)
E2 (pg/ml)
time (hours)


61 M
LH (ng/ml)
E2 (pg/ml)
time (hours)

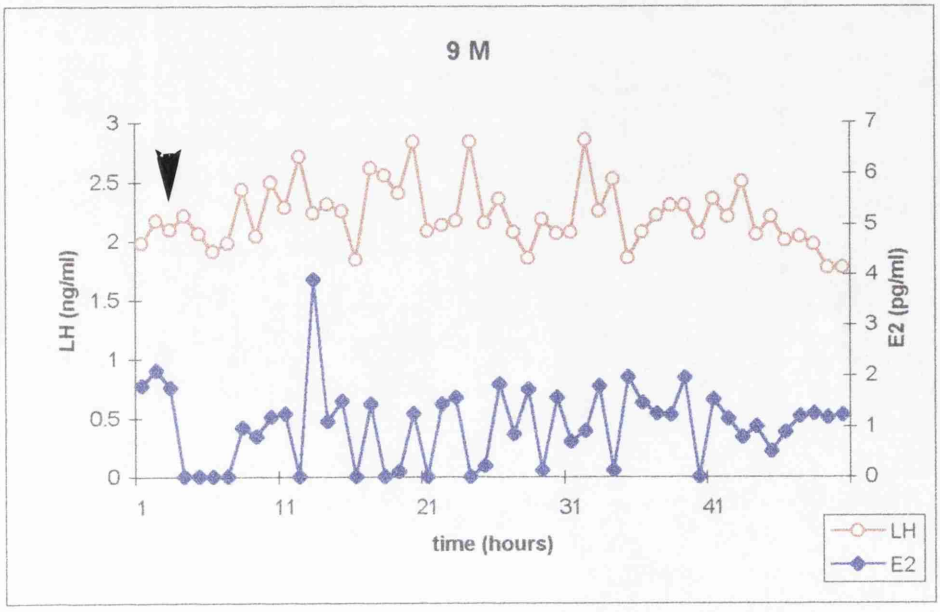
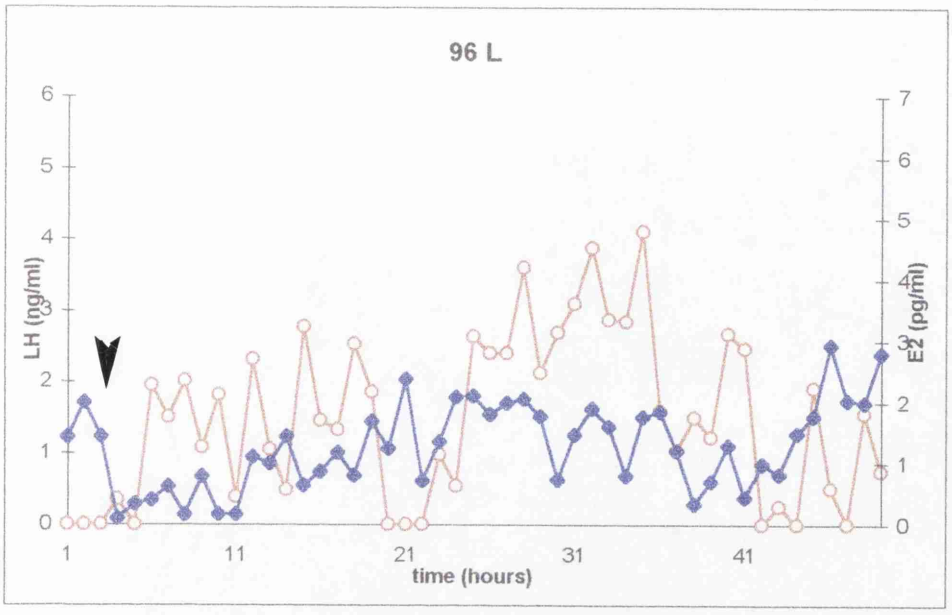
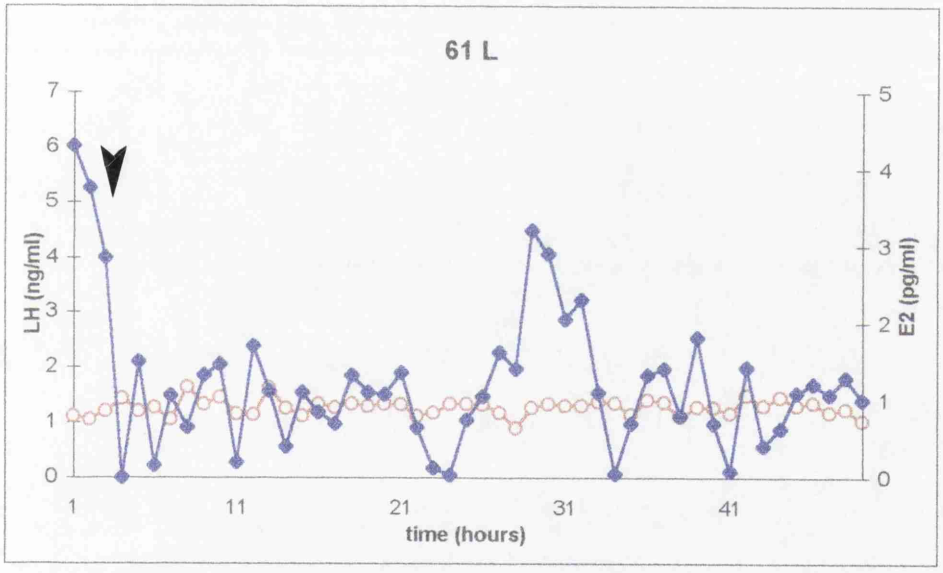

9 M
LH (ng/ml)
E2 (pg/ml)
time (hours)
LH
E2

Figure 31 Plasma oestradiol and LH in hourly samples from three cows aspirated during the late luteal phase. (Arrow indicates the time of aspiration)


96 L
LH (ng/ml)
E2 (pg/ml)
time (hours)
0
1
2
3
4
5
6
7
1
11
21
31
41


61 L
LH (ng/ml)
E2 (pg/ml)
time (hours)
0
1
2
3
4
5
6
7
1
11
21
31
41

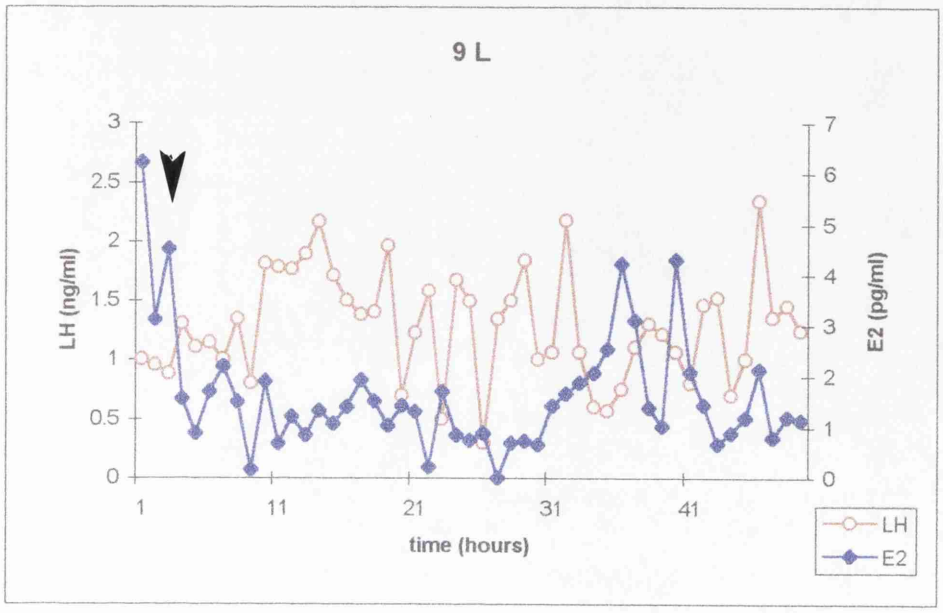
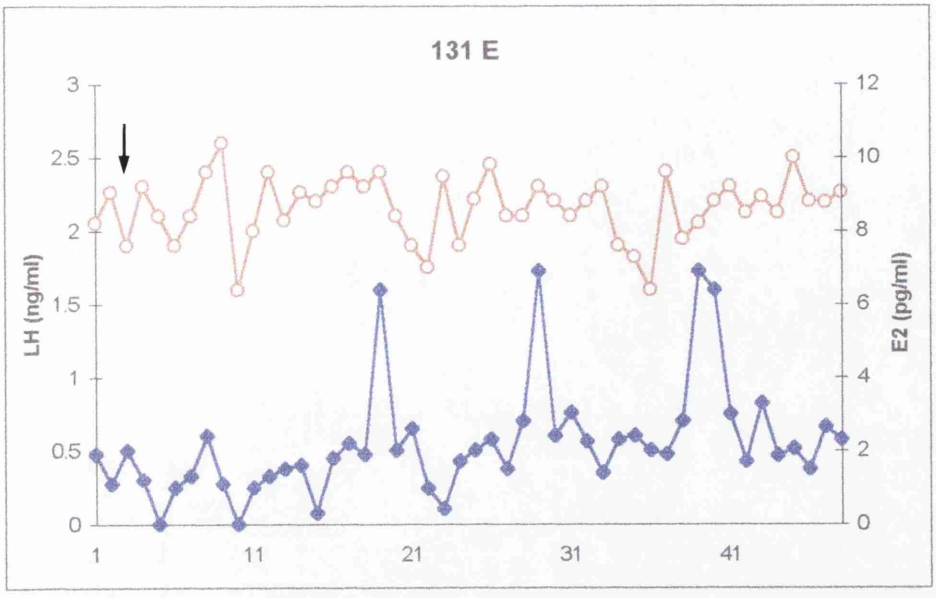
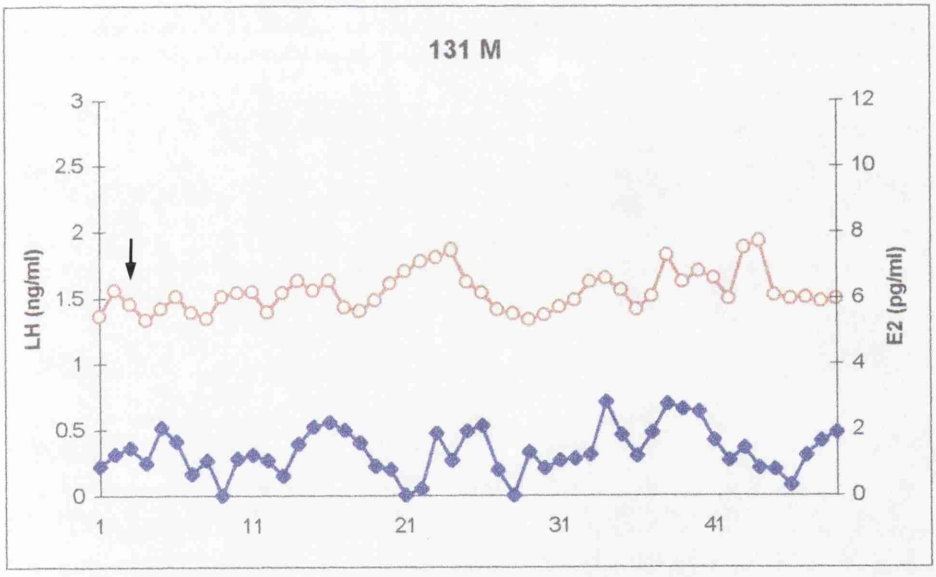
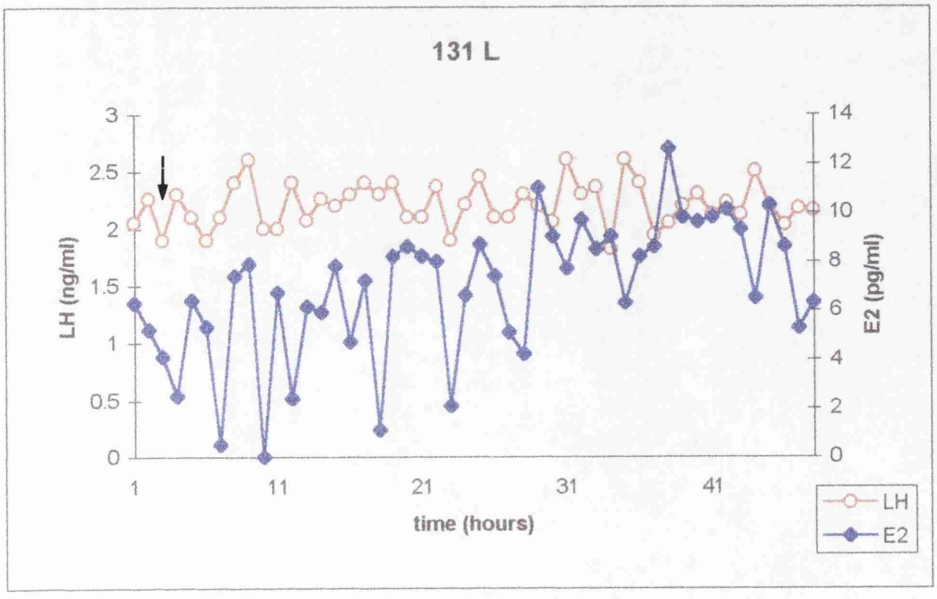

9 L
LH (ng/ml)
E2 (pg/ml)
time (hours)
0
0.5
1
1.5
2
2.5
3
1
11
21
31
41
LH
E2

Figure 32 Plasma oestradiol and LH in hourly samples from one control cow during the early, mid and late luteal phases.


131 E
LH (ng/ml)
E2 (pg/ml)
3
2.5
2
1.5
1
0.5
0
12
10
8
6
4
2
0
1
11
21
31
41


131 M
LH (ng/ml)
E2 (pg/ml)
3
2.5
2
1.5
1
0.5
0
12
10
8
6
4
2
0
1
11
21
31
41


131 L
LH (ng/ml)
E2 (pg/ml)
3
2.5
2
1.5
1
0.5
0
14
12
10
8
6
4
2
0
1
11
21
31
41
time (hours)
LH
E2

PG administration in the aspirated cow and 53 hours afterwards in the control (fig 33)

3.3.4 Effect of repeated multiple follicular aspiration on follicular turn-over and follicular growth rate.

3.3.4.1 Follicular population

Among the control animals two oestrus cycles were found to comprise two follicular waves and six cycles had three follicular waves. The length of the two wave-cycles was equal to that of two cycles comprising three follicular waves (21 days)

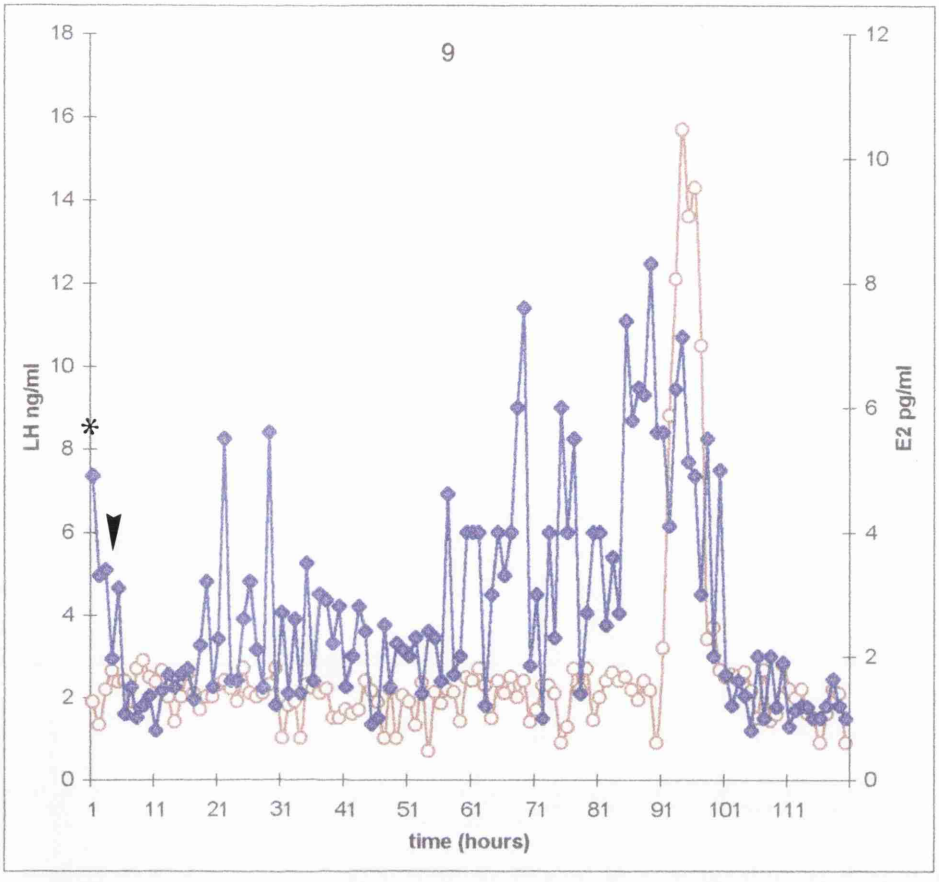
During the early luteal phase the mean number of follicles available for aspiration was similar between aspirated and control cows (4.66 ± 1.49 and 5.15 ± 2 respectively). However, the follicular population in the aspirated group increased to 7.95 ± 1.33 and 5.62 ± 1.44 during the mid and late stages of the luteal phase respectively, but remained essentially unchanged in the control animals (5.62 ± 1.44 and 6.15 ± 1.25 respectively).

3.3.4.2 Follicular size and re-growth

The size of the largest follicle and its growth rate as measured by transrectal ultrasonography at two day intervals throughout the luteal phase is shown in table 14.

An interesting observation was that by one day after multiple follicle aspiration some ovaries were found to be bearing follicles of comparable size to those found before aspiration. A spherical echogenic structure representing a blood clot in the

Figure 33. Plasma LH and oestradiol in an aspirated cow (upper panel) and a sham-aspirated cow (lower panel) following prostaglandin injection. Asterisk indicates the time of PG injection and the arrow the time of aspiration.


9
LH ng/ml
E2 pg/ml
time (hours)
0
2
4
6
8
10
12
14
16
18
1
11
21
31
41
51
61
71
81
91
101
111

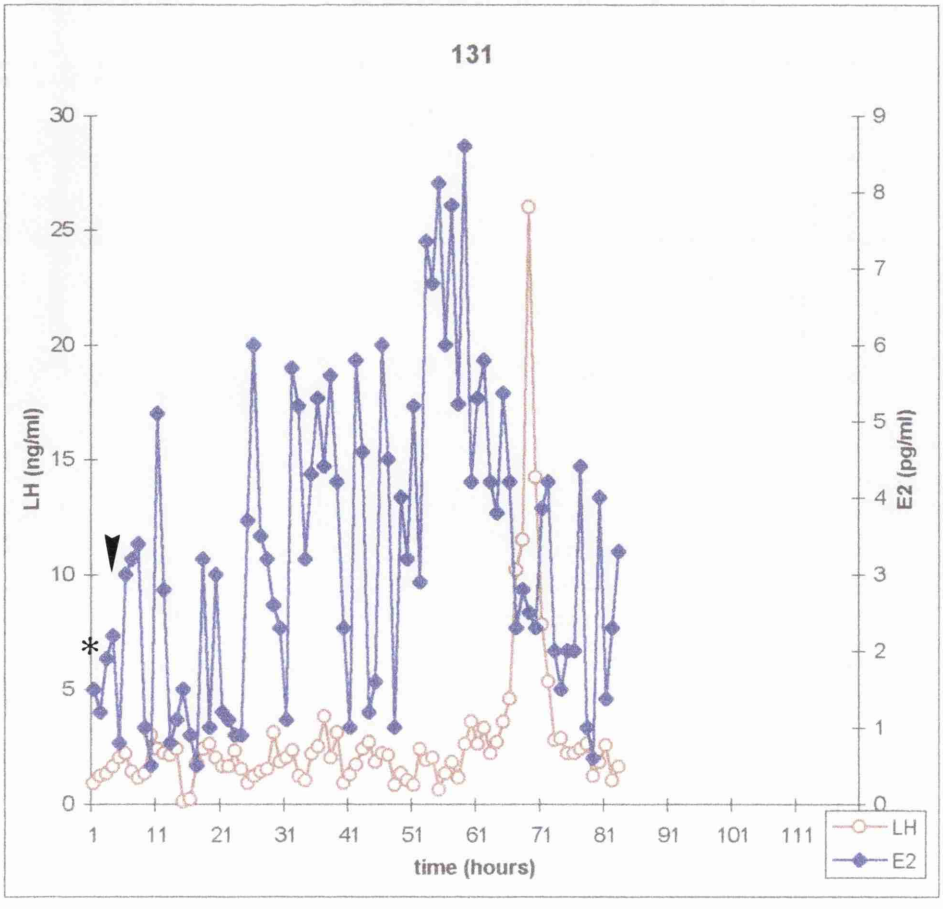
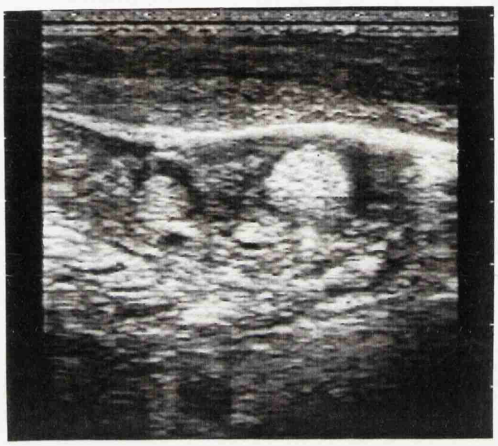
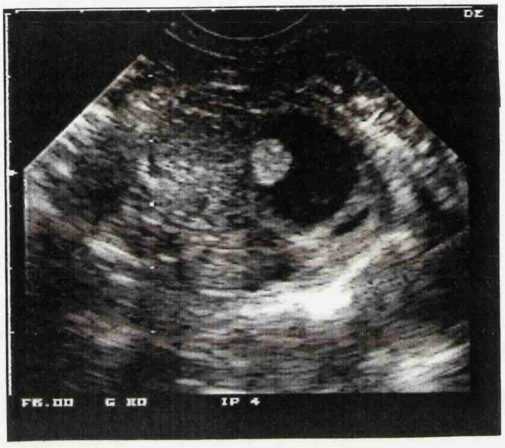
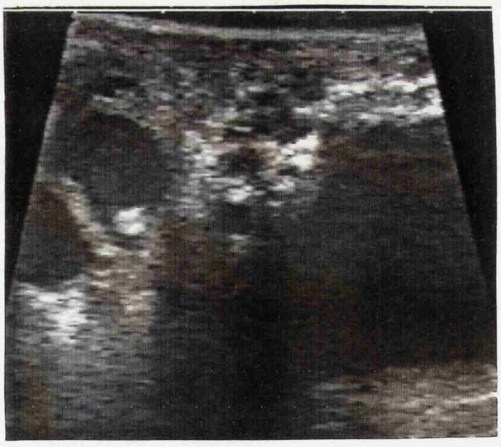
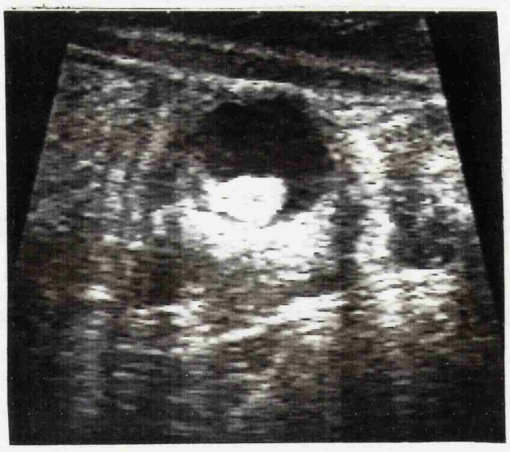

131
LH (ng/ml)
E2 (pg/ml)
time (hours)
LH
E2
0
5
10
15
20
25
30
1
2
3
4
5
6
7
8
9
1
11
21
31
41
51
61
71
81
91
101
111

Figure 34 Re-growth of aspirated follicles.
Upper left Blood clots in the cavities of aspirated follicles as detected by transrectal ultrasound scanning the day after aspiration.
Upper right: A blood clot seven days later: The structure has decreased in size but is still visible in the follicle which attained a diameter of almost 1.5cm.
Lower left and Right: Blood clots in re-filled follicles seven days after aspiration.




DE
F6.00 G 80 IP 4





follicular cavity was also observed on approximately 12 occasions, each a day or two after aspiration. In some cases this structure filled the follicle completely but more commonly it appeared as a small bright (echogenic) spot attached to the follicular wall. (fig. 34).

Table 14. Diameter of the largest follicle (Mean ±SD) and its growth rate assessed at two day intervals.

	Aspirated		**Controls**	
Stage of cycle	Diameter (cm)	Growth rate (mm/2d)	Diameter (cm)	Growth rate (mm/2d)
Early	0.98±0.056	2.81±1.8	0.94±0.13	2.16±1.61
Mid	1.11±0.3	2.42±2	1.2±0.2	1.63±1.1
Late	1.31±0.37	3.15±2.3	1.2±0.17	2.16±0.98

3.3.4.3 Correlation between plasma oestradiol concentration and the number of follicles aspirated

During 30 aspiration sessions 226 follicles were punctured. A mean of 7.52 ± 2.7 follicles were aspirated per session ranging from 2 to 12 follicles. The degree of reduction in plasma oestradiol concentration after the follicular ablation showed a linear correlation to the number of aspirated follicles (r= 0.84, fig. 35)

3.4 A possible link between dominant follicle ablation and development of an accessory luteal structure.

3.4.1 Introduction.

Repeated aspiration of all ultrasonically visible follicles, as described in Chapter 3.3, resulted in development of suspected accessory luteal structures in four cows as described below. An accessory structure was confirmed in one cow and this is the subject of section 3.4.2, below. The results described in the remainder of this chapter refer to experiments aimed at studying the effect of aspirating just the dominant follicle.

The oestrous cycles of eight cows (numbers 47, 61, 68, 96, 97 100, 131and 156) were synchronised to allow aspiration of just the dominant follicle once during the early, once during the mid and once during the late stages of the luteal phase. Blood samples for hormone analysis were collected every 15 minutes starting four hours before aspiration and continuing for eight hours after aspiration. It was considered unnecessary to include control (sham-aspirated) cows since the four hour pre-aspiration bleeding period allowed sufficient opportunity to compare pre- and post-aspiration blood samples.

3.4.2 Accessory Luteal Structure

Four cows developed suspected accessory luteal structures

Cow 9: A structure with the appearance of a developing corpus luteum was detected on the left ovary five days after multiple follicle aspiration carried out during the late luteal phase. The structure was examined *post mortem* when it was confirmed to be a real corpus luteum (fig. 37). However, blood sample analysis revealed the presence of a substantial LH peak occurring seven hours after aspiration as shown in fig. 36.

Figure 35 Correlation between number of follicles aspirated per session and the relative reduction in plasma oestradiol concentration.

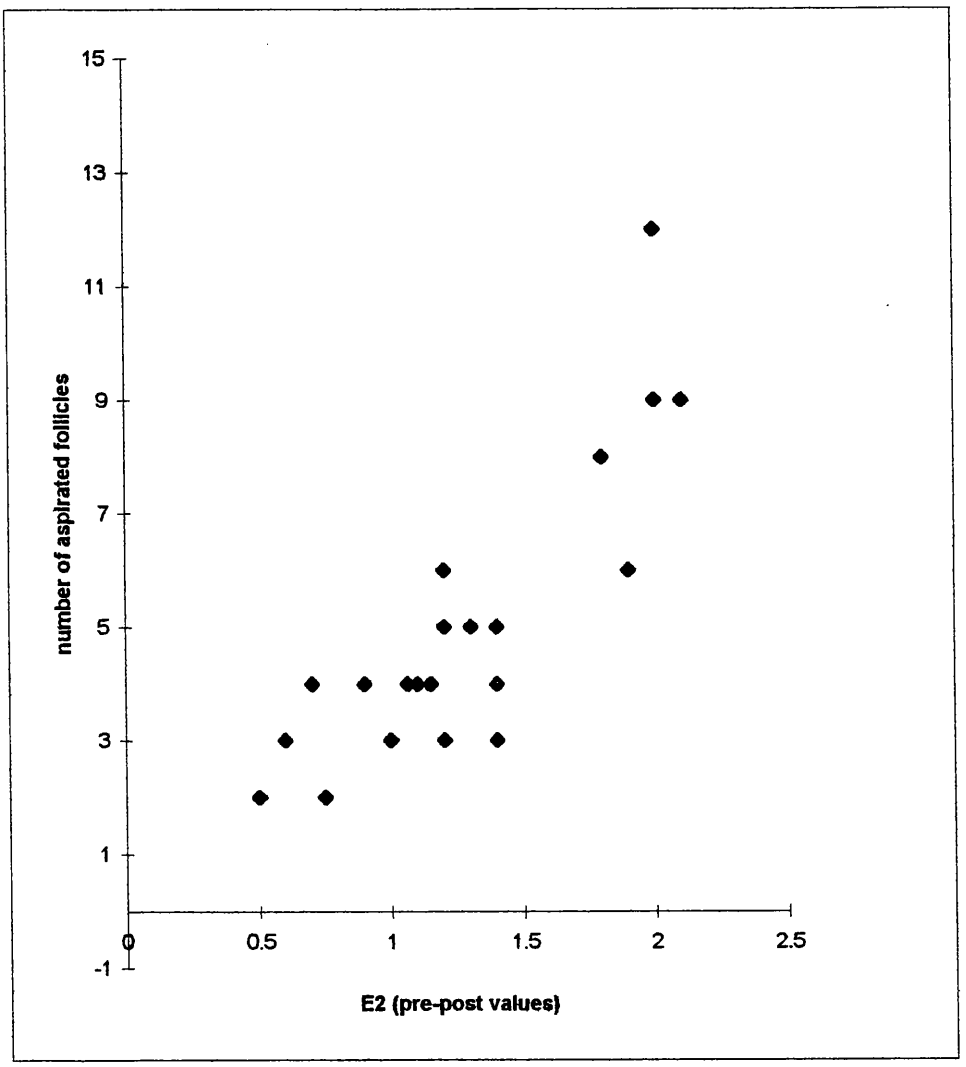

15
13
11
9
7
5
3
1
-1
number of aspirated follicles
0
0.5
1
1.5
2
2.5
E2 (pre-post values)

Figure 36. Plasma concentration of LH and oestradiol in hourly samples and progesterone concentration(asterisks) in daily samples from cow 9 aspirated during the early follicular phase. The arrow indicates the time of aspiration.

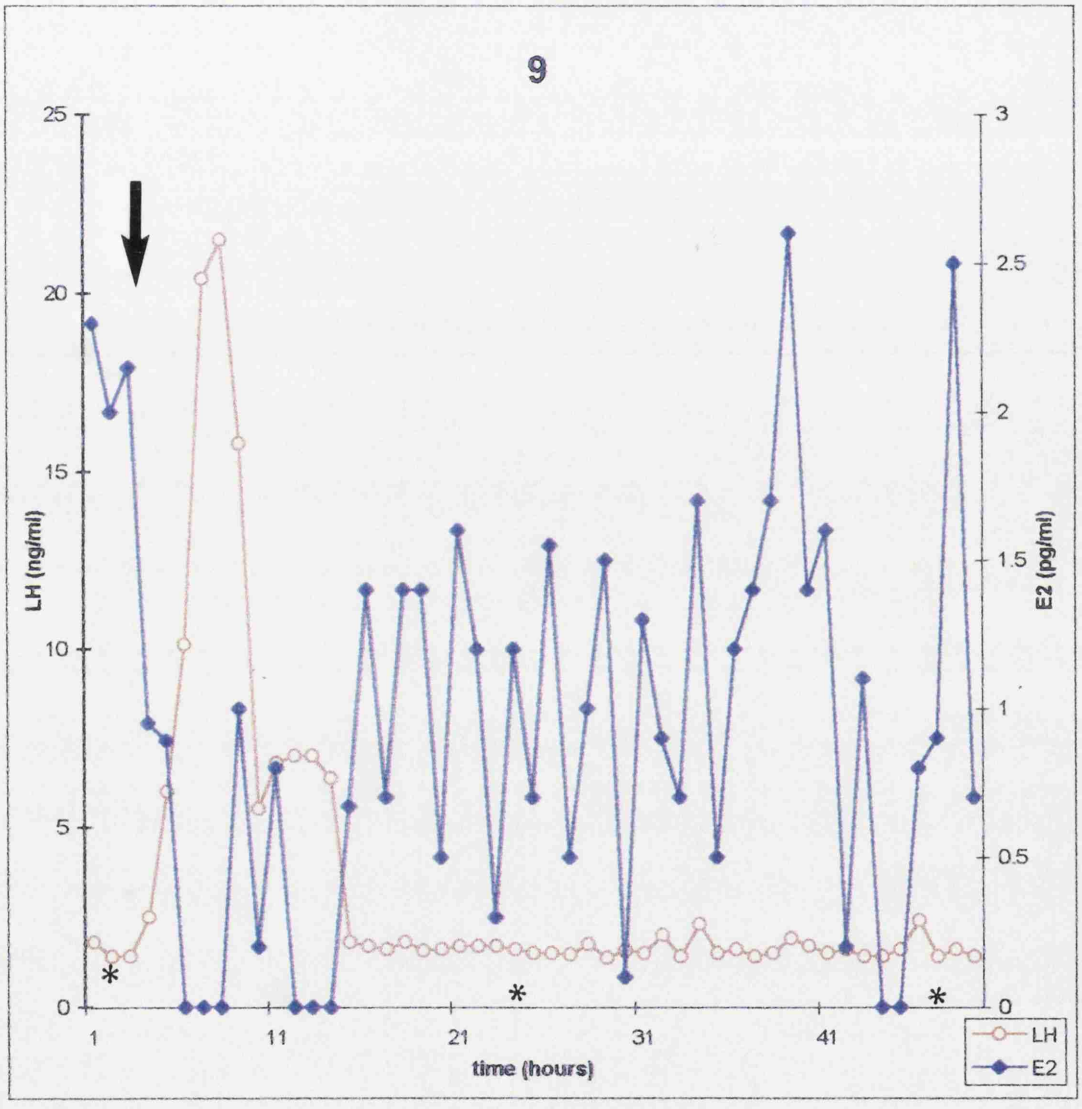
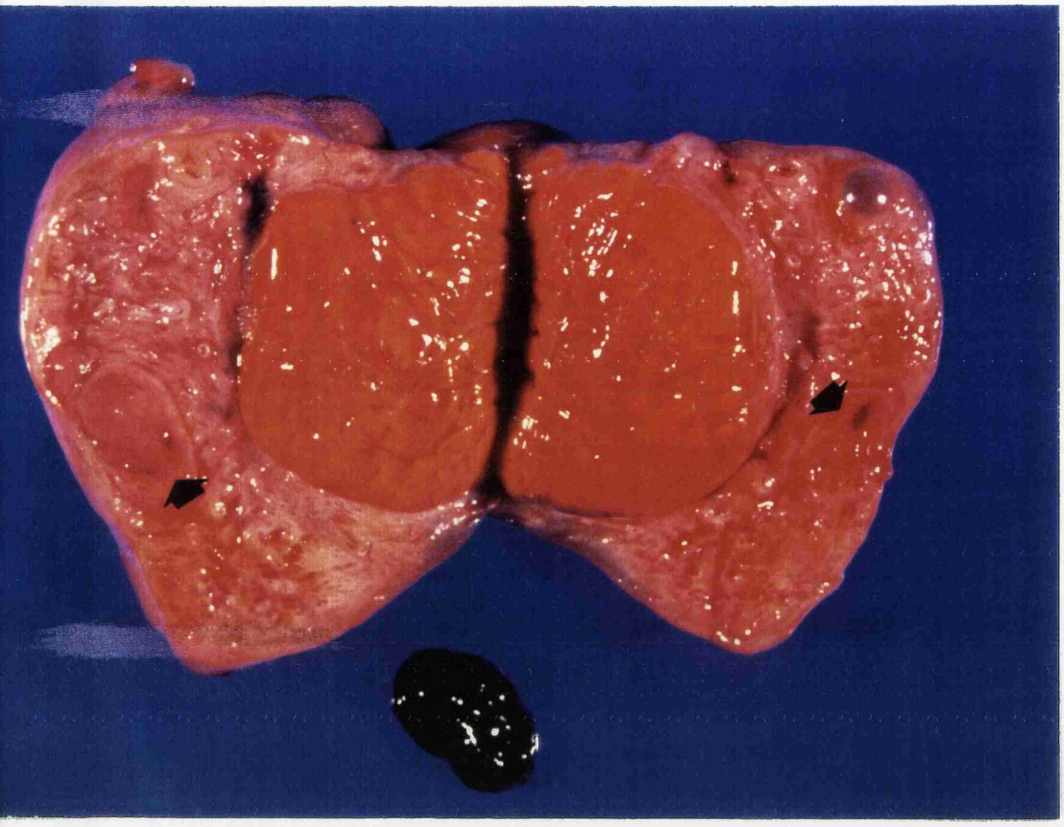

9
LH (ng/ml)
E2 (pg/ml)
time (hours)
LH
E2
25
20
15
10
5
0
3
2.5
2
1.5
1
0.5
0
1
11
21
31
41
*
*
*

Figure 37 **Upper**: The left ovary of cow 96 six days after aspiration in the early luteal phase. The follicle on the left of the picture (arrow) presumed to be an accessory CL contained the blood clot.

Lower: The ovaries of cow 9 . The corpus luteum presumed to be an accessory structure was formed after a normal LH surge.



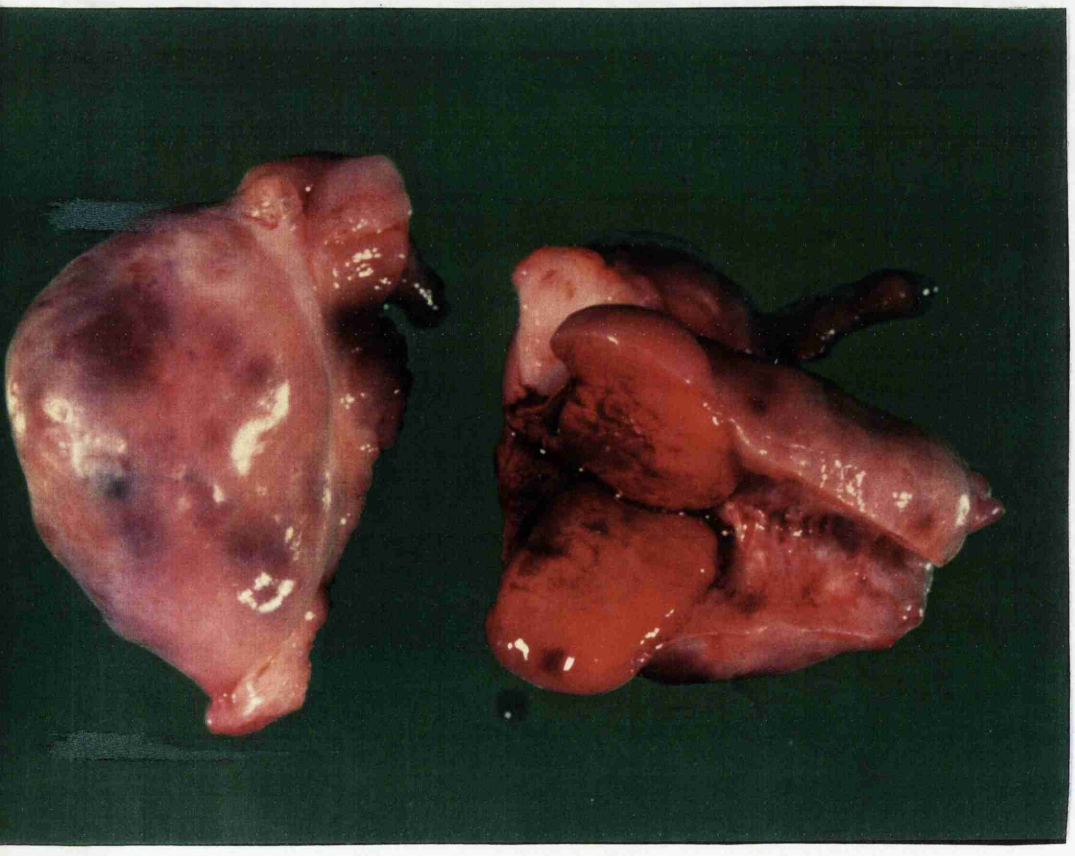
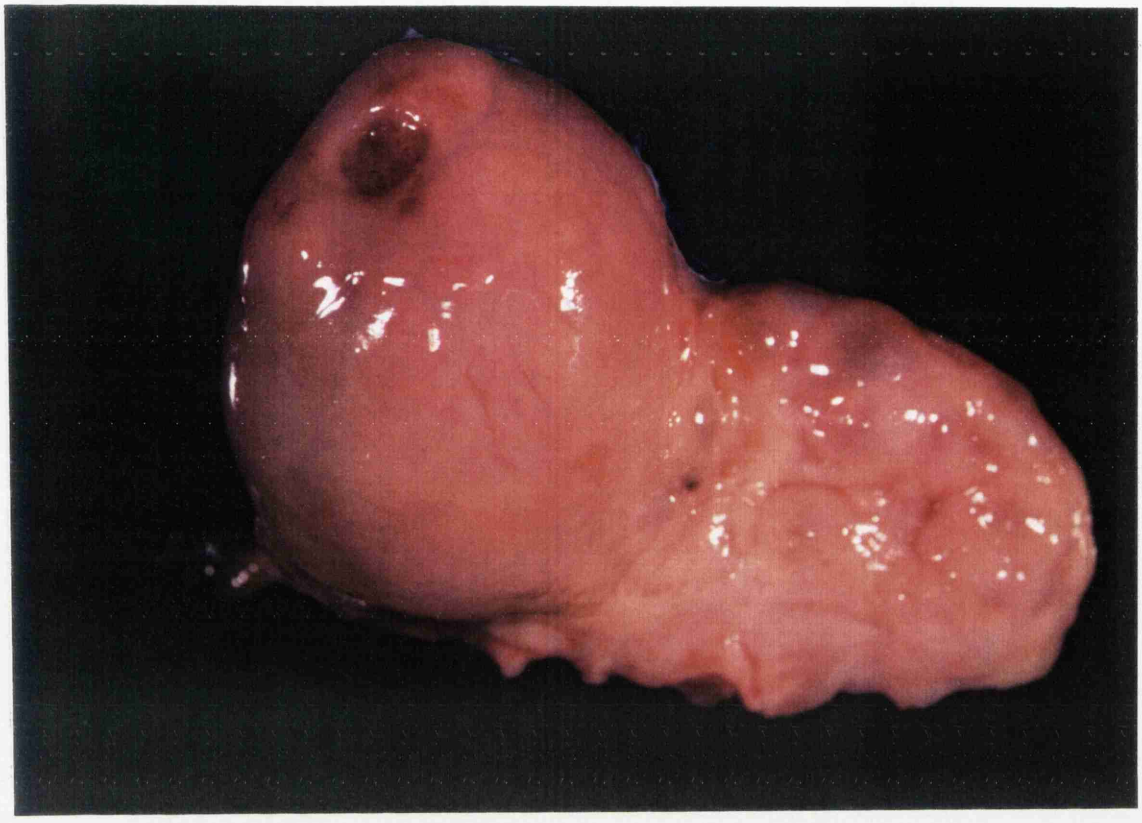
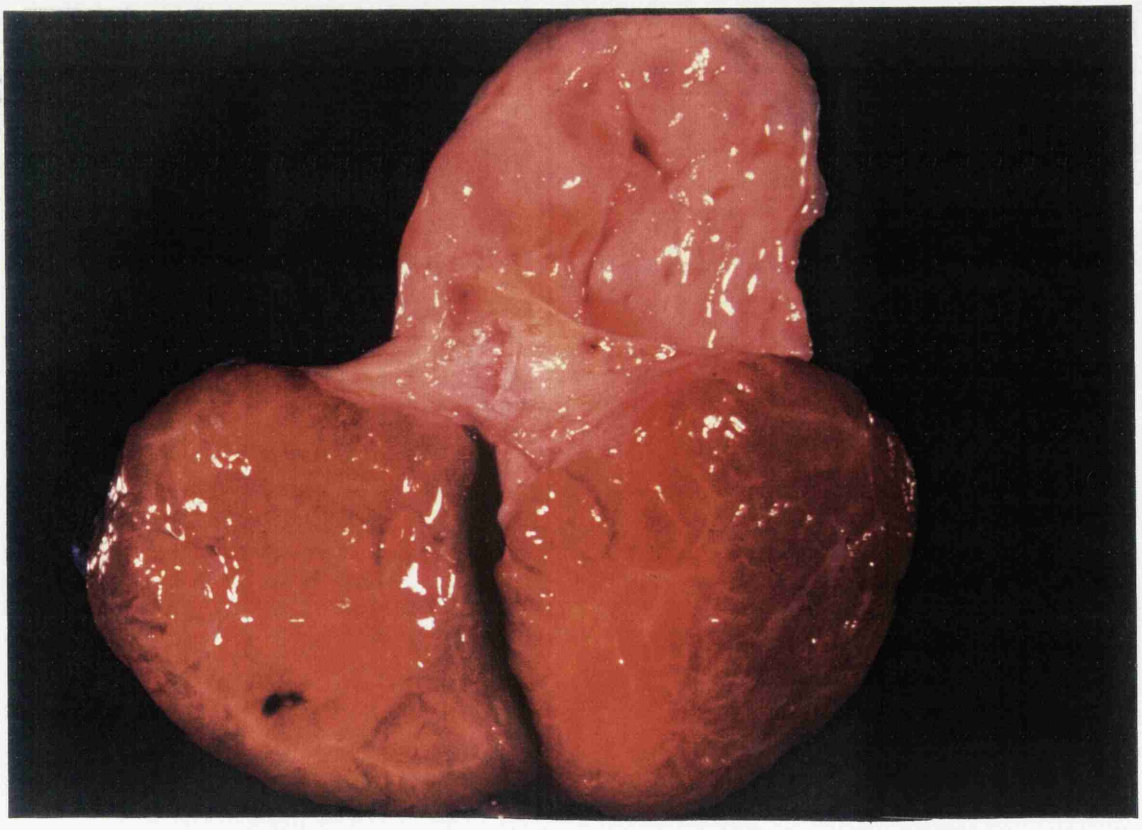


Figure 38 The right ovary of cow 47 bearing a large CL.





Cow 96: An echogenic structure appeared in the left ovary two days after multiple follicle aspiration carried out during the early stage of the luteal phase. The ultrasonographic appearence of the structure remained unchanged for a further five days. The structure was examined *post mortem* and it was revealed to be a blood clot that had filled the cavity of an aspirated follicle as shown in fig. 37.

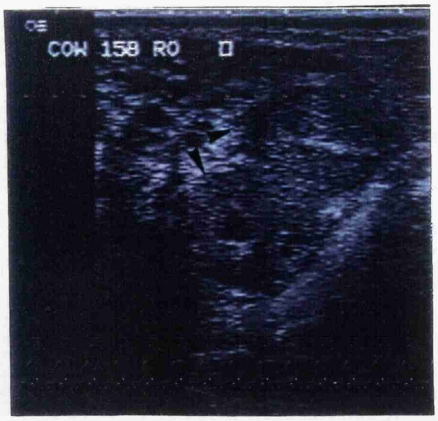
Cow 47: An abnormal structure was palpable on the right ovary seven days after follicular aspiration during the early stage of the luteal phase. It felt like two structures protruding from the surface of the ovary. Corresponding ultrasonographic images were obtained. Upon *post mortem* examination a very large corpus luteum was found to occupy the entire right ovary (fig. 38).

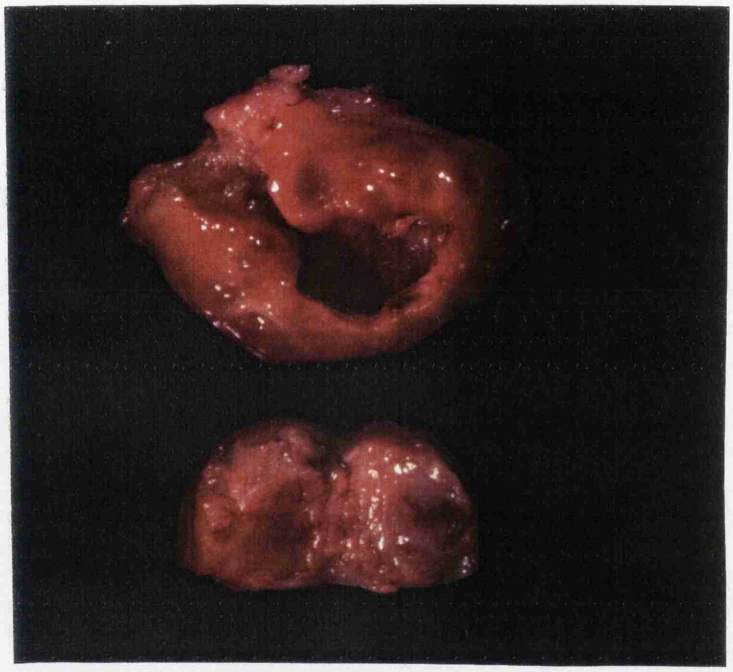
Cow 158: A recently formed structure was revealed seven days after follicle aspiration carried out during the early luteal phase. This structure had similar echogenicity to the existing corpus luteum (fig. 39). *Post mortem* examination revealed that this structure had a similar appearence to a corpus luteum (fig. 39) but it was lighter in weight than the normal structure weighing 4.97g compared with 6.02g. This structure was assumed to be an accessory corpus luteum.

3.4.2.1 Microscopic examination

The histological appearance of the secondary corpus luteum from cow 158 (above) did not differ from that of the normal cyclic corpus luteum. Small and large luteal cells were readily distinguished. The natural corpus luteum was histologically denser than the accessory corpus luteum (158 ± 23 vs 121 ± 15 cells per field) and its cells contained more lipid droplets (fig. 40, 41).

Figure 39 **Top**: Transrectal ultrasound image of the right ovary of cow 158. Two adjacent corpora lutea are noted (arrows)
Middle: The two corpora lutea excised from the ovary
Lower: The natural CL (excised) and the accessory (*in situ* on the ovary)


COW 158 RO



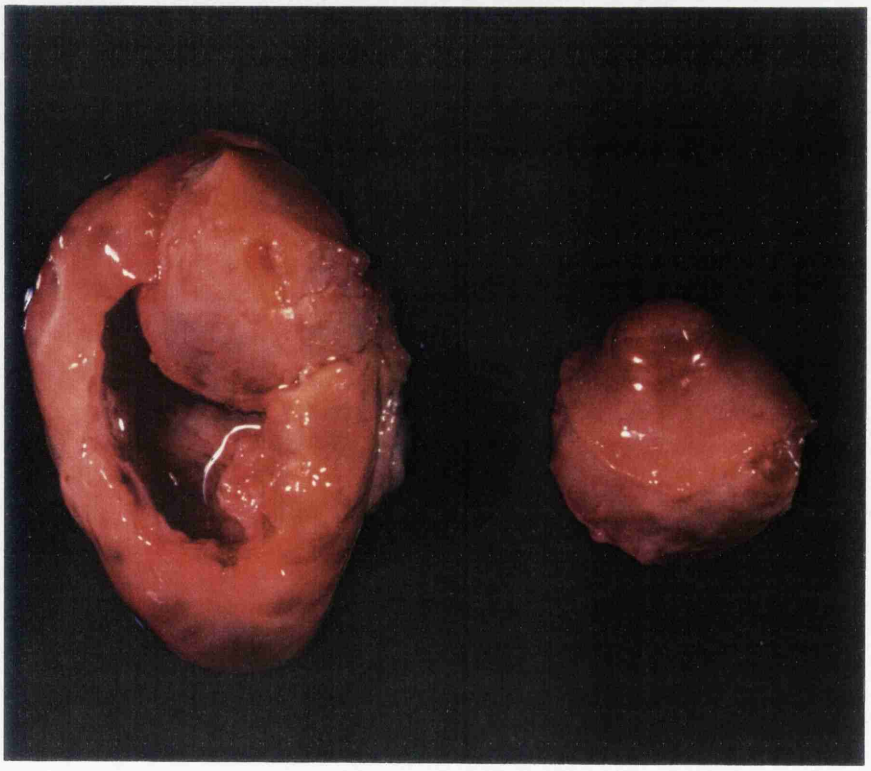
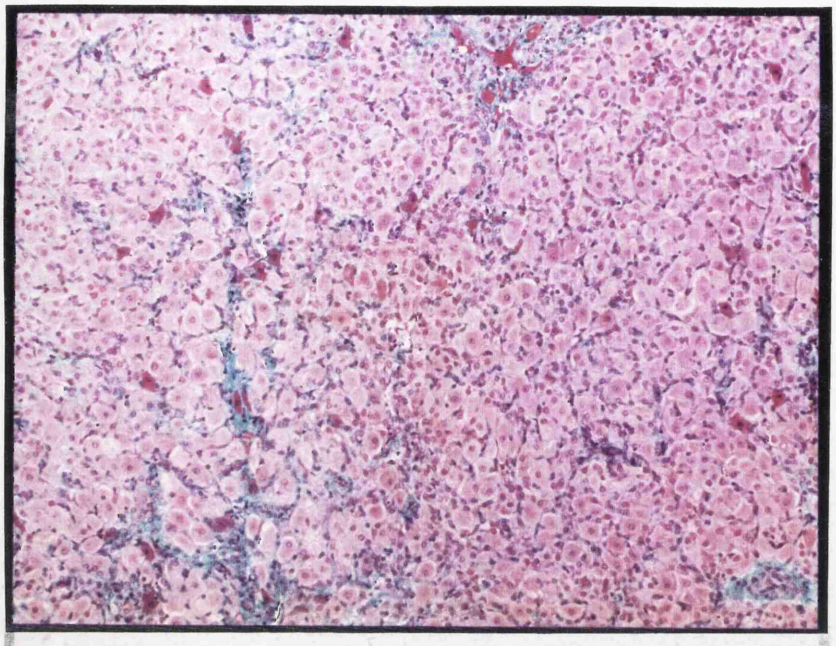


Figure 40 Histological section of the natural CL (upper) and the accessory structure(lower) (X 100)



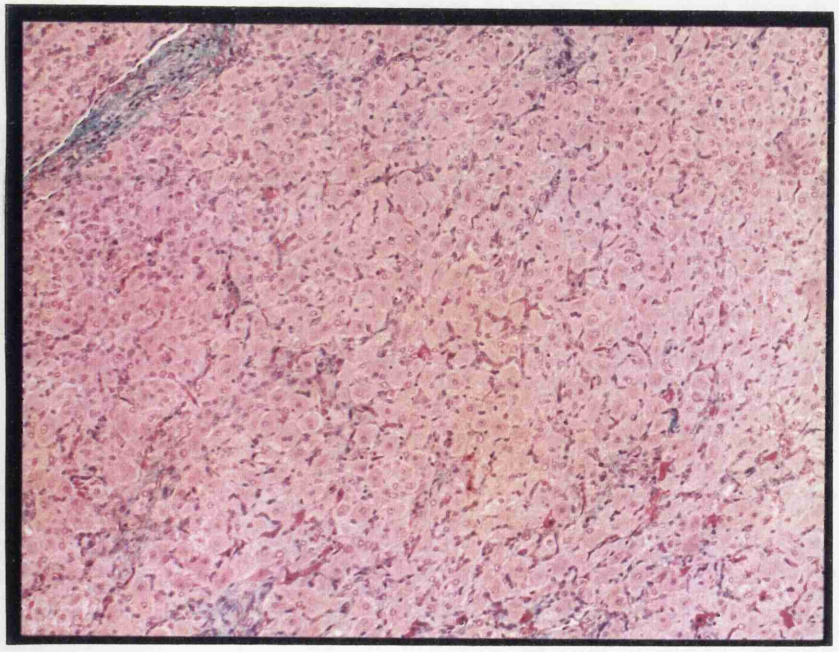
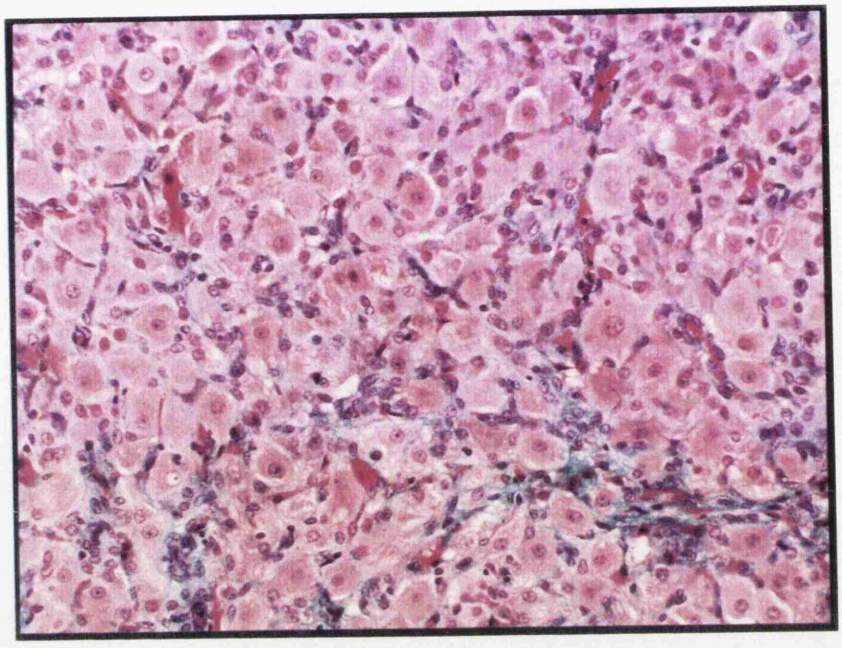


Figure 41 Histological section of the natural CL (upper) and the accessory structure(lower) (X 400). Note the difference in tissue density between the two structures.



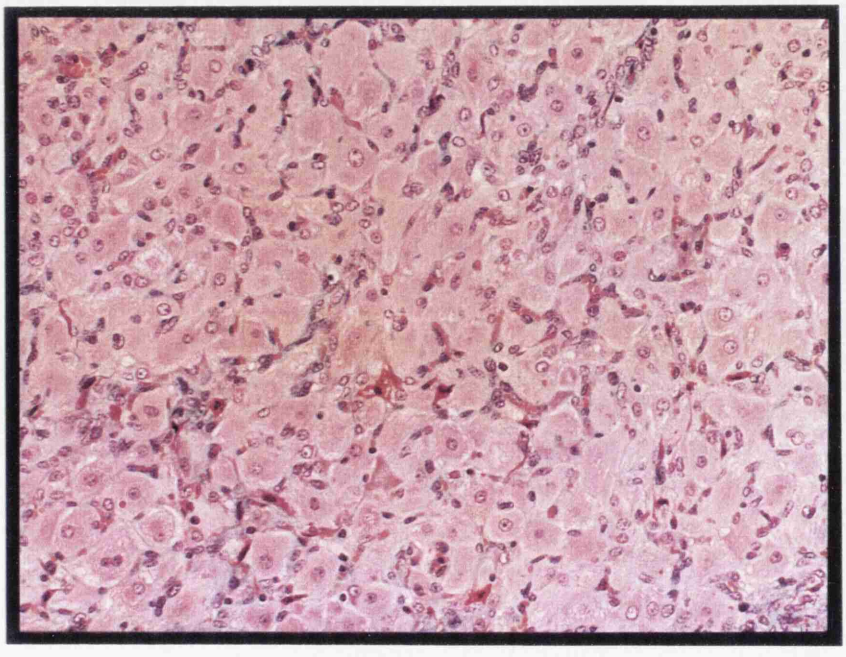
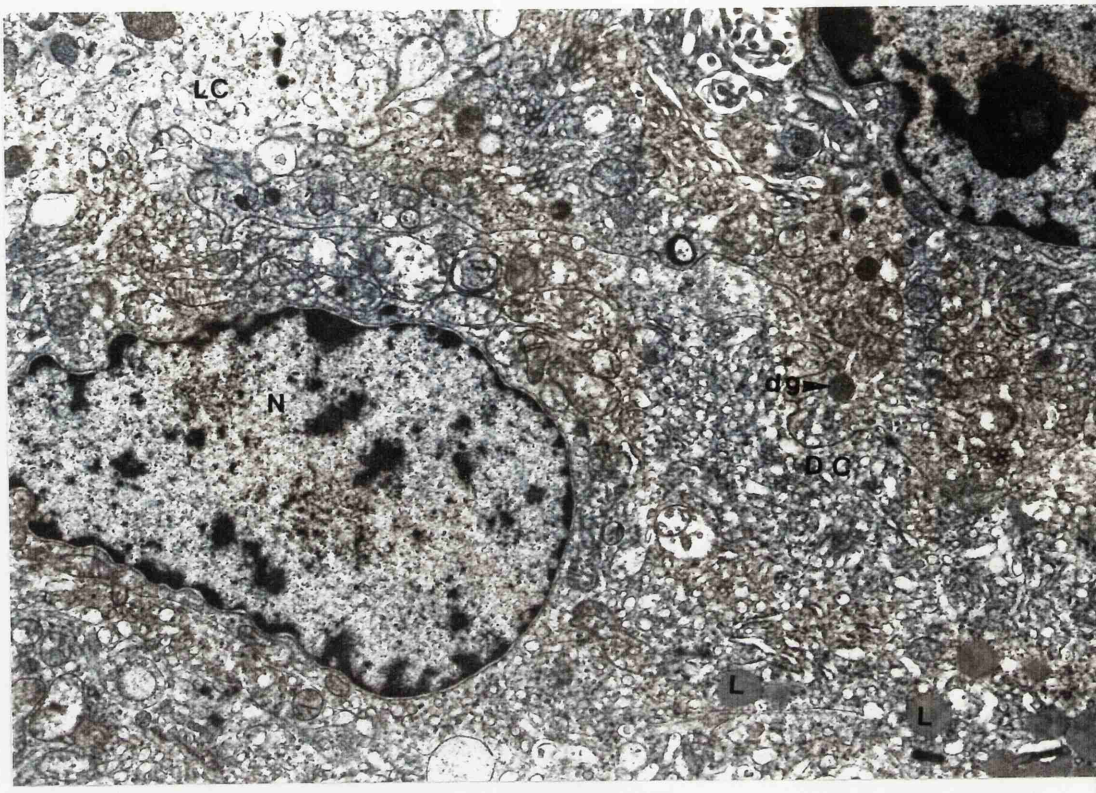


Figure 42 Transmission electron microscopy of the accessory structure.
Upper: Small luteal cell
Lower: Large luteal cell

LC: Light cell
DC: Dark cell
N: Nucleus
dg: dark secretory granule
M: mitochondria
L: lipid droplets


LC
N
dg
D.C
L
L

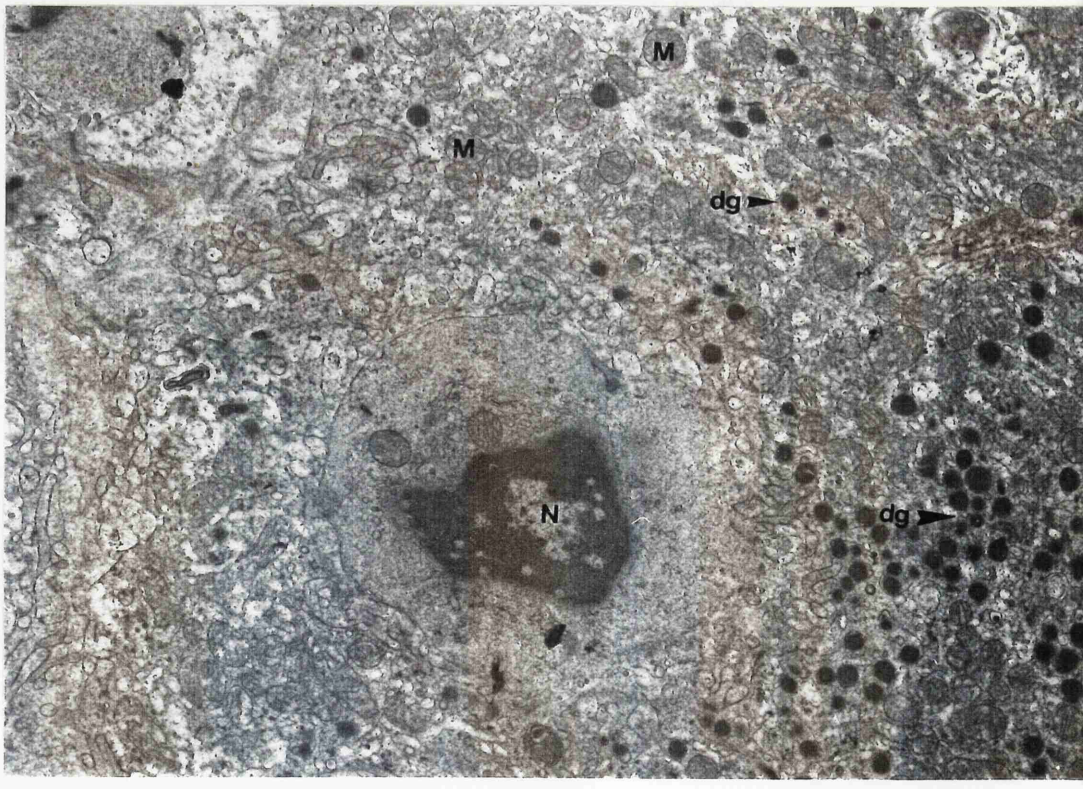
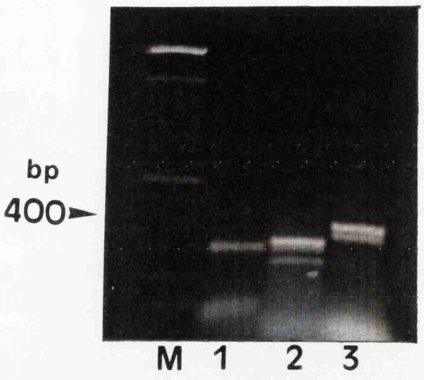

M
M
dg
N
dg

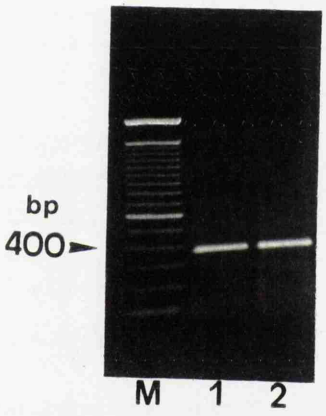
Figure 43 PCR amplification of CDNA from the accessory and the natural CLs

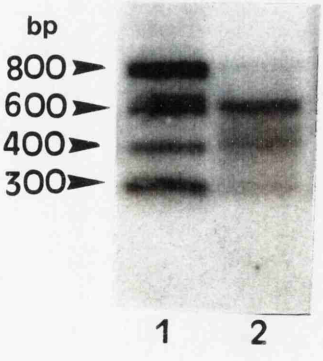
Top: Amplification by RT-PCR, using primers for GAPDH (1), P450scc(2) and P450arom (3) of RNA isolated from the accessory structure. Lane M contained 100bp DNA ladder.

Middle: Amplification by RT-PCR, using primers for P450 17α, of RNA isolated from the accessory (lane 1) and the natural (lane 2) CL

Bottom: Southern blot hybridisation of PCR products using FSH receptor primers. cDNA was prepared from the accessory (lane 1) and the natural (lane 2) CL.


bp
400
M 1 2 3


bp
400
M 1 2


bp
800
600
400
300
1 2

Transmission electron microscopy revealed that the secondary corpus luteum had ultrastructural features typical of luteal tissue (fig. 42). Large and small luteal cells contained abundant smooth endoplasmic reticulum, polymorphic mitochondria, lipid droplets and dark granules The dark secretory granules were present in both small and large luteal cells.

3.4.2.2 Tissue hormone concentrations

Mean progesterone and oestradiol-17β concentrations were determined for five tissue samples from each corpus luteum. Mean tissue progesterone content in samples from the accessory corpus luteum was 31.15 ± 3.11μg/g compared with 58.29 ± 6.32 μg/g tissue for the natural corpus luteum ($P<0.001$) Mean tissue oestradiol was significantly higher ($P<0.05$) than that found in the secondary corpus luteum (108 ± 11.6 compared with 74.2 ± 7.81 pg/g tissue).

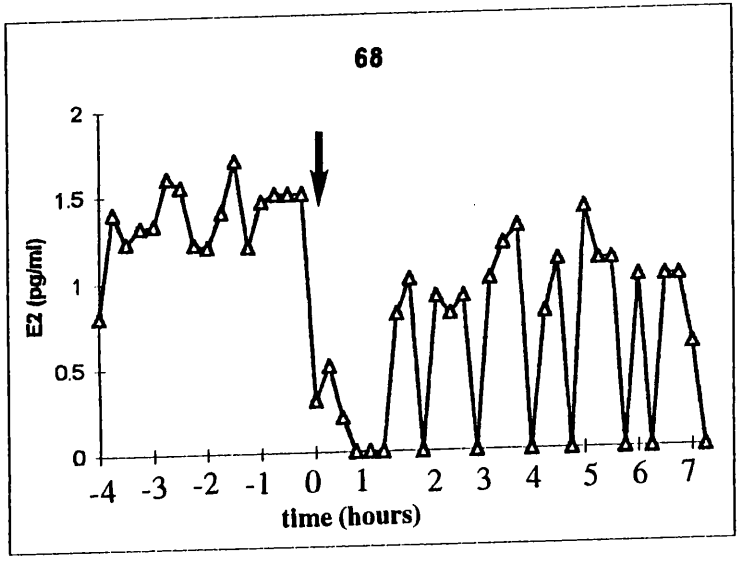
3.4.2.3 Enzyme studies

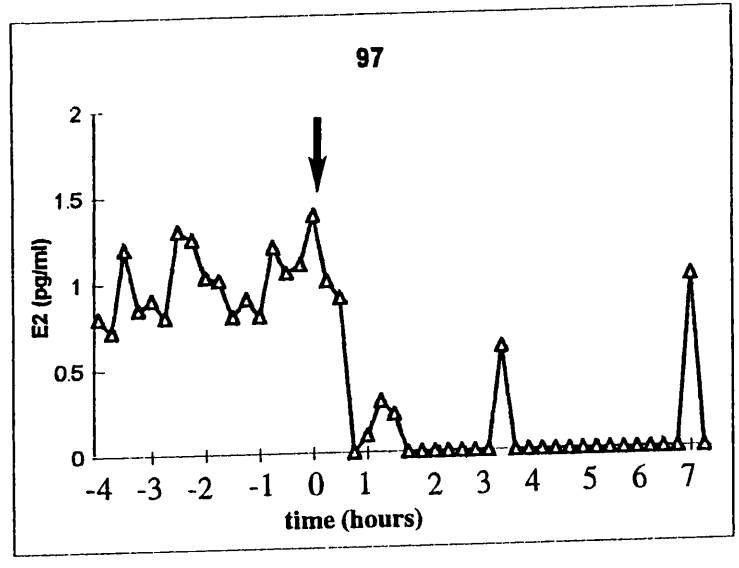
Using PCR, cDNA corresponding to P450scc and $P450_{17\alpha}$ was detected in both the natural and the secondary corpus luteum. Complementary DNA corresponding to $P450_{arom}$ mRNA was not detected in the natural corpus luteum but was present in the secondary structure (fig.. 43).

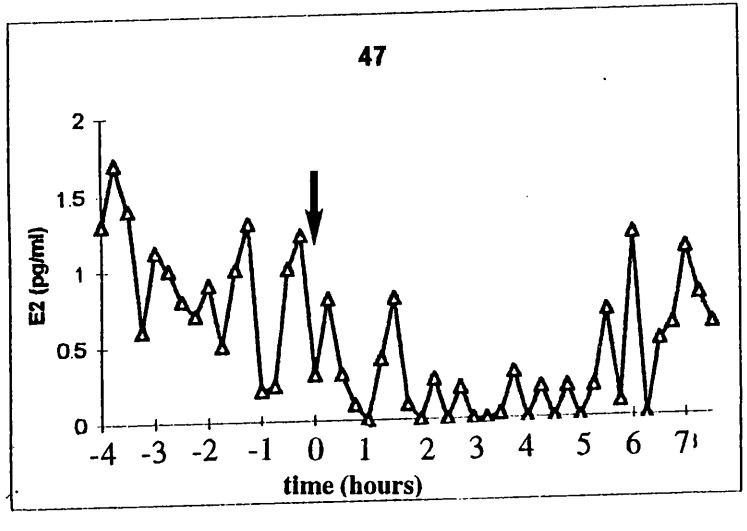
3.4.2.4 Receptor studies

Southern blotting for FSH-receptor cDNA revealed it to be expressed in full length in the accessory structure but not to be expressed in the natural corpus luteum (fig 43)

Figure 44 Plasma oestradiol concentration in samples collected every 15 min, for four hours before and eight hours after aspiration of the dominant follicle during the early luteal phase(arrow) from four representative cows


68
E2 (pg/ml)
2
1.5
1
0.5
0
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)


97
E2 (pg/ml)
2
1.5
1
0.5
0
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)


47
E2 (pg/ml)
2
1.5
1
0.5
0
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)

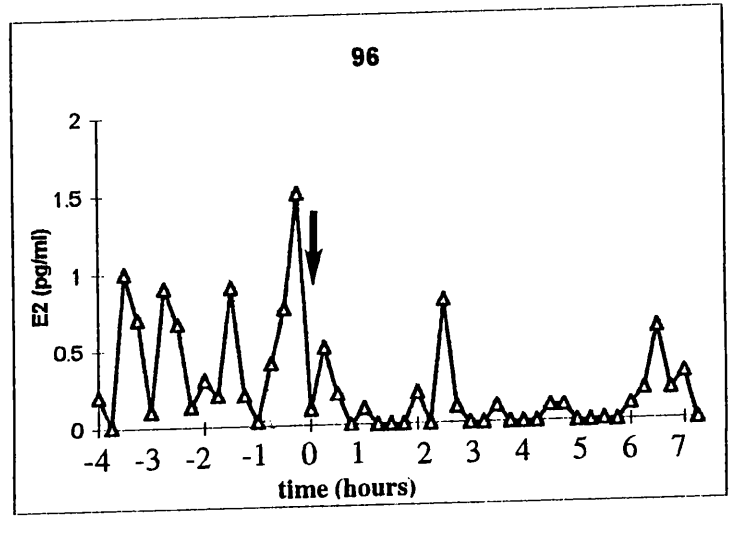
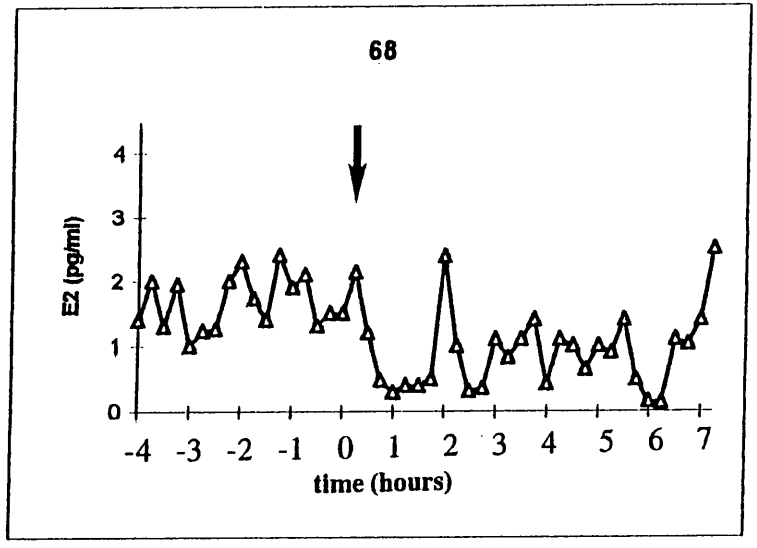
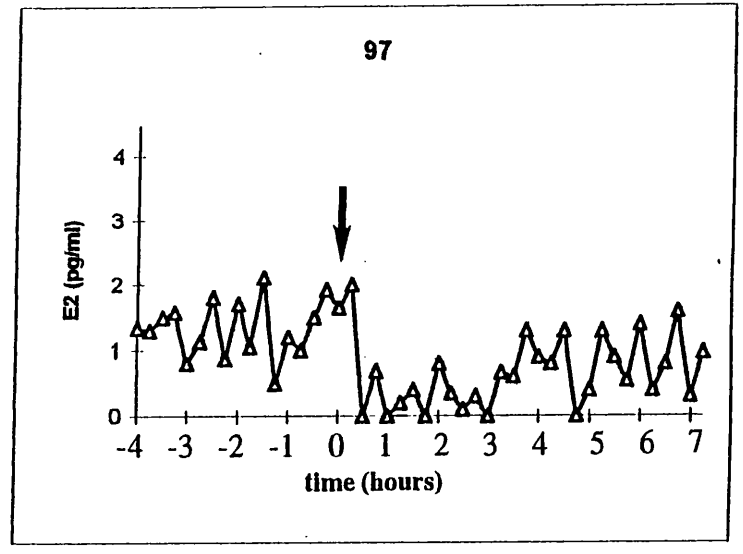
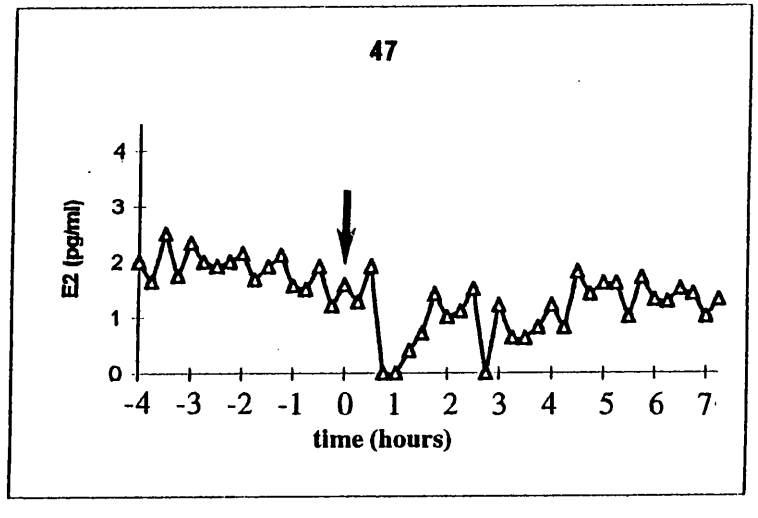

96
E2 (pg/ml)
2
1.5
1
0.5
0
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)

Figure 45 Plasma oestradiol concentration in samples collected every 15 min, for four hours before and eight hours after aspiration of the dominant follicle during the mid luteal phase(arrow) from four representative cows


68
E2 (pg/ml)
0
1
2
3
4
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)


97
E2 (pg/ml)
0
1
2
3
4
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)


47
E2 (pg/ml)
0
1
2
3
4
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)

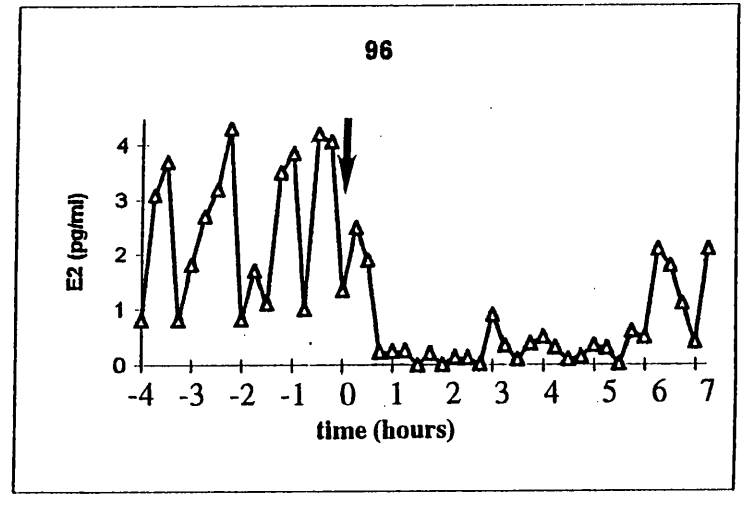
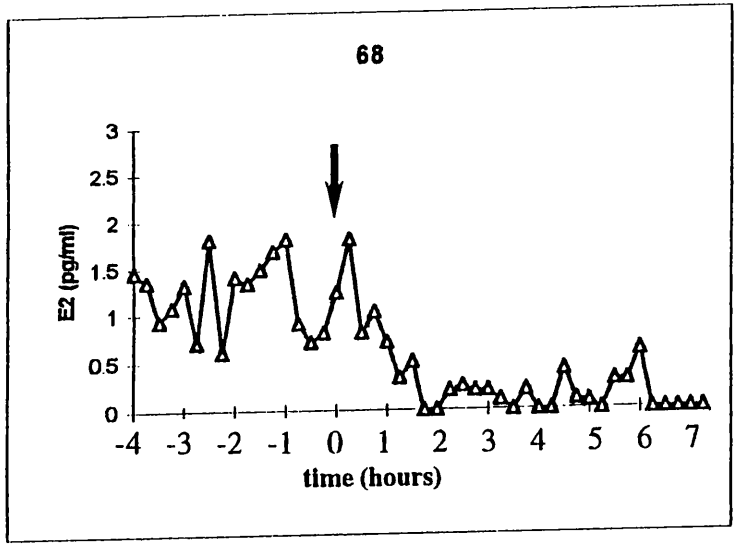
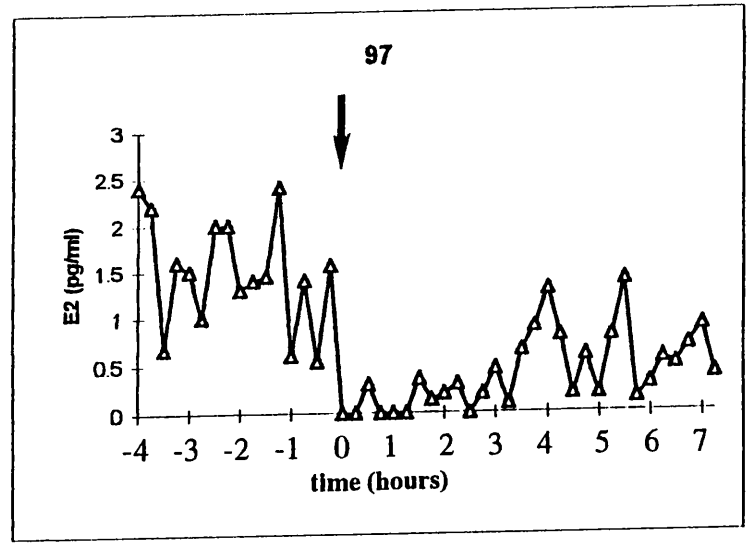
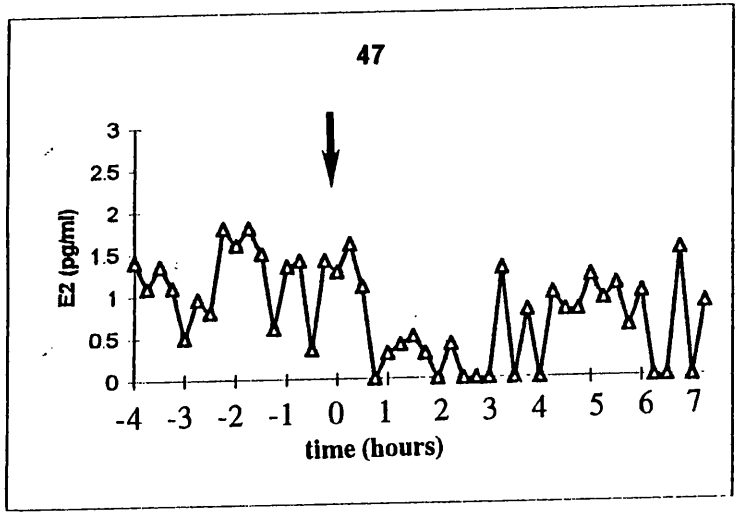
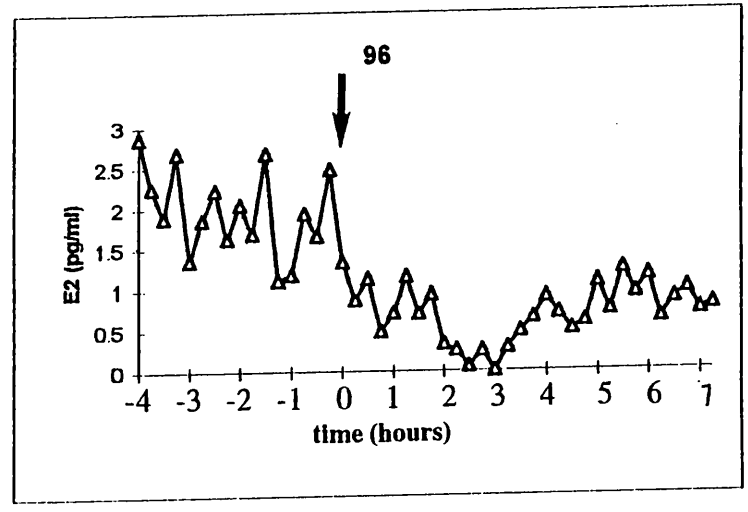

96
E2 (pg/ml)
0
1
2
3
4
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)

Figure 46 Plasma oestradiol concentration in samples collected every 15 min, for four hours before and eight hours after aspiration of the dominant follicle during the late luteal phase(arrow) from four representative cows


68
E2 (pg/ml)
3
2.5
2
1.5
1
0.5
0
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)


97
E2 (pg/ml)
3
2.5
2
1.5
1
0.5
0
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)


47
E2 (pg/ml)
3
2.5
2
1.5
1
0.5
0
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)


96
E2 (pg/ml)
3
2.5
2
1.5
1
0.5
0
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)

3.4.3 Plasma oestradiol after dominant follicle aspiration

A large variation between individual cows in plasma oestradiol concentration was detected during each of the three stages of the luteal phase. Mean plasma oestradiol concentrations in the period before aspiration were found to be 1.38 ± 0.94pg/ml, 1.8 ± 1.22pg/ml and 1.7 ± 1.05pg/ml in the early, mid and late stages of the luteal phase respectively with ranging from 0.6 to 2.5 pg/ml, 0.3 to 3pg/ml and 0.74 to 3.7pg/ml in the early, mid and late stages respectively. No significant difference was detected between pre-aspiration plasma oestradiol levels at any stage of the luteal phase. The plasma oestradiol responses to the aspiration of the dominant follicle during the three luteal phase stages of four cows are shown in figures 44, 45, 46.and summarised in table 15.

Dominant follicle aspiration resulted in a substantial decrease in mean plasma oestradiol concentration regardless of the stage of the cycle. Thus, the overall mean plasma oestradiol concentration in the eight hour period after aspiration was found to be reduced to 0.72 ± 0.26, 0.96 ± 0.2 and 0.76 ± 0.28pg/ml in the early, mid and late luteal stages respectively ($P<0.001$, $P<0.01$and $P<0.05$ respectively). The mean plasma oestradiol concentration during each hourly interval (four samples) was compared to the mean of the pre-aspiration concentration using the two-tailed Tukey's procedure for pairwise comparison of means, followed by analysis of variance for a single factor experiment with repeated measurements. This analysis revealed that in the early and late stages of the luteal phase, the mean plasma oestradiol during the first hour post-aspiration was significantly lower than the pre-aspiration values, while in the mid-luteal stage, the oestradiol concentration two hours after aspiration was significantly lower than the pre-aspiration mean. During the early luteal phase the dramatic decline in plasma oestradiol (to around

Figure 47 FSH, LH and oestradiol after aspiration of the dominant follicle during the early stage of the luteal phase. Each point is the mean of the aspirated cows balanced by subtraction of the pre-aspiration mean value.

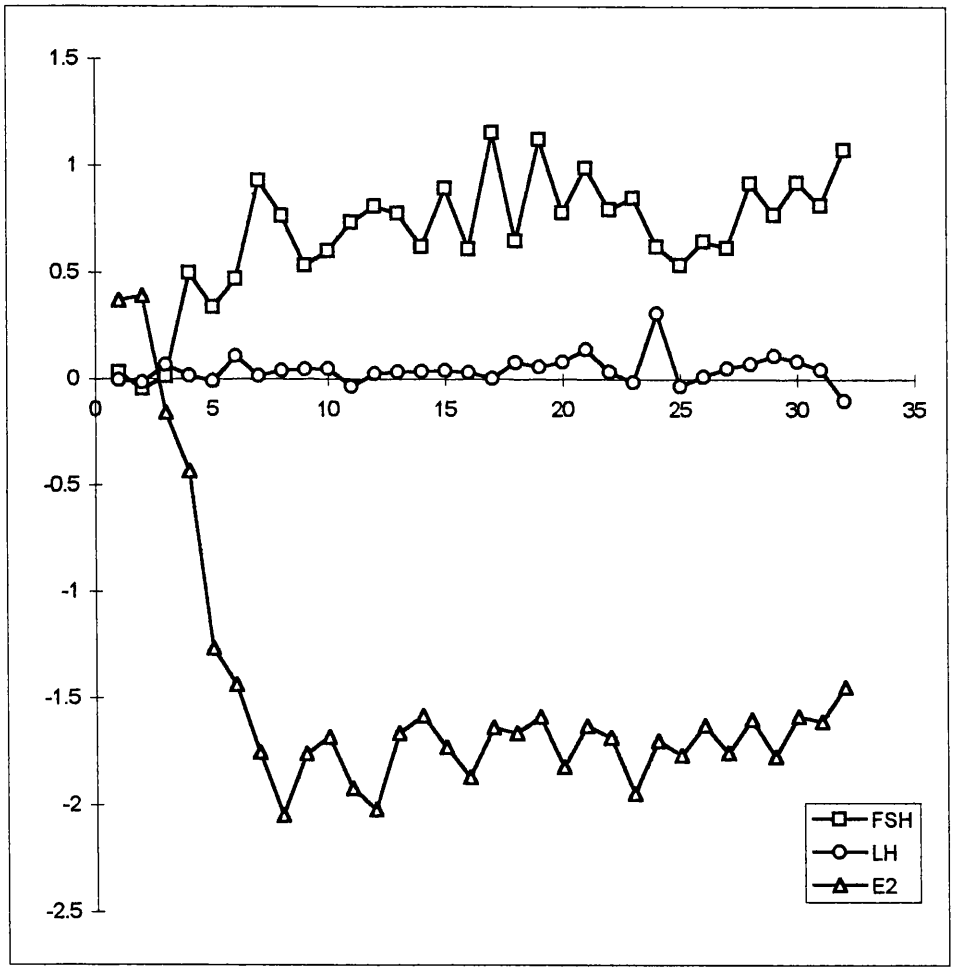

1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
0
5
10
15
20
25
30
35
FSH
LH
E2

Figure 48 FSH, LH and oestradiol after aspiration of the dominant follicle during the mid stage of the luteal phase. Each point is the mean of the aspirated cows balanced by subtraction of the pre-aspiration mean value.

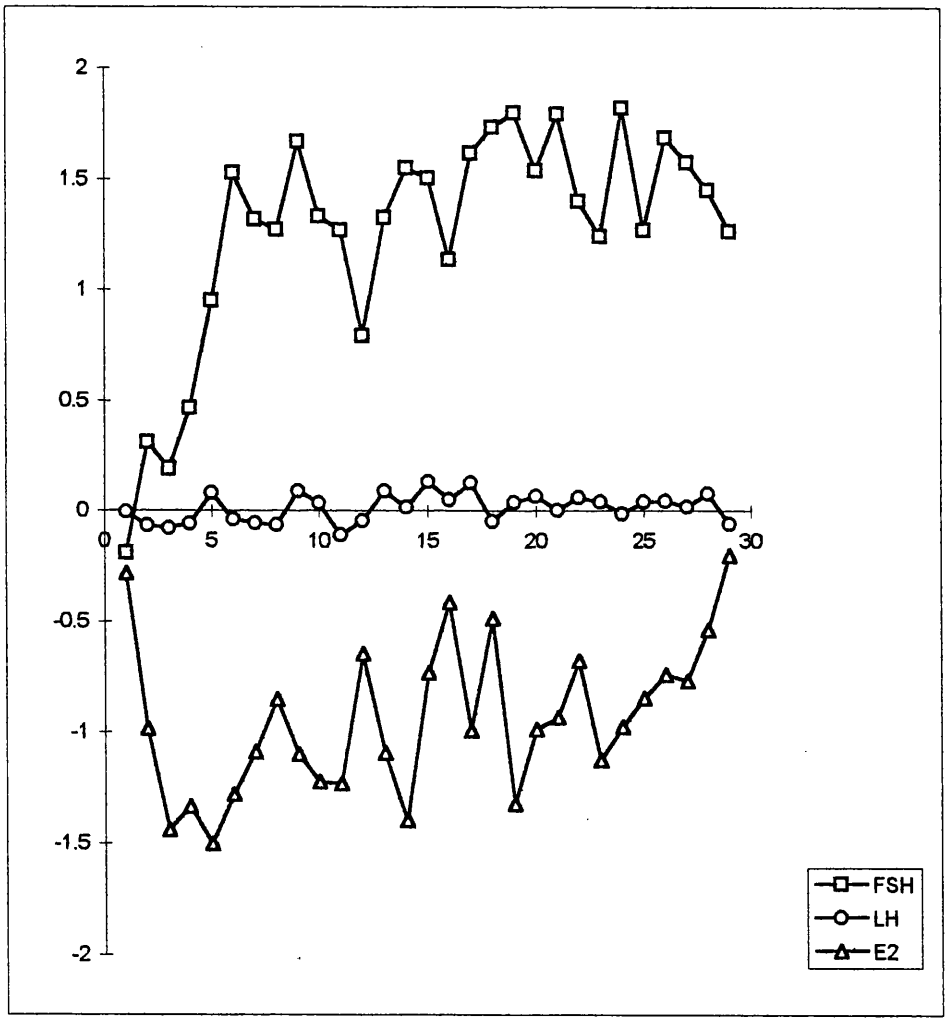
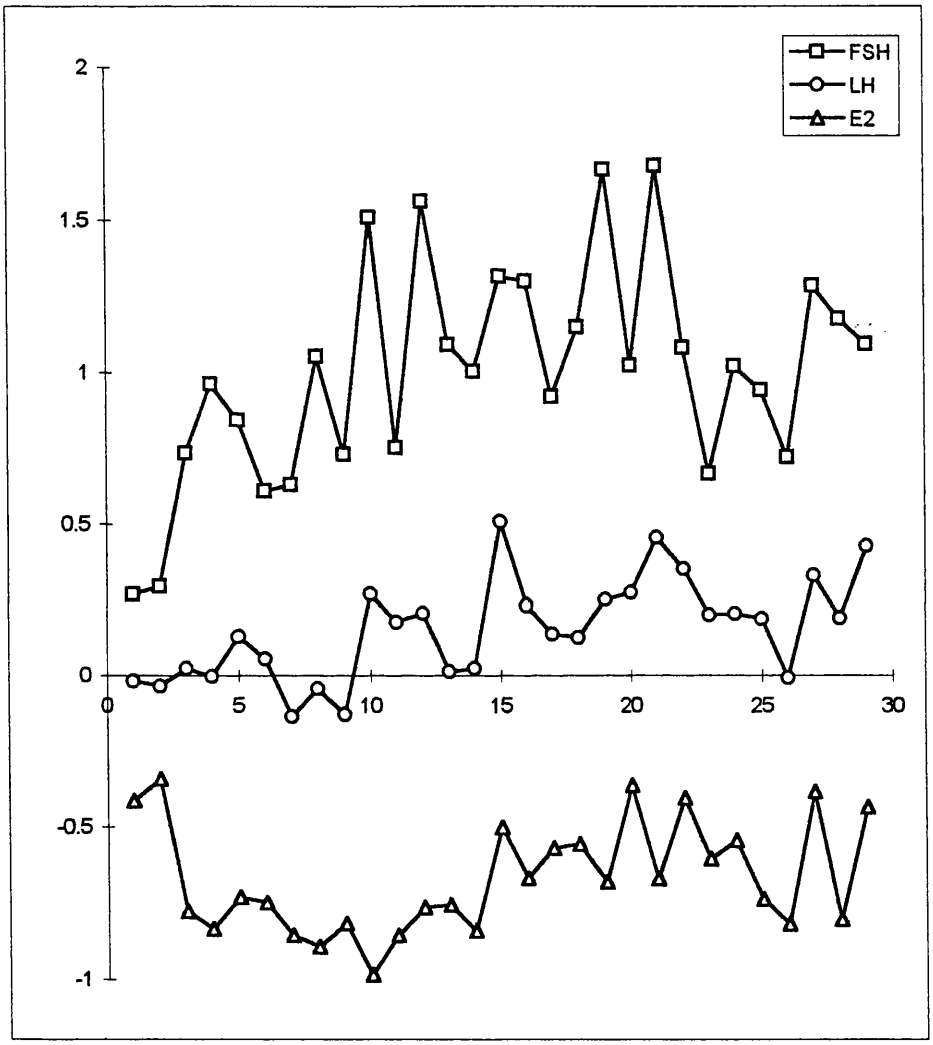

2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
0
5
10
15
20
25
30
FSH
LH
E2

Figure 49 FSH, LH and oestradiol after aspiration of the dominant follicle during the late stage of the luteal phase. Each point is the mean of the aspirated cows balanced by subtraction of the pre-aspiration mean value.


FSH
LH
E2
2
1.5
1
0.5
0
-0.5
-1
0
5
10
15
20
25
30

0.7 pg/ml) concentration was maintained throughout the bleeding period, while in the mid luteal stage, there was a tendency for a gradual increase in mean plasma oestradiol concentration to 1.25 ± 0.88pg/ml six hours after aspiration, which was not significantly lower from the pre-aspiration levels. During the late luteal phase, there was a tendency for an increase in plasma oestradiol concentration five hours after aspiration but the mean plasma concentration was still significantly lower than the pre-aspiration levels (figs. 47, 48, 49).

In one cow (No 47) following an aspiration in the mid luteal stage, no significant difference was found between plasma oestradiol during the pre and post-aspiration periods (fig. 45)

Mean oestradiol pulse frequency decreased significantly following aspiration During the mid and late stages of the luteal phase, i.e. from 5.42 ± 1.5 to 3.57 ± 1.13 and from 4.57 ± 0.97 to 3.14 ± 0.7 pulses/8hours respectively. In contrast, aspiration did not seem to influence oestradiol pulse frequency in the early luteal stage (4.0 ± 1.15 and 4.5 ± 0.97 pulses/8hours in the pre- and post-aspiration samples respectively)

3.4.4 Plasma FSH after dominant follicle aspiration

Hourly mean plasma FSH concentration in pre- and post- aspiration samples is summarised in Table 15. In each of the three stages of the luteal phase, post-aspiration plasma FSH concentrations were significantly higher compared to the pre-aspiration values in all eight cows studied (fig. 50, 51, 52). Thus, mean plasma FSH increased from 1.05 ± 0.72 to 1.78 ± 0.88ng/ml, from 1.65 ± 0.87 to 2.9 ± 0.73ng/ml and from 0.84 ± 0.8 to 1.86 ± 0.85ng/ml in the early, mid and late stages repectively. However, the response to dominant follicle aspiration varied between

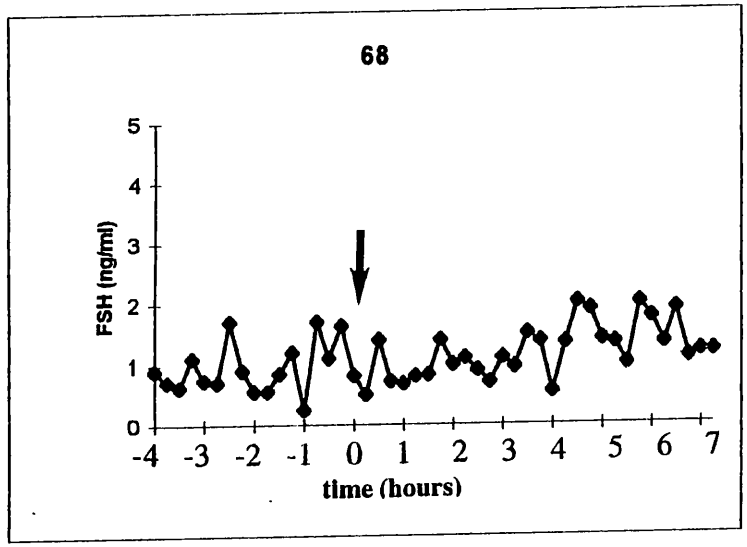
cows and also depended on cycle stage (fig. 50). In the early stage of the luteal phase a small but significant increase (from 0.94 ± 0.15 before aspiration to 1.18 ± 0.29ng/ml after aspiration) was observed in cow 68, while in cow 96 an immediate and more sustained increase in plasma FSH was detected (from 2.35 ± 0.76 to 3.44 ± 0.48ng/ml (fig. 50). Tukey's analysis revealed that during the early and mid stage, the first significant increase in mean plasma FSH concentration, occured two hours after aspiration. During the late stage of luteal phase, plasma FSH concentration rose but this was only apparent by three hours after aspiration except in cow 68 in which the post-aspiration rise in FSH concentration started almost an hour earlier than in the other animals.

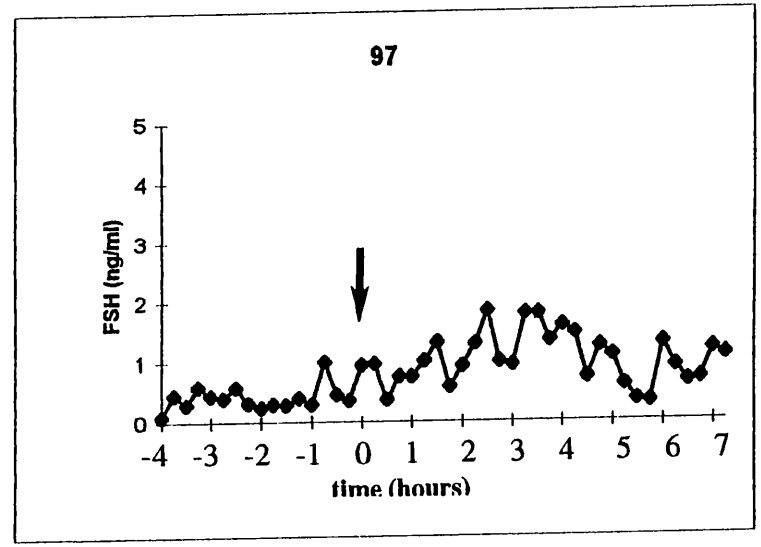
During the early stage of the luteal phase there was a tendency for FSH pulse frequency to increase after aspiration from 3.25 ± 1 to 3.5 ± 0.5 pulses /8 hours. In the late luteal phase there was no significant change in FSH pulse frequency after aspiration, while in the mid luteal phase, the FSH pulse frequency was slightly decreased in the post-aspiration period (from 4 ± 1 to 3.25 ± 0.4 pulses/8hours, P=0.09).

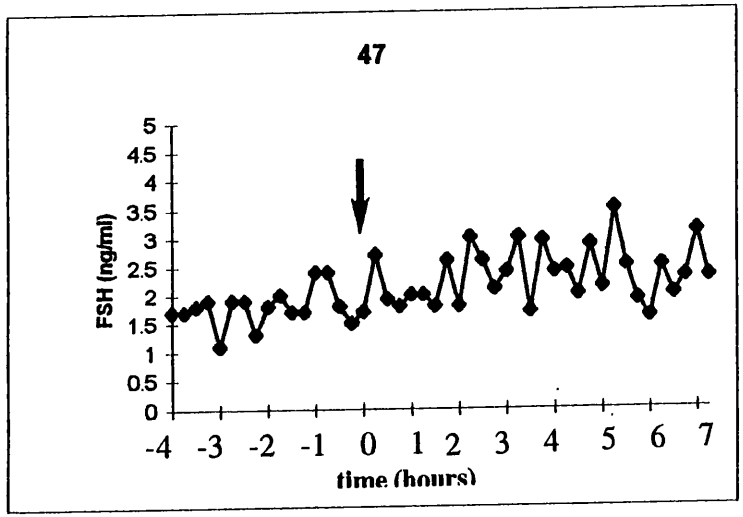
3.4.5. Plasma LH after dominant follicle aspiration

A remarkable uniformity was observed between the animals in mean plasma LH concentration before aspiration regardless of cycle stage. During the early stage of the luteal phase mean plasma LH concentration was 1.62 ± 0.37 ng/ml ranging from 1.2 (cows 100 and 131) to 1.99ng/ml (cows 156 and 97). During the mid luteal stage a small non-significant increase to 1.72±0.4ngLH/ml was observed ranging from 1.3 (cow 131) to 2.25ng/ml (cow 47). A further slight increase to

Figure 50 Plasma FSH concentration in samples collected every 15 min during the early luteal phase, for four hours before and eight hours after aspiration (arrow) from four representative cows


68
FSH (ng/ml)
0
1
2
3
4
5
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)


97
FSH (ng/ml)
0
1
2
3
4
5
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)


47
FSH (ng/ml)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)

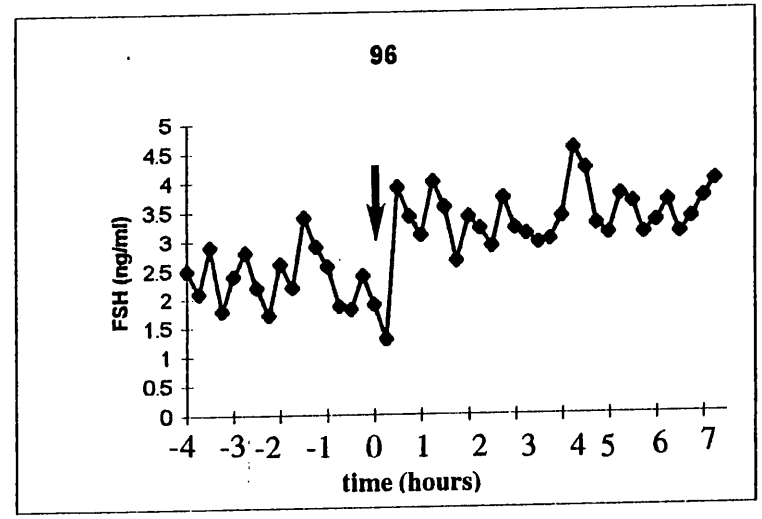
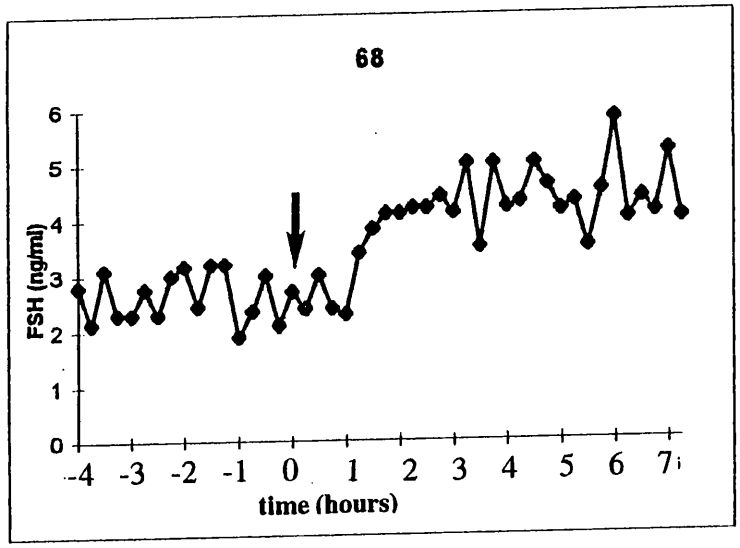
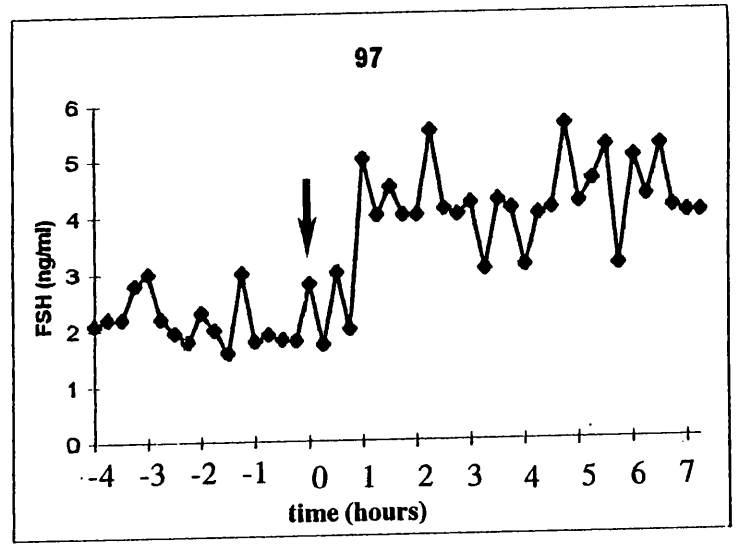
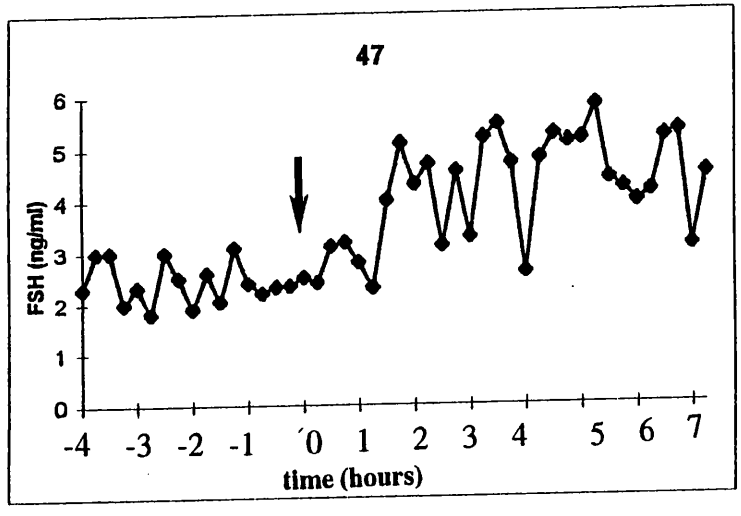

96
FSH (ng/ml)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)

Figure 51 Plasma FSH concentration in samples collected every 15 min during the mid luteal phase, for four hours before and eight hours after aspiration of the dominant follicle (arrow) from four representative cows


68
FSH (ng/ml)
0
1
2
3
4
5
6
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)


97
FSH (ng/ml)
0
1
2
3
4
5
6
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)


47
FSH (ng/ml)
0
1
2
3
4
5
6
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)

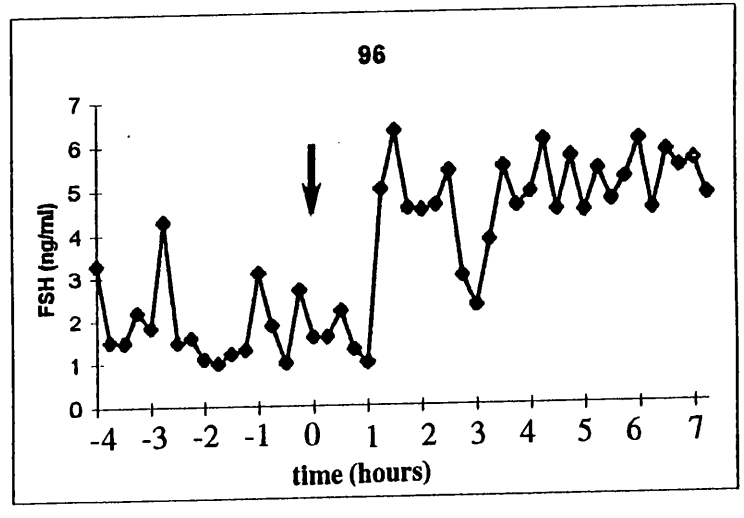
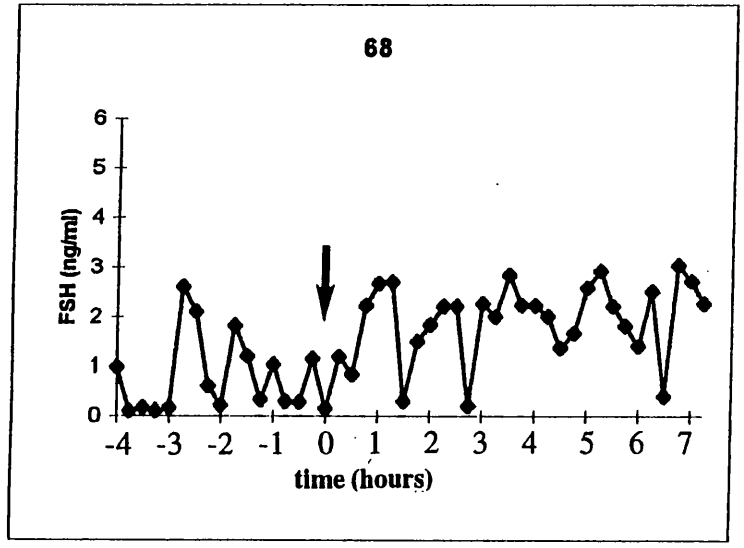
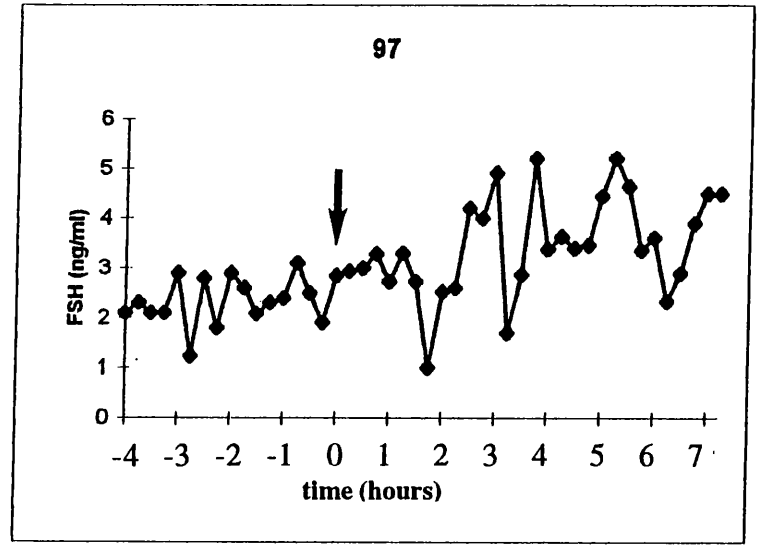
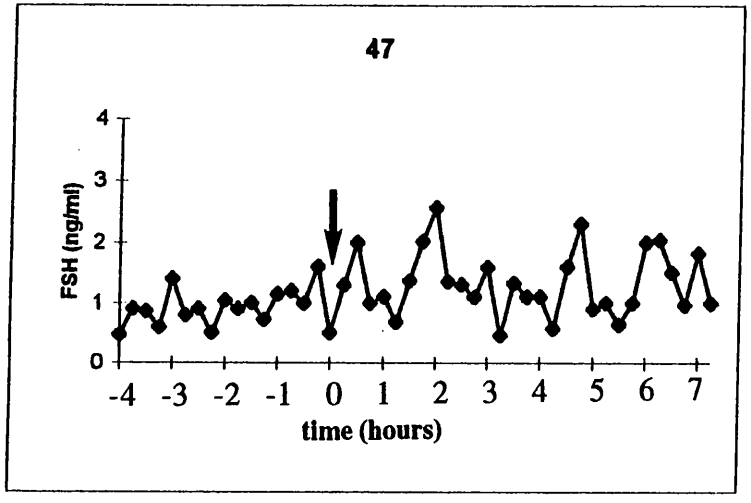
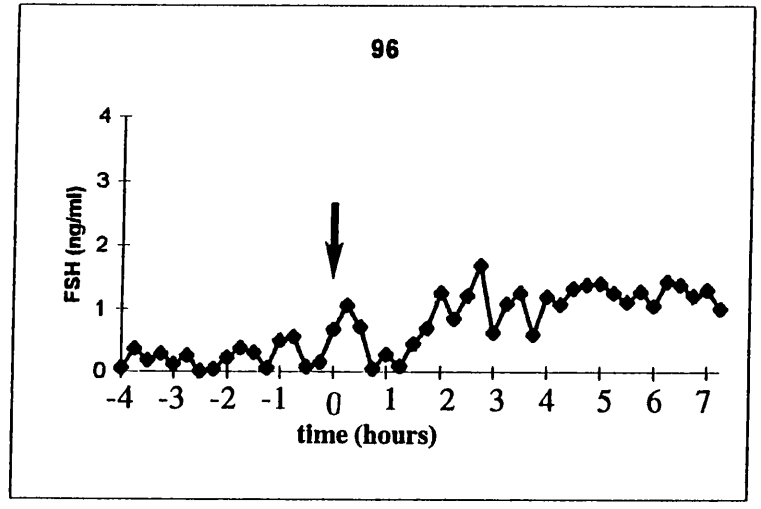

96
FSH (ng/ml)
0
1
2
3
4
5
6
7
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)

Figure 52 Plasma FSH concentration in samples collected every 15 min during the late luteal phase, for four hours before and eight hours after aspiration of the dominant follicle (arrow) from four representative cows


68
FSH (ng/ml)
0
1
2
3
4
5
6
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)


97
FSH (ng/ml)
0
1
2
3
4
5
6
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)


47
FSH (ng/ml)
0
1
2
3
4
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)


96
FSH (ng/ml)
0
1
2
3
4
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)

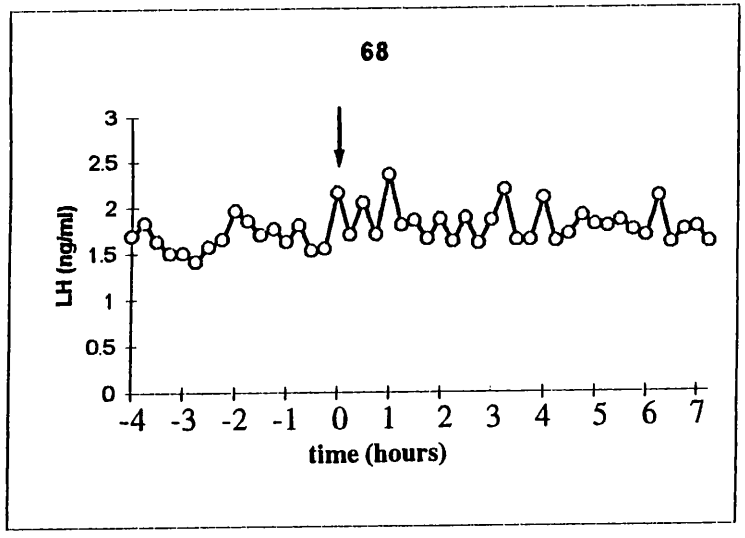
1.82±0.56ng/ml was detected during the late stage of the luteal phase ranging from 1.3 (cow 131) to 2.56ngLH/ml (cow 97).

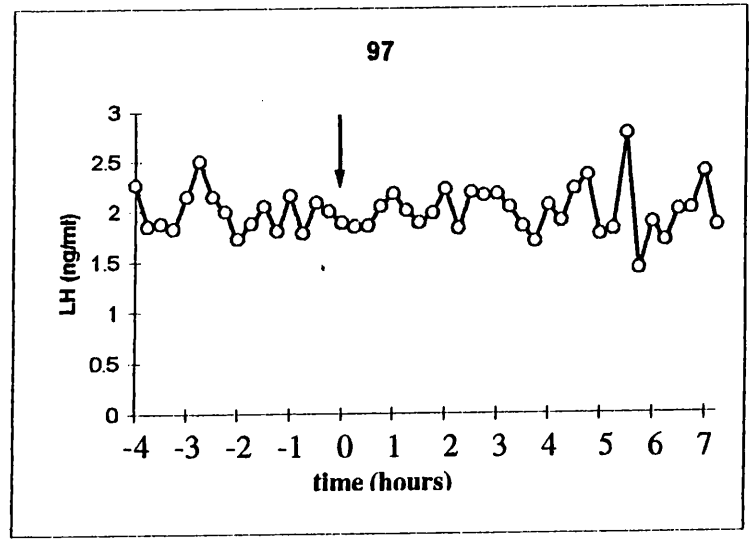
Aspiration of the dominant follicle at any stage of the luteal phase did not appear to influence the mean plasma LH concentration. During the early and mid stages, no change between pre- and post-aspiration values was detected (fig. 47, 48) even when data from individual cows were considered (fig.53, 54, 55, Table 15)

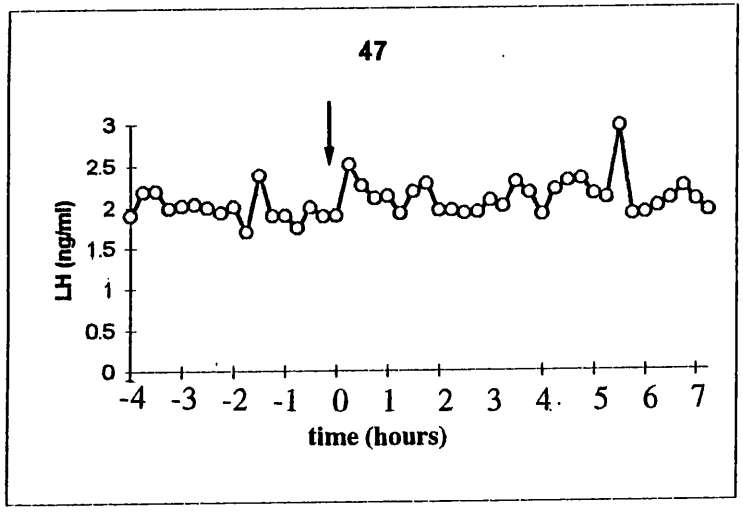
During the late luteal phase there was a clear but not significant tendency for mean plasma LH concentration to increase approximately five hours after aspiration and in cow 97 this achieved statistical significance increasing from 2.56ng/ml before aspiration to 4.2ng/ml six hours later.

LH pulse frequency was affected by aspiration, increasing from 2 to 3.1±1 pulses/8 hours($P<0.05$) during the early luteal stage, and decreasing during the mid and late stages (from 3.42 ± 1.5 and 3.14 ± 1.5 to 3 ± 1.15 and 2.7 ± 0.7 pulses/8hours respectively)

Figure 53 Plasma LH concentration in samples collected every 15 min during the early luteal phase, for four hours before and eight hours after aspiration of the dominant follicle (arrow) from four representative cows


68
LH (ng/ml)
3
2.5
2
1.5
1
0.5
0
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)


97
LH (ng/ml)
3
2.5
2
1.5
1
0.5
0
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)


47
LH (ng/ml)
3
2.5
2
1.5
1
0.5
0
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)

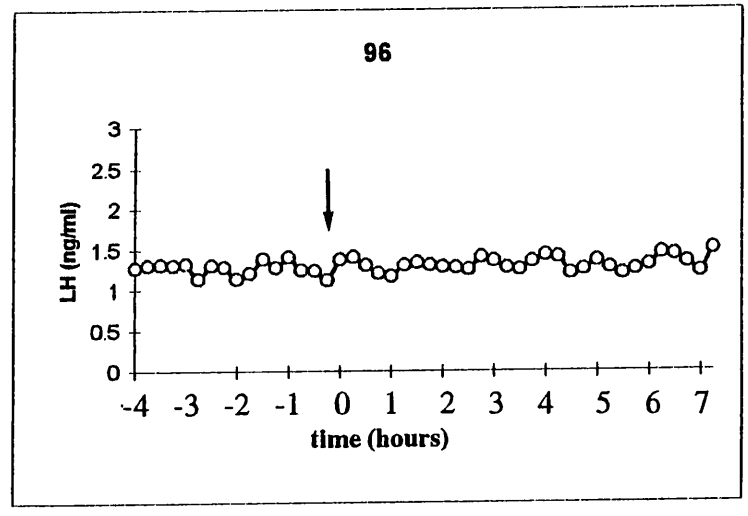
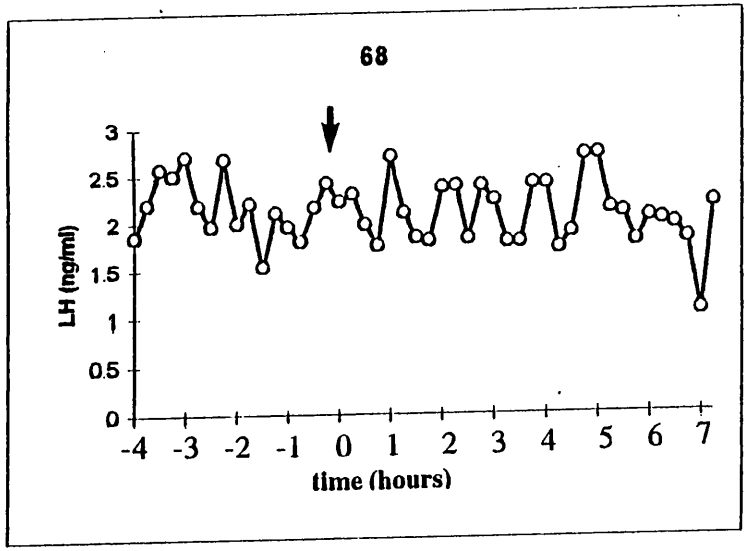
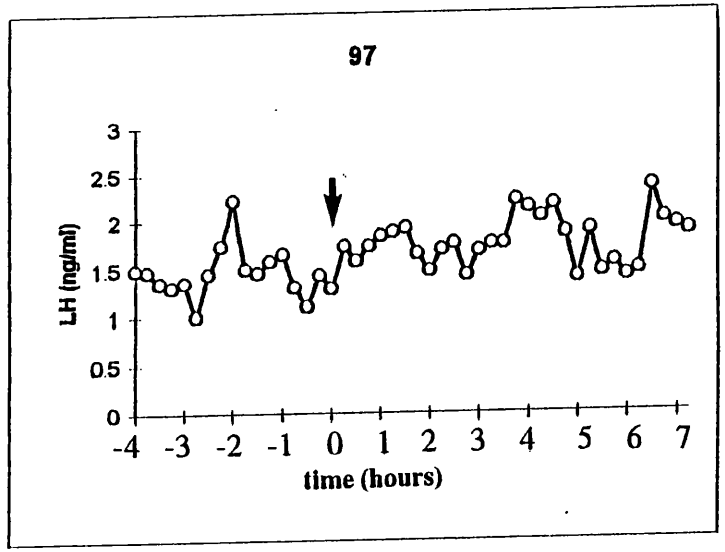
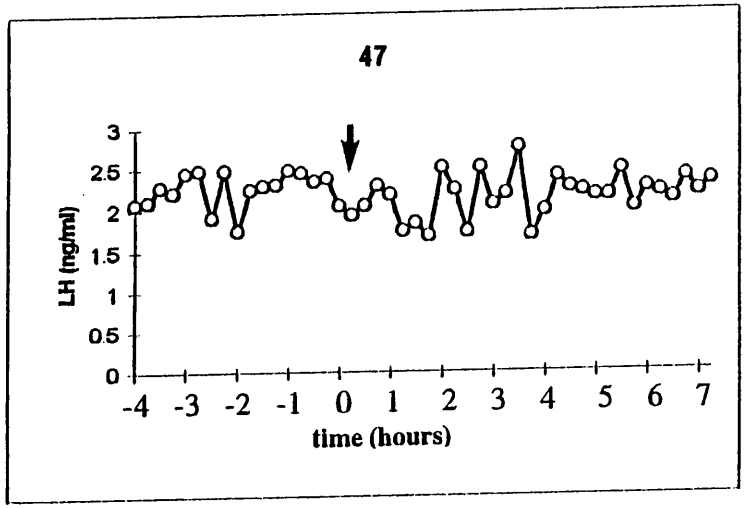

96
LH (ng/ml)
3
2.5
2
1.5
1
0.5
0
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)

Figure 54 Plasma LH concentration in samples collected every 15 min during the mid luteal phase, for four hours before and eight hours after aspiration of the dominant follicle (arrow) from four representative cows


68
LH (ng/ml)
3
2.5
2
1.5
1
0.5
0
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)


97
LH (ng/ml)
3
2.5
2
1.5
1
0.5
0
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)


47
LH (ng/ml)
3
2.5
2
1.5
1
0.5
0
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)

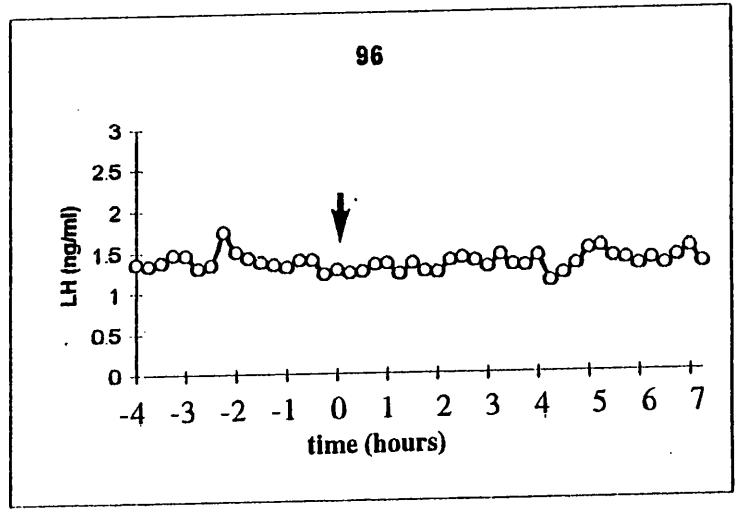
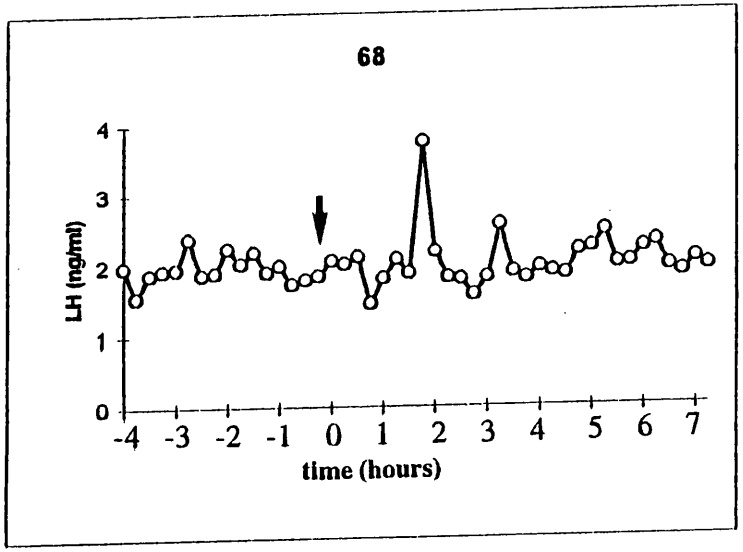
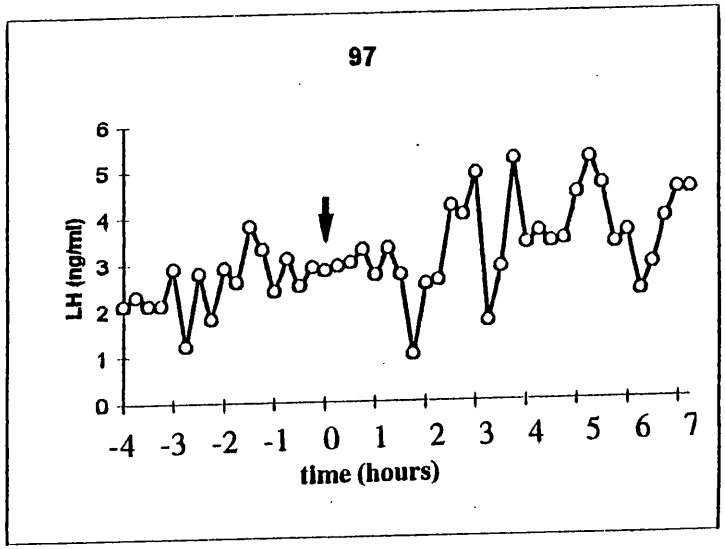
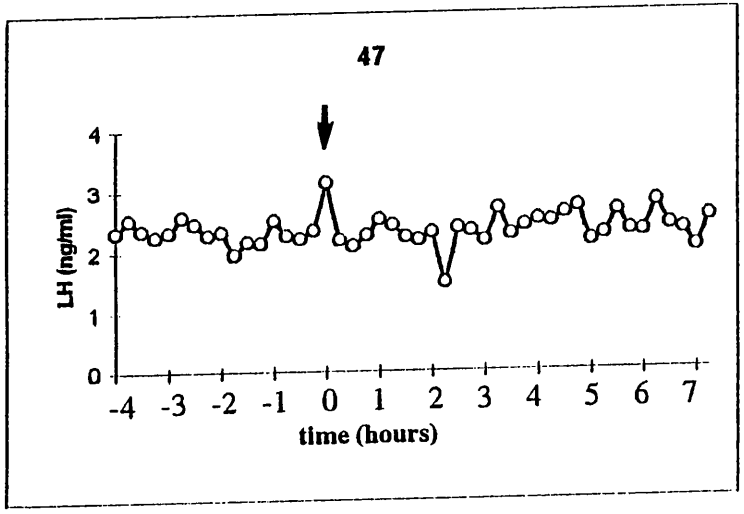

96
LH (ng/ml)
3
2.5
2
1.5
1
0.5
0
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)

Figure 55 Plasma LH concentration in samples collected every 15 min during the late luteal phase, for four hours before and eight hours after aspiration of the dominant follicle (arrow) from four representative cows


68
LH (ng/ml)
0
1
2
3
4
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)


97
LH (ng/ml)
0
1
2
3
4
5
6
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)


47
LH (ng/ml)
0
1
2
3
4
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)

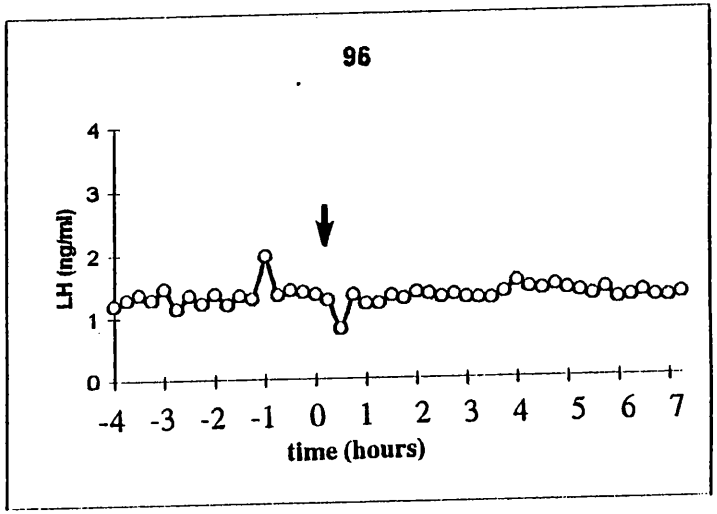

96
LH (ng/ml)
0
1
2
3
4
-4 -3 -2 -1 0 1 2 3 4 5 6 7
time (hours)

Figure 56 Mean progesterone (open bars) and oestradiol (closed bars) concentration in follicular fluid of dominant follicles aspirated during the early, mid and late luteal phases.

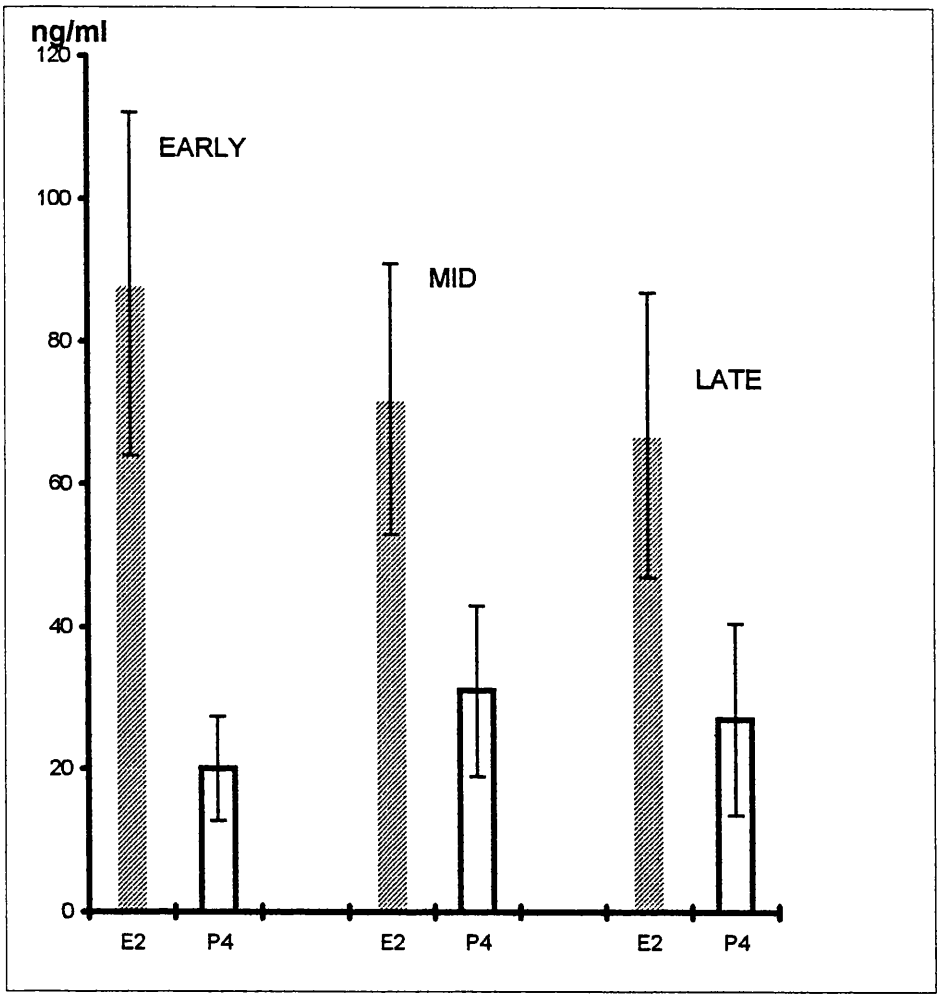

ng/ml
120
100
80
60
40
20
0
EARLY
MID
LATE
E2
P4
E2
P4
E2
P4

Table 15. Mean ±SD (even rows) plasma oestradiol, FSH and LH concentrations before and at hourly intervals after aspiration.

	Stage of cycle	Time (hours)								
		Pre-	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
E2	**Early**	**2.62**	1.27	0.57	0.31	0.76	0.66	0.73	0.78	0.75
		2.1	1.4	0.72	1	1.2	1.17	1	1	0.71
	Mid	**1.8**	1.18	0.64	0.77	0.91	0.87	0.91	1.25	1.15
		1.22	1.65	0.61	0.75	0.79	0.72	0.76	0.88	0.74
	Late	**1.7**	1.23	0.87	0.67	0.19	0.73	0.86	0.68	0.7
		1	0.89	0.79	0.79	0.56	0.67	0.68	0.64	0.85
FSH	**Early**	**1.05**	1.38	1.76	1.78	1.87	1.945	1.75	1.79	1.99
		0.72	0.48	0.95	0.81	0.88	0.26	0.9	0.57	1.22
	Mid	**1.65**	1.88	2.89	2.89	3.01	3.13	3.26	3.13	3.06
		0.87	0.95	1.51	1.38	1.47	1.5	1.64	1.6	1.62
	Late	**0.84**	0.87	1.5	1.62	2.22	2.22	2.39	1.98	2.1
		0.8	0.81	1	0.64	1.21	1.1	1.22	0.9	1.14
LH	**Early**	**1.62**	1.64	1.65	1.65	1.65	1.67	1.67	1.72	1.65
		0.35	0.43	0.38	0.36	0.37	0.31	0.45	0.39	0.45
	Mid	**1.72**	1.71	1.7	1.72	1.71	1.8	1.76	1.74	1.73
		0.42	0.4	0.3	0.4	0.35	0.39	0.44	0.34	0.35
	Late	**1.82**	1.85	1.87	1.77	2	2.1	2.07	2.23	2.16
		0.56	0.58	0.68	0.64	0.9	0.87	0.79	0.94	0.8

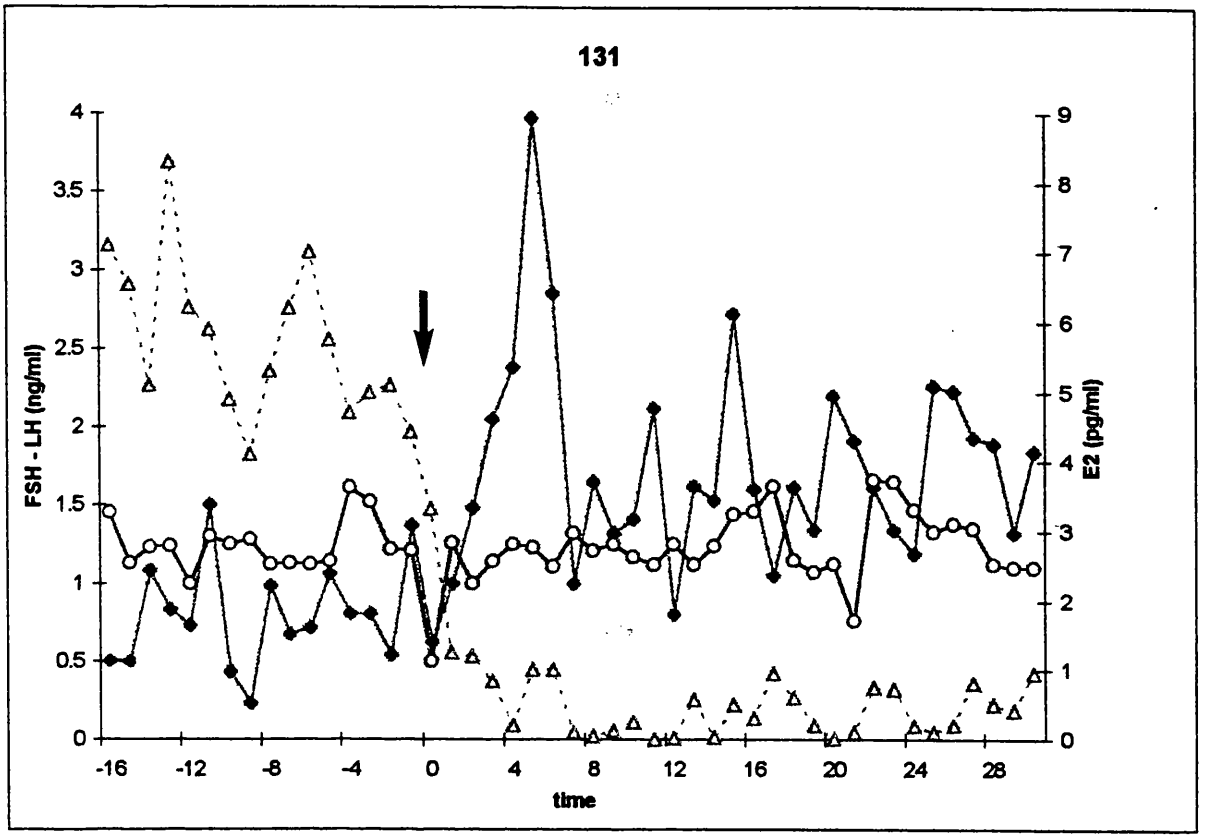
3.4.6. Follicular fluid oestradiol and progesterone concentrations

The ratio of oestradiol : progesterone concentrations in follicular fluid is a safe criterion to determine if a follicle is healthy or atretic. To that end follicular fluid obtained after aspiration of the dominant follicle was analysed for oestradiol concentrations after serial dilutions ranging from 1:50 to 1:1000. Progesterone concentration was measured in follicular fluid either undiluted or diluted 1:1 in assay buffer. In all cases but one (cow 47 in mid luteal phase) the ratio of oestradiol : progesterone concentration was greater than two. In cow 47 oestradiol : progesterone was 1.2. The highest follicular oestradiol concentration and the highest follicular progesterone concentrations were found during the early and mid luteal stages respectively (Table16, fig. 56) but overall, three were no significant differences in follicular fluid steroid content at different stages of the luteal phase.

Table 16. Mean (± sd) of oestradiol and progesterone concentrations in follicular fluid during the three stages of the luteal phase.

	Oestradiol (ng/ml)	**Progesterone** (ng/ml)
Early	88.1±23.9	20±7.3
Mid	72±19.2	31±12.1
Late	66.8±20.4	27.5±13.5

Figure 57 Plasma FSH, LH and oestradiol concentrations in samples collected every 15 min during the early luteal phase, for four hours before and eight hours after aspiration of the dominant follicle (arrow) from two representative cows


131
FSH - LH (ng/ml)
E2 (pg/ml)
time
4
3.5
3
2.5
2
1.5
1
0.5
0
9
8
7
6
5
4
3
2
1
0
-16
-12
-8
-4
0
4
8
12
16
20
24
28

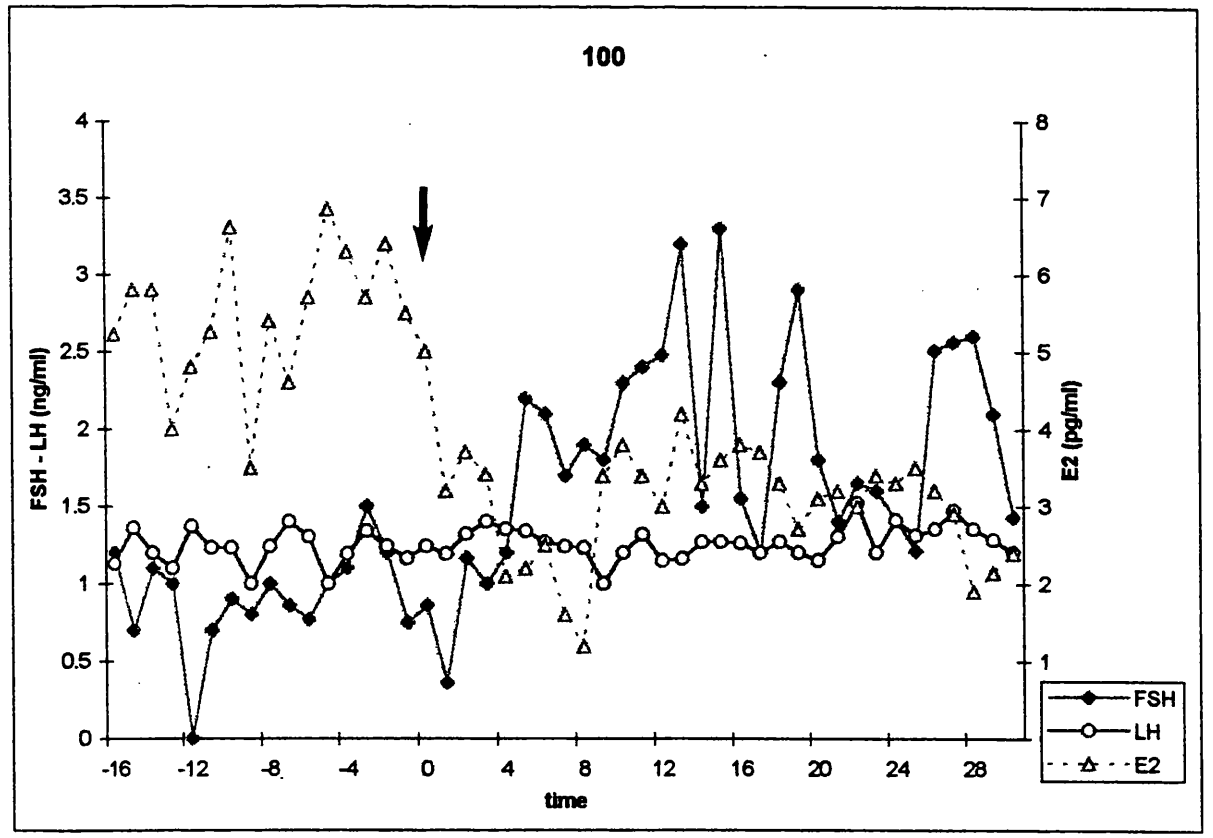
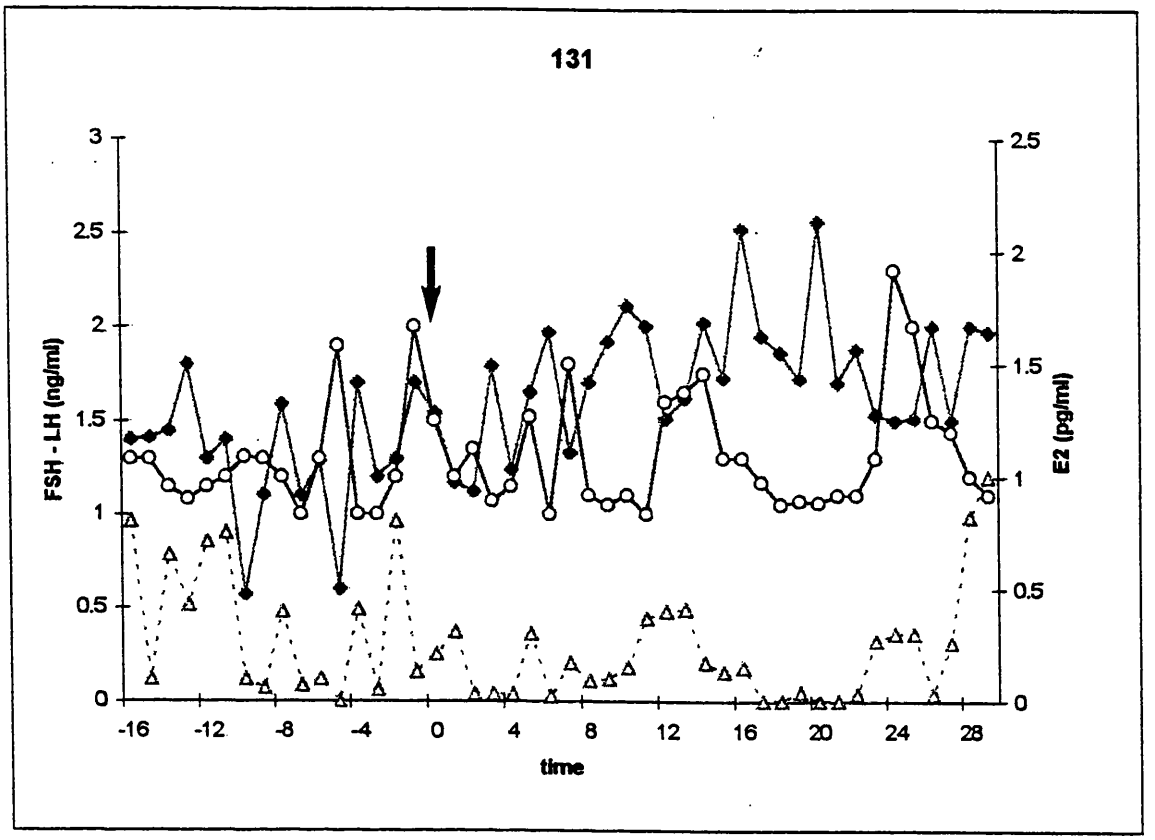

100
FSH - LH (ng/ml)
E2 (pg/ml)
time
4
3.5
3
2.5
2
1.5
1
0.5
0
8
7
6
5
4
3
2
1
-16
-12
-8
-4
0
4
8
12
16
20
24
28
FSH
LH
E2

Figure 58 Plasma FSH, LH and oestradiol concentrations in samples collected every 15 min during the mid luteal phase, for four hours before and eight hours after aspiration of the dominant follicle (arrow) from two representative cows


131
FSH - LH (ng/ml)
E2 (pg/ml)
time
3
2.5
2
1.5
1
0.5
0
-16
-12
-8
-4
0
4
8
12
16
20
24
28

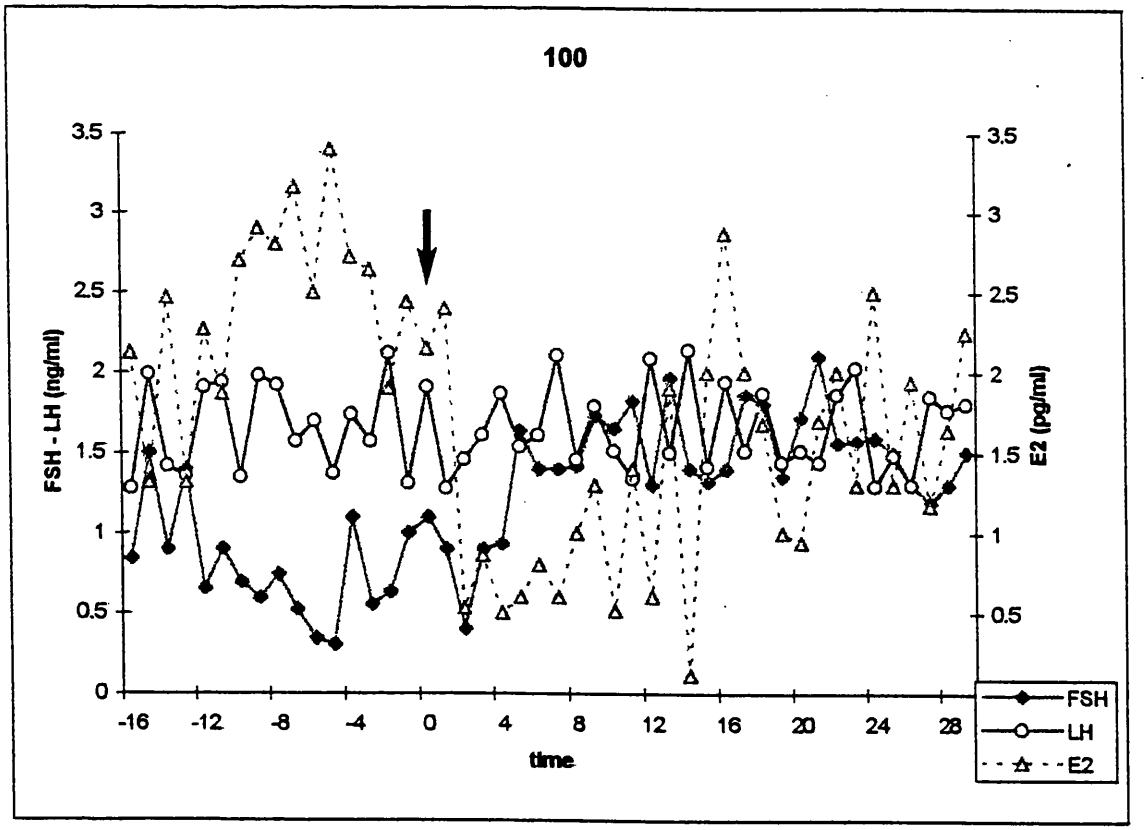
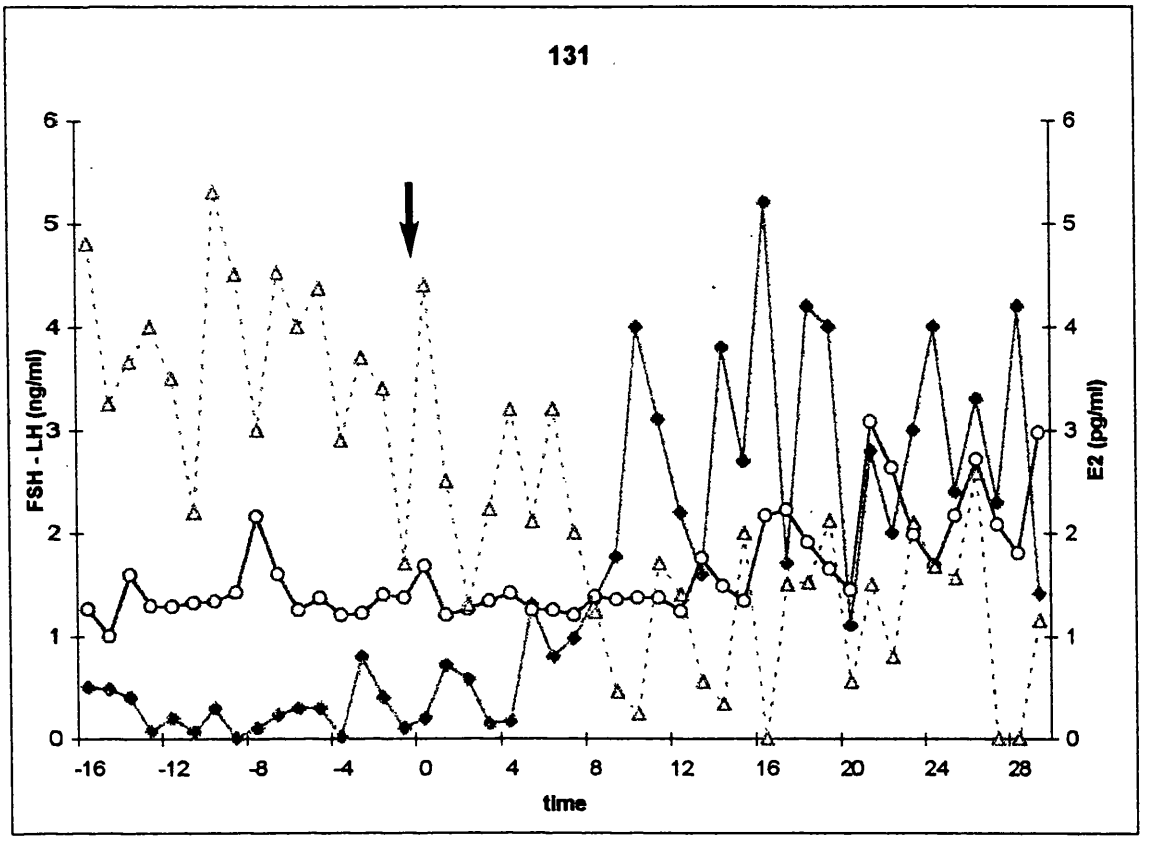
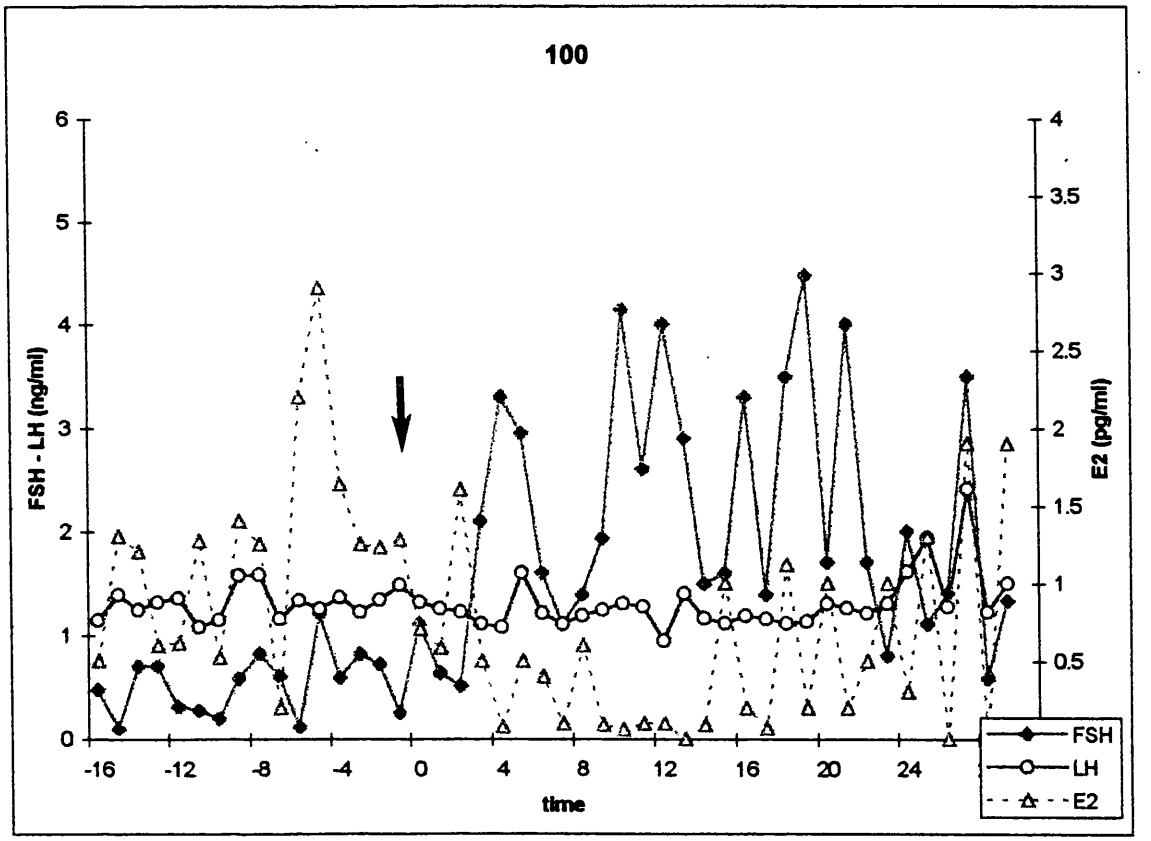

100
FSH - LH (ng/ml)
E2 (pg/ml)
time
3.5
3
2.5
2
1.5
1
0.5
0
-16
-12
-8
-4
0
4
8
12
16
20
24
28
FSH
LH
E2

Figure 59 Plasma FSH, LH and oestradiol concentrations in samples collected every 15 min during the late luteal phase, for four hours before and eight hours after aspiration of the dominant follicle (arrow) from two representative cows


131
FSH - LH (ng/ml)
E2 (pg/ml)
time
6
5
4
3
2
1
0
-16
-12
-8
-4
0
4
8
12
16
20
24
28


100
FSH - LH (ng/ml)
E2 (pg/ml)
time
6
5
4
3
2
1
0
4
3.5
3
2.5
2
1.5
1
0.5
-16
-12
-8
-4
0
4
8
12
16
20
24
FSH
LH
E2

3.4.7 Enzyme studies

Follicular luteinisation is characterised by the shift from oestradiol to progesterone secretion by the granulosa cells. The production of the two steroids is regulated by certain enzymes which are produced after the expression of particular genes.

A clear GAPDH band of approximately 320 bp was obtained in the agarose gell electrophoresis, confirming effective transcription of the mRNA to complimentary DNA (fig. 57).

Using primers for P450arom and P450scc, single bands corresponding to 400 and 350 bp respectively, were detected in agarose gell electrophoresis following reverse transcription-PCR amplification of granulosa cell RNA, (fig. 57).

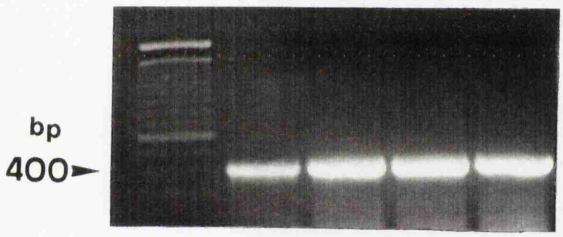
FSH receptor

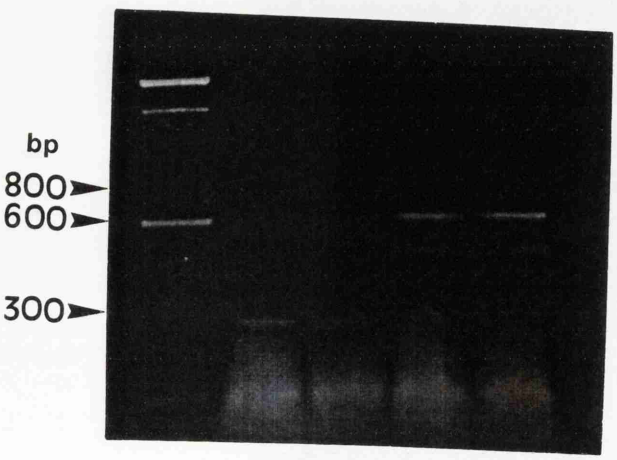
FSH receptor cDNA was amplified by PCR, following granulosa cells mRNA reverse transcription. At least four PCR products were detectable in the agarose gel, ranging from 800 to 300 bp.

Figure 60 PCR amplification of cDNA from granulosa cells collected after aspiration of the dominant follicle.

Amplification by RT-PCR, using primers for
P450scc (top panel)
P450arom (middle panel)
FSH receptor (bottom panel). Bant at 800bp is very faint.


bp
400


bp
400


bp
800
600
300

Chapter 4

DISCUSSION

4. Discussion

The aims of the present study were **a:** to improve existing techniques for follicular aspiration, to increase the oocyte recovery rate, **b:** to describe the effects of follicular aspiration on interoestrus intervals and on circulating hormone concentrations and **c:** to investigate the hypothesis that under certain conditions follicular aspiration can cause follicular luteinisation.

4.1. a. Follicular aspiration

A number of technical problems were encountered during the experiments.

The crush used for the aspiration was not suitably designed for the procedure. Although the animal was sedated and the crush was fitted with side padding, the cow was still able to move slightly from side to siden and this disrupted the accuracy of the needle handling. Furthermore the crush was too long and this created additional problems since the operator had to work in the crush and very little space was left for the assistant to handle the needles efficiently. This was even worse when small cows were aspirated since it was impossible for the operator and the assistant to work next to each other, and the latter had to manipulate the needles from a position behind the operator. A different crush type with adjustable side panels, of shorter length and with floor at ground level might have helped to overcome these problems. Alternatively, the cow might be kept between two parallel horizontal bars fixed in the ground and fitted with a pair of adjustable bars to restrain the animal. The front panel of a crush might be used in this case to immobilise the cow's head.

Certain problems were encountered using the different types of needles. The 45 cm long, 19g needles tended to bend very easily and they were replaced by the 18g 32 and 45 cm long ones. The 32cm needles were found to be more effective, since it was easier to pass them through the stylette and they bent less frequently. The use of a stylette was a modification of the technique initially introduced by our group (Scott et al., 1994). It helped to overcome the problems of bent and blunt needles and made the procedure less traumatic, since it enabled aspiration of a number of follicles with only one puncture of the vaginal wall. The manipulation of the needle was much easier, enabling very delicate movements of the needle to be performed, since the latter was not in contact with the vaginal wall.. However, even when using a stylette and when the aspiration needle was only required to puncture the ovarian tissue it still blunted quickly. Sterilisation and re-use of the same needles probably reduced their sharpness.When blunt needles were used, the force applied by the assistant to overcome the resistance of the ovarian tissue resulted in sudden rupture of the follicle giving no time for aspiration and in some instances additional damage was done to the ovary and the intestine. Sharpening the needles never gave the effectiveness of new ones. To that end an improvised system was tested, consisting of the 3cm tip of a 19g disposable needle attached with epoxy glue to the blunt end of a 18g, 32 cm needle. After the first attempt though this system was abandoned because it was obvious that the connection was not strong enough, and there was a danger that the 19g tip might break off in the peritoneal cavity.

Another problem was related to successful aspiration of follicular fluid. The initial plan was to apply suction as soon as the operator felt the popping sensation of the needle puncturing a follicle and when the tip of the needle was visible in the follicle by ultrasound scanning. However, time inevitably elapsed between the operator's

signal and activation of the pump by the assistant, resulting sometimes in failure to collect the follicle contents since the puncture caused the follicle to burst. A burst occurred more frequently in large follicles having high intrafollicular tension and also in very small follicles. The effectiveness of the technique was improved when suction was constantly applied from the moment of insertion of the aspiration needle in the stylette, but follicular fluid from small follicles (3-4 mm) still could not be recovered in some cases. It seems probable that an 18g needle was too big in relation to the size of the smaller follicles. The Watts - Marrow 501 peristaltic pump caused some problems related to generation of a pulsatile vacuum.

In many cases, although the needle tip was clearly visible in the follicle and the suction was applied the follicle did not collapse. Sometimes aspiration was re-established after rotation and gentle forward and backward movements of the needle. In such cases it was assumed that the needle was blocked by a fold of the collapsing follicular wall. However in other cases it was impossible to resume aspiration without replacement of the needle since it was found to be blocked by either a small plug of ovarian tissue or a blood clot.

Co-ordination between the operator and the assistant was of paramount importance for the successful aspiration. However, it was very difficult for the assistant to follow the operator's instructions exactly on time. During the final experimental sessions, a transvaginal transducer modified for use in embryonic fluid recovery (Makondo 1996) was tested for use in follicular aspiration. The handle of the transducer was extended to 44cm with a rigid plastic tube. This extension enabled the operator to stabilise the transducer with his chest and with his free hand to manipulate the needle (Salaheddine personal communication). This technique allowed more accurate manipulation of the needle and shortened the duration of the aspiration session.

There was a variability in the oocyte recovery rate between individual cows. This can probably be attributed to factors such as the docility of the cow and to anatomical differences. Despite the drug treatment, some cows were still nervous in the crush and constantly moved from side to side. Increasing the dose of detomidine hydrochloride caused some cows to fall in the crush and endangered the operator. The broad ligament was very short in some cows and this made the manipulation of the ovary very difficult especially when the ovary was small.

The initial oocyte recovery rate was quite discouraging (around 9%) but this was mainly attributed to operator inexperience and the need for some modification of the equipment. After gaining experience, the recovery rate increased to 17% in the third session and remained above 30% during the successive sessions exceeding 50% during the last two sessions. Improved oocyte recovery rate was highly correlated to operator experience. This becomes apparent when looking at the results published from the Utrecht group who improved their recovery rate from 18% in 1988 to 55% in 1994 (Pieterse et al., 1988; Kruip et al., 1991; Kruip et al., 1994). Most of the recent studies report average oocyte recovery rates well above 40% (Kruip et al., 1991, 50.5%; Bols et al., 1995, 42%; Bungartz et al., 1995, 65%). Overall recovery rate in this study was 30.5%. However, if the very poor results of the initial trials are omitted, then the average does not differ substantially from that published by other groups. The highest recovery rate was achieved during the early luteal stage in agreement with results published by Pieterse et al., (1991). Although more follicles were available for aspiration in the mid stage of the luteal phase, the recovery rate was lower when compared to the early stage. In the mid and late stages of the luteal phase, the corpus luteum is fully developed and occupies a large proportion of the ovarian tissue. In order to avoid puncturing the corpus luteum, it was attempted to aspirate follicles from different angles and this reduced the success rate.

The size of the follicles from which oocytes were collected was not recorded in this study. Establishment of the origin of each oocyte would require replacement of the collection tube and flushing of the needle after every aspiration, causing serious delay to the completion of the procedure. The majority of the aspirated follicles were 4-6mm in diameter and it is reasonable to expect that they yielded the majority of the oocytes. This would be an advantage for the IVM/IVF protocols since there would be a relative uniformity between the maturity of the oocytes.

Aspiration of larger follicles (>12mm) was sometimes difficult since although some follicular fluid was initially withdrawn, aspiration might be suddenly interrupted, presumably because the needle became blocked by a fold of the collapsing follicular wall. This tendency of the large follicles to collapse around the needle is probably the reason for the low oocyte yield from these follicles and it is also believed likely that the oocyte might remain trapped in follicular folds (Pieterse et al., 1991; Vos et al., 1994).

The majority of the oocytes collected were categorised as grade three or four which is consistent with previous reports (Kruip et al., 1991; Looney et al., 1994; Scott et al., 1994). Other research groups (e.g. van der Schams et al., 1991; Pieterse et al., 1991 and Gibbons et al., 1994) have reported very high yields of good oocytes but this may reflect less strict criteria set by these latter groups. It is believed that passage of the oocyte through the needle and the aspirating tubing results in partial or even complete removal of its cumulus layer thus drastically reducing its quality since the cumulus cells are vital for cytoplasmic maturation (Chian et al., 1994) and for the formation of a functional monolayer during the maturation process. Nevertheless, oocytes obtained by follicular aspiration exhibited similar (Bugartz et al., 1995) or even better (Kruip et al., 1990) yields of transferable embryos compared to oocytes collected from abattoir material.

A follicular wave is defined as the contemporaneous appearance of a cohort of follicles, one of which becomes dominant several days later (Fortune et al., 1991; Savio et al., 1993). In the cow, two or three follicular waves usually occur in every oestrous cycle. Subordinate follicles of the first follicular wave maintain the capacity to develop further, but only until the fifth day of the cycle unless exogenous FSH is administered, or the dominant follicle is removed (Ko, et al., 1991; Adams et al., 1993). Such interventions prevent atresia from occurring. It might be assumed that two aspirations performed on a weekly basis, would reduce the incidence of advanced atresia, since a dominant follicle could only recently have become established. Furthermore, it has been found that when aspiration was performed every 3-4 days, more grade-one oocytes were collected and excellent embryos were generated, presumably because the oocytes had been derived from non atretic follicles at a similar stage of development (Gibbons et al., 1994; Bugartz et al., 1995). Addition of granulosa cells to oocytes in culture medium enhances the developmental competence of grade three and four oocytes derived from follicular aspiration, indicating that despite their morphological imperfections, these oocytes maintain the potential to develop (Konishi et al., 1996).

More follicles were available for aspiration during the mid luteal phase (70 vs. 42 in the early and 65 in the late stages of the luteal phase). These results are in agreement with Stubbings & Walton (1995) who reported an increased number of follicles in unstimulated cows following follicular aspiration, as well as results from Bergfelt et al. (1994), who demonstrated that follicle ablation at any stage of the cycle increased the number of the follicles in the subsequent follicular wave. The explanation for these effects concerns the fact that ovarian follicles grow after FSH stimulation, and that oestradiol and inhibin produced by the growing follicles exert negative feedback on FSH secretion (Ireland, 1987; Fortune et al., 1991). There is empirical evidence that an increase in plasma FSH concentration precedes the

emergence of each follicular wave, while the selection of the dominant follicle on day 3 and 5 of the cycle is associated with the suppression of plasma FSH concentration (Adams et al., 1992). In this study we have demonstrated that, follicular aspiration causes a dramatic drop in plasma oestradiol concentration lasting for several hours regardless of the stage of the cycle. Furthermore, ablation of just the dominant follicle caused an increase in plasma FSH concentration soon after the drop of oestradiol concentration. It was assumed that this increase in FSH concentration initiated the development of a new cohort of follicles, just as exogenous FSH induces superovulation. Inhibin is another factor that appears to contribute directly, or indirectly through FSH modulation, to the regulation of the ovarian follicular population, since immunisation against inhibin enhanced the number of follicles (Glencross et al., 1990). Badinga et al. (1992) reported that after unilateral ovariectomy of cows, there was an increase in the number of small follicles in the remaining ovary associated with an increase in plasma FSH. Pieterse et al. (1988) reported that the follicular population was greater in FSH-stimulated cows when compared to that of unstimulated cows. However, in the latter experiment, it is difficult to judge whether the increase in follicular number was caused by the exogenous PMSG or by elevated levels of FSH caused by follicular aspiration before the administration of the PMSG. Recent reports reveal that no differences in the number of follicles available for aspiration were observed between unstimulated and superovulated cows (Stubbings and Walton, 1995, Bungartz et al., 1995). This being the case, it must be assumed that the increase in FSH concentration following aspiration pre-empts the effects of exogenous FSH or PMSG.

Repeated follicular aspirations did not appear to disrupt the cyclicity of the cows, as indicated by continuation of a mean interoestrous interval of 21.62±1.45 days. An exception to this was cow 156 which only returned to oestrus after

prostaglandin injection to cause regression of a persistent corpus luteum. It is generally accepted that inflammatory lesions, such as endometritis and pyometra, usually extend the lifespan of the corpus luteum by disrupting the normal luteolytic mechanisms initiated by the uterus (Noakes et al., 1990; Gilbert et al., 1990). No explanation can be found fora persistent corpus lutetium in this particular animal, since no pathological condition was diagnosed. These findings are in agreement with those of (Pieterse et al., 1991a; Kruip et al., 1994; Gibbons et al., 1994 and Bungartz et al., 1995), but they are in contrast to the findings of Stubbings & Walton (1995) who reported that a follicular aspiration of all visible follicles performed only once during the mid luteal phase was sufficient to extend the interoestrous interval by four days. There is enough evidence that if follicular aspiration is conducted twice weekly it results in the interruption of the cows cyclicity, apparently because no dominant follicle can develop and subsequently no ovulation can occur (Kruip et al., 1994; Bungartz et al., 1995). However, a five-day interval between aspirations is sufficient time for the selection of a dominant follicle and its subsequent development to preovulatory size (Kruip et al., 1994). Even if all follicles larger than 5 mm are destroyed on day 18 of the cycle (when luteolysis has begun) oestrus occurs at the expected time, indicating that selection and growth rate of the ovulatory follicle is a fairly rapid process (Matton et al., 1981). Our results indicate that cows could delay to return to oestrus only after aspiration conducted during the follicular phase. From our observations and others (Cattoni 1991) oestrus follows prostaglandin injection by approximately 80 hours. In two animals, when follicular aspiration was performed immediately after PG, the cows came in heat after 120 and 124 hours, indicating that less than two days are sufficient time for the selection and development of the preovulatory follicle.

4.1 b. Hormonal studies

Despite the main purpose of this section of the study being to study the endocrine consequences of multiple follicle ablation, the experimental design allowed detailed study of endocrine profiles in cows in the early, mid- and late stages of the luteal phase before aspiration. This section will discuss the results obtained before aspiration at different stages of the cycle and then cover the effects of multiple follicular aspiration.

To our knowledge this is the first study to examine the impact of follicular aspiration on the mode of secretion of reproductive hormones. The methodology used in this study introduces an alternative to the techniques of ovariectomy, immunisation and cautery of the follicle for studying ovarian endocrinology at different stages of the oestrous cycle.

Pre-aspiration blood sampling revealed that during the early luteal phase, mean plasma oestradiol concentration did not differ in aspirated and control cows. During the mid stage of the luteal phase, plasma oestradiol concentration in the aspirated cows was found to be lower than in the early luteal phase and slightly lower than in the control cows, possibly because the follicular population was younger and had not attained the same maturity as the control animals. Plasma oestradiol concentrations in the control cows did not differ from that of the early luteal phase in agreement with previous reports (Walters et al., 1984; Schallenberger et al., 1985). During the late luteal phase an increase in plasma oestradiol was detected in the pre-aspiration samples in both groups when compared to the mid and early stages. Of interest was the finding that in this stage of the cycle, although the diameter of the dominant follicle was greater in the

aspirated cows, mean plasma oestradiol was higher in the control animals. This suggests that follicular size and follicular oestradiol contents are not necessarily proportional.

It has long been known that ovarian follicles are the main source of oestrogens in the female animal and that follicular fluid is a rich source of these steroids. Following aspiration, a dramatic decrease in plasma oestradiol concentration was detected regardless the stage of the luteal phase. The decrease in mean plasma oestradiol was detected within one hour from aspiration indicating that diffusion of oestradiol into the blood stream is an unhampered process. A major feature of the plasma oestradiol response to aspiration was the variability between individual cows (Fig3.3a,b,c). Thus, in cow 100, plasma oestradiol was reduced to undetectable levels after aspiration during the mid luteal phase, while in cow 61 after aspiration in the early luteal phase, a moderate decrease in plasma oestradiol was detected. This variation might be due to incomplete aspiration of the dominant follicle, or the failure to aspirate some smaller follicles. Oestradiol concentration in small subordinate follicles is remarkably lower than in the dominant follicle (Ireland & Roche, 1982a;1983) but it seems that small follicles contribute to the overall peripheral concentration (Ireland & Roche, 1987). This concept is supported by the results of the present study indicating a positive correlation between the number of follicles aspirated and the degree of oestradiol reduction. Around day seven and between days 9 to 13 of the cycle most of the heifers were found to have only a single oestrogenically active follicle but this study was carried out at stages of the cycle when dominance had already been established and the subordinate follicles would have been in advanced atresia (Ireland & Roche, 1983). In the case of follicular aspiration performed on a weekly basis, it is likely that more than one dominant follicle would develop and in any case the subordinate follicles would not have progressed to atresia within this

time. In addition, the capacity of granulosa cells to synthesise oestradiol is dependent on the location of the cell within the follicle. Thus, granulosa cells aspirated along with follicular fluid from small follicles maintain their capacity to secrete oestradiol in culture for longer periods than cells obtained after scraping the follicular wall (Roberts & Echternkamp 1994). This being the case, it could be hypothesised that any remaining small follicles after aspiration are an additional source of oestradiol.

In seeking to explain the observation that plasma oestradiol concentration rebounded about six hours after aspiration, and that this renewed oestradiol secretion was maintained in the longer term, two observations are of importance. Firstly, atresia does not seem to be an irreversible process at least in the early stages of the luteal phase, since follicles that are recognised as subordinates on day three of the cycle, on the basis of size in comparison to an existing larger follicle, are capable of attaining dominance (to become oestrogenically active) if the dominant follicle is removed or they are exposed to exogenous FSH (Ko et al., 1991; Adams et al., 1993). Secondly, it seems unlikely that the steroidogenic properties of mural granulosa cells within the dominant follicle are lost after aspiration, since in many cases large follicles were found to be refilled the day after aspiration, indicating that the follicular components left in the follicle are at least partially functional. Combining these two observations would suggest that the increased plasma oestradiol concentration observed six hours post-aspiration was initially attributed to oestradiol secretion by the mural granulosa cells of the aspirated dominant follicle and that the significant increase in plasma oestradiol concentration, which was observed approximately 30 hours after aspiration, was due to oestradiol secreted by a new wave of follicles which were rescued from atresia after aspiration of the dominant follicle. This indicates that the emergence

of the subsequent follicular wave after aspiration occurs sooner than the two days that has been reported by other researchers (Bergfelt et al., 1994).

During the early luteal stage of the cycle, the observed increase in plasma oestradiol concentration 30 hours after aspiration resulted in significantly higher plasma concentrations than were seen pre-aspiration. It seems unlikely that this increase was derived from granulosa cells remaining in the aspirated dominant follicle, because although these follicles in many cases were found refilled the day after aspiration they never regained dominance, suggesting that the new wave produced the extra amounts of oestradiol. Since these follicles were still small in size, it is assumed that follicular aspiration induced the formation of more than one oestrogenically active follicle. Alternatively, the increase in plasma oestradiol seen 30 hours after aspiration might have been facilitated by the elevated LH concentration which was observed at any stage of the cycle. LH itself cannot directly increase oestradiol synthesis, but it may act by supplying the granulosa cells with increased androgen substrates after stimulation of the theca cells (McNeilly et al., 1991). The rebound increase in plasma oestradiol concentration occurring after aspiration during the mid and late stages of the luteal phase never exceeded pre-aspiration levels, probably because the preceding aspiration seven days previously had induced multiple oestrogenically active follicles, unlike the situation in the early luteal phase when there had been no previous aspiration during the same cycle. Unfortunately, the plasma samples from this series of experiments were not analysed for FSH concentration because radioimmunoassay was not available at the time. However, results from a subsequent experiment on dominant follicle ablation (which started 16 months later) revealed that a substantial increase in plasma FSH concentration does occur after aspiration. There are no reasons to doubt that a similar increase in plasma FSH would have occurred after the aspiration of all visible follicles from both ovaries and it was

probably this FSH increase that triggered the development of highly oestrogenic follicles, similar in some respects to the stimulation of ovarian follicles by superovulatory drugs.

Gonadal steroids are often thought of as having an inhibitory effect on gonadotrophin secretion as exemplified by the dramatic increase of plasma gonadotrophin concentration which follows castration in both female and male animals (Roche et al., 1970; Nett et al., 1974; Wise et al., 1979).

The effects of follicular aspiration in the present study also demonstrate certain interesting aspects of ovarian feedback control of gonadotrophins. The LH results reveal that oestradiol, or another unknown follicular factor, is an important component of the negative feedback mechanism on LH secretion. At all three stages of the luteal phase, follicular aspiration resulted in an increase in plasma LH concentration, which was maintained throughout the bleeding period (figs. 29, 30, 31). It is thought that the mechanism of regulation of pituitary gonadotrophin secretion by the gonadal steroids differs according to the stage of the cycle. Thus oestradiol acts to induce the LH surge by positive feedback on hypothalamus in follicular phase, (Karsch et al., 1992), while during the mid-luteal phase, it exerts a negative feedback on gonadotrophin release (Schallenberger & Prokopp, 1985). In addition, during the luteal phase oestradiol synergises with progesterone to suppress LH secretion (Tamanini et al., 1986). Even during the follicular phase when oestradiol exerts positive feedback in LH secretion, it is believed that oestradiol concentration must be transiently suppressed for a few hours to allow a high amplitude LH and FSH surge to occur (Walters & Schallenberger, 1984).

The present study showed that the highest increase in plasma LH concentration occured as a result of follicular aspiration during the early luteal phase, either because it coincided with the most dramatic drop of oestradiol concentration or

because at this stage of the cycle progesterone circulates in very low concentrations. However, in the present study, it appears that changes in plasma LH could be attributed to decreased oestradiol secretion after aspiration. This speculation is made because the increase in plasma LH occurred regardless of differences in the concentration of plasma progesterone in individual cows. Progesterone's role in controlling LH secretion in cattle by decreasing LH pulse frequency and increasing its pulse amplitude is well established (Rahe et al., 1980). Oestradiol exerts a negative feedback effect on LH secretion resulting in reduced pulse amplitude (Goodman & Karsch, 1980). The combined effect of oestradiol and progesterone on LH secretion appears to be greater than either hormone alone (Beck et al., 1976; Hauger et al., 1977). Results from ovariectomised cows and ewes indicate that oestradiol has a biphasic effect on LH secretion: initially suppressing mean plasma LH concentration for upto 12 hours and then inducing a preovulatory-like LH surge lasting for around eight hours. However, administration of a combination of oestradiol and norgestomet causes a substantial decrease in plasma LH concentration without any subsequent rise (Bolt et al., 1990, Caraty et al., 1989).

The present LH data obtained after aspiration of all follicles demonstrate that there is a close relationship between oestradiol and LH secretion. It is assumed that after aspiration and ablation of the follicular contents, the negative feedback exerted by oestradiol on LH is removed. This caused higher amplitude LH pulses to occur and this raised the mean plasma LH concentration. This increased LH might have indirectly triggered the subsequent increase in plasma oestradiol, by enhancing secretion of androgens by the theca cells of both aspirated and small follicles that escaped ablation. Gradually elevated oestradiol levels would mediate the maintenance of the relatively high LH concentration probably by acting on the pituitary. It seems unlikely that oestradiol would exert an inhibitory effect on LH

secretion through an action on pituitary GnRH receptors since treatment of ovariectomised ewes and cows with oestradiol results in increased numbers of GnRH receptors and increased pituitary responsiveness to GnRH (Moss et al., 1981; Schoeneman et al., 1985). *In vitro* studies provide compelling evidence that oestradiol acts on dispersed pituitary cells enhancing basal LH production and evoking a preovulatory-like LH surge by increasing the sensitivity of the cells to GnRH (Padmanabhan et al., 1978). However, presence of progesterone in such a culture, blocks both basal and GnRH-induced LH release (Padmanabhan & Convey, 1981).

It appears that an additional critical factor for the action of oestradiol on the hypothalamus-pituitary axis is the rate of change of plasma oestradiol concentration. The disagreement between studies investigating the effects of gonadal steroids on gonadotrophin secretion can possibly be attributed to such considerations, so although similar doses of oestradiol were used, it may have been administered by different means e.g. ranging from subcutaneous implants with slow oestradiol release (Imakawa et al., 1986), to multiple intravenous oestradiol injections with rapid effects on circulating oestradiol concentration (Bolt et al., 1990). In the present study, animals with different degrees of oestradiol reduction after follicular ablation showed similar increases in plasma LH indicating that rapid changes in oestradiol concentration could be equaly effective in LH regulation regardless of the absolute oestradiol reduction.

In the present study, the number of LH pulses recorded in the 42 hours after aspiration at any stage of the luteal phase was much lower than that described by other researchers (e.g. Rahe et al., 1980; Walters et al., 1984; Scallenberger et al., 1985). This indicates that the sampling frequency was not high enough to pick up all the pulses. This was the reason why the association between LH and oestradiol pulses was not investigated. Nevertheless, during the mid and late stages of the

luteal phase, LH pulse frequency in the control cows was lower than that in the early luteal phase, which is similar to that showed by Rahe et al.(1980) and Walters et al.(1984). LH pulse frequency between the pre- and post- aspiration periods cannot be compared as only four samples were taken before aspiration. Thus studies on pulse frequency were exclusively based on the comparison between aspirated and control cows.

4.1.c. Aspiration-induced follicular luteinisation

The experiment using selective ablation of the dominant follicle was conducted to investigate the hypothesis that accessory luteal structures could form in the presence of the natural corpus luteum after the aspiration of the dominant follicle. However, the ablation protocol also proved to be a novel approach to studing the role of the dominant follicle in regulation of gonadotrophin secretion.

The first dominant follicle can be identified by day four of the cycle using ultrasonography (Pierson & Ginther, 1987; Savio et al., 1988) and subordinate follicles that are considered to be oestrogenically inactive at this time can begin to synthesise oestradiol around day eight or nine (Ireland & Roche 1982a). In the present study, however, the reduction in plasma oestradiol when all follicles were aspirated was higher than when only the dominant follicle was ablated, suggesting that follicles classified as subordinates and therefore destined to undergo atresia are actually producing some oestrogen.

During the early luteal phase aspiration of the dominant follicle resulted in a dramatic decrease in plasma oestradiol concentration which lasted for the entire bleeding period. In the mid and late stages of the luteal phase, a decrease in plasma oestradiol was also observed but not to the same extent as in the early

luteal phase. Additionally, there was a tendency for plasma oestradiol to increase after a lag of five to six hours after aspiration during the mid and late stages of the luteal phase. This difference in the oestradiol response to aspiration at the three stages of the luteal phase indicates that the increase in plasma oestradiol concentration might be derived from a source other than the aspirated dominant follicle. It could be hypothesised that during the early luteal phase oestradiol secretion by the subordinate follicles either had been boosted after the end of the bleeding period or the subordinate follicles were incapable to respond to the signal of aspiration by increasing their oestradiol production. It is believed that each follicle develops spontaneously to a certain size beyond which it must be stimulated by FSH to initiate further development, otherwise it undergoes atresia (Cahill et al., 1981; Quirk & Fortune 1986) but that each follicle has its own threshold of responce to FSH. The dominant follicle is believed to have the lowest FSH threshold and its development can therefore start early in a low FSH milieu. Oestrogens subsequently produced by the dominant follicle decrease FSH secretion to concentrations too low to sustain development of the subordinate follicles (Hillier 1994). Increased FSH levels after ablation of the dominant follicle probably permitted renewed development of follicles with higher FSH thresholds

Of considerable interest from the present work was the observation that aspiration of the dominant follicle alone did not affect LH secretion during the subsequent eight hours, while aspiration of all ultrasonically visible follicles from both ovaries did alter LH secretion This may be linked to the finding that aspiration of all ultrasonically visible follicles resulted in a higher degree of oestradiol reduction compared to that seen after aspiration of the dominant follicle only. It could be suggested that the decline in plasma oestradiol after aspiration of the dominant follicle was not sufficient to alter the mode of LH secretion. Furthermore, if

oestradiol is not the only factor regulating LH secretion, but another unidentified substance is also responsible; then this putative factor might originate from the subordinate follicles and might act to suppress LH either alone or in synergy with oestradiol. Of interest was the significant increase of plasma LH concentration after the dominant follicle was aspirated during the late stage of the luteal phase in cow 97. However, from a careful inspection of figure 46 it becomes apparent that in this cow unlike the others, there was a dramatic drop in oestradiol concentration immediately after aspiration, in levels below the detection limit of the assay, which lasted for the subsequent three hours.

Studies in ovariectomised heifers have revealed that administradion of low doses of oestradiol had little if any effect on mean LH concentration, while higher doses of oestradiol significantly suppressed both mean LH concentration and pulse amplitude in a dose dependent manner (Price & Webb, 1988; Wolfe et al., 1992). Moreover, Price and Webb (1988) have demonstrated that, in the absence of progesterone, there is a ceiling to oestradiol induced LH suppression, above which oestradiol is unable to exert any further negative feedback on LH, but this ceiling does not apply to inhibition of LH pulse frequency. However, the action of oestradiol on LH pulse frequency was enhanced in the presence of progesterone.

On the other hand, plasma FSH increased significantly shortly after aspiration of the dominant follicle at any of the three stages of the luteal phase (figs.57, 58,59). Gonadotrophs of the anterior pituitary are the cells that synthesise FSH and LH in response to stimulation of the hypothalamic GnRH (Fink 1988). However, it appears that the regulatory mechanisms for synthesis and secretion of FSH and LH are different. Thus in the ewe, GnRH antagonists acutely suppressed LH secretion but had no effect on FSH concentration (Campbell et al., 1990), while GnRH agonists induced minor increases in FSH concentration in cows (Foster et al.,

1980). The fact that in the present study, LH concentrations remained unaltered after aspiration of the dominant follicle indicates that ablation of the dominant follicle does not induce changes in GnRH secretion. It therefore seems reasonable to hypothesise that factors other than GnRH triggered the increase in plasma FSH concentration.

It has been known for many years that oestradiol is the main inhibitor of FSH secretion. Thus immunisation against oestradiol increased FSH concentration in ewes (Scarramuzzi et al., 1980), while oestradiol injections caused suppression in FSH concentration in cows and ewes (Butler et al., 1983; Salamonsen et al., 1973). Inhibin is another factor that actively suppresses FSH secretion in the female animal (Robertson et al., 1985; Demoulin et al., 1987; Findlay et al., 1991). The source of inhibin is the large antral follicles, that are not necessarily oestrogenically active (Baird et al., 1991). In the ewe, inhibin production appears to be independent of gonadotrophic stimulation (McNeilly & Baird, 1989) and exogenous gonadotrophins, in a dose equivalent to high physiological levels, failed to cause any significant increase in the secretion of inhibin (Campbell et al., 1989). Higher amounts of FSH can cause increased inhibin secretion, probably due to increased numbers of developing follicles, rather than to enhanced production by individual follicles (McNeilly et al., 1991).

Injections of charcoal-treated follicular fluid caused a significant drop in plasma FSH concentration (Ireland et al., 1983; Quirk & Fortune, 1986). The relative contribution of the two hormones oestradiol and inhibin to suppression of FSH is not clear. However, from experiments in ovariectomised ewes it was revealed that low doses of oestradiol and inhibin injected together, were able to prevent the FSH rise that occurs after ovariectomy, while each hormone alone failed to suppress the rise so effectively (Martin et al., 1988). In addition, immunisation against both hormones induced a higher increase in plasma FSH concentration than did

immunisation against either hormone alone (Mann et al., 1990). The temporal correlations between oestradiol and FSH on one hand and inhibin and FSH on the other at different stages of the ovine oestrous cycle have led to the proposal that oestradiol regulates day to day fluctuations in the pattern of FSH secretion while inhibin exerts a long-term negative feedback in FSH secretion (Baird et al., 1991). This being the case, it could be suggested that the increase in FSH concentration observed in the present experiment after aspiration was probably attributed to the withdrawal of oestradiol rather than inhibin from the dominant follicle, firstly because the changes of FSH concentration occurred almost two hours after aspiration, and secondly because aspiration of the dominant follicle resulted in depletion of the oestrogenic reserves of the ovary but probably only in partial reduction of inhibin.

Our results indicate that progesterone plays only a minor role in the regulation of FSH secretion since mean FSH concentrations were maintained at high levels post-aspiration regardless of the stage of the cycle and therefore regardless of the concentration of plasma progesterone. This is in agreement with previous reports where FSH concentrations were found to remain unaltered after administration of progesterone to intact or ovariectomised cows (Ireland & Roche, 1982; Price & Webb, 1988).

Another interesting observation is that during the mid and late stages of the luteal phase there was a tendency for a delayed increase in mean oestradiol concentration several hours after aspiration yet there was no tendency for mean plasma FSH concentration to decrease at this time. This cannot be explained by the proposal of Baird et al. (1991) that oestradiol establishes the fine tuning of FSH secretion while inhibin sets the long-term FSH concentration. However, in some cases (eg cow 96 aspirated in the mid and late luteal stages, and cow 97 in the late luteal phase) oestradiol pulses during the post-aspiration period appeared to be followed by

temporal suppression of FSH secretion. These data may indicate that the ratio of oestradiol and inhibin concentration in the blood is more important in regulating FSH secretion than the concentration of each hormone alone. Moreover, FSH secretion appeared to be acutely affected by the sudden disruption of the oestradiol : inhibin ratio but FSH did not seem to respond to gradually increasing oestradiol, probably until this ratio had been re-established. Further, aspiration of the dominant follicle of cow 47 during the mid luteal phase would provide some support to the hypothesis of the critical role of oestradiol : inhibin ratio. In the case of cow 47 aspirated during the mid luteal phase, post-aspiration plasma oestradiol concentration was not significantly different than that of the pre-aspiration period and in addition the ratio of oestradiol : progesterone concentration in the follicular fluid was the smallest observed in the experiment (1.2), indicating that although the follicle appeared dominant it had lost its functional dominance (Fortune et al., 1991). It must be assumed that the main source of oestrogens in this case was a smaller follicle which had apparently remained intact. However, in the same case, aspiration of the largest follicle resulted in a substantial increase in plasma FSH concentration, in a way that did not differ from that of the other animals during the same stage of the cycle, suggesting that either a short-term decrease in plasma oestradiol, or disruption of the equilibrium between oestradiol and inhibin was sufficient to increase FSH secretion.

Follistatin, another ovarian peptide has been isolated from bovine follicular fluid (Robertson et al., 1987) where it selectively suppresses FSH synthesis. Follistatin has also been detected in the peripheral blood of sheep, but it may not originate exclusively from the ovary (Findlay et al., 1992). The physiological role and contribution of follistatin in FSH secretion is still unclear. Nevertheless, Wood et al. (1993) have reported that steroid free bovine follicular fluid injected in either

normal or inhibin-immune heifers resulted in suppression of FSH concentration to a similar extent in each case, indicating that follicular non-steroidal components other than inhibin play a role in the regulation of FSH secretion, acting either alone or in synergy. It therefore seems that the physiolocal role and contribution of follistatin in FSH secretion is still unclear.

4.3 Accessory corpus luteum.

Despite finding that cow 9 developed a corpus luteum after aspiration of all follicles during the late luteal phase this structure was not considered to be accessory corpus luteum since its formation followed a preovulatory-like LH surge. In addition plasma progesterone concentration had already begun decreasing (fig. 36) Nevertheless, there are a series of events indicating that the aspiration hastened the formation of the new corpus luteum. Firstly, standing heat was not detected although the animals were observed for oestrus signs each day. Secondly, plasma progesterone levels were found to be decreasing, but they were still not as low as they are normally found to be around oestrus. Thirdly, oestradiol concentration in the pre-aspiration period was relatively high but it would be risky to judge if it was increasing or decreasing at the time. However, it seems likely that plasma oestradiol levels were increasing since oestradiol concentration normally reaches its peak at the time when progesterone concentration is at its nadir. If this was the case, the LH surge occurred earlier than expected since the preovulatory surge of gonadotrophins takes place when oestradiol levels start declining. Further, Walters & Schallenberger (1984) have reported that oestradiol

concentration needs to be depressed some hours before the gonadotrophin surge in order to evoke positive feedback on both gonadotrophins. Maybe in the present study, the decline in oestradiol induced by the follicular aspiration imitated the naturally occurring drop in oestradiol levels and triggered the LH surge.

In another case (cow 158) an accessory corpus luteum developed after aspiration of all follicles during the early luteal phase. Hormonal profiles are not available in this example, and therefore the explanation of the accessory corpus luteum will be based on the data collected from other animals treated in an identical manner as described in chapter 3.2.

The current understanding of the mechanisms of follicular luteinisation is that corpus luteum formation is dependent on a surge of LH in a hormonal milieu characterised by low plasma progesterone and high levels of oestradiol. In the present case an accessory corpus luteum was seen to develop after follicular aspiration on day three of the oestrous cycle and in the presence of a new natural corpus luteum. While the role played by the LH surge in ovulation and luteinisation is well documented, the role of the contemporary FSH surge is not clear. Experiments in hypophysectomised rats show that FSH injections can cause ovulation and luteinisation in the absence of detectable levels of LH. In addition, the number of follicles which underwent luteinisation was dependent on the FSH dose (Hubbard & Erickson, 1988) and in separate studies, intrafollicular injection of FSH induces ovulation in sheep (Murdoch et al., 1981). Early studies by Nalbandov showed that removal or death of the oocyte led to luteinisation of rat granulosa cells, and cultured granulosa cells in the vicinity of oocytes failed to luteinise, while those cultured in a distance from the oocyte showed clear signs of luteinisation (Nekola & Nalbandov 1971; Stolskowa & Nalbandov 1972). However, other researchers have failed to detect such an inhibitory action of the

oocyte (Linder et al., 1974) and others have suggested that the follicular fluid does contain a luteinisation inhibitor, but not of oocyte origin (Channing & Tsafriri, 1977). Similarly, follicular fluid prevented luteinisation of granulosa cells *in vitro* (Bernard & Psychoyos, 1977), but luteinisation occurred if granulosa cells were previously exposed to high gonadotrophin concentrations or if high concentrations of gonadotrophins were added to the culture medium (Berndtson et al., 1995).

Results from the present study demonstrate that follicular aspiration at any stage of the luteal phase causes a dramatic drop in plasma oestradiol concentration and an increase in plasma LH levels. However, this LH increase never resembled the amplitude of the preovulatory LH surge. Further, an increase in plasma FSH concentration was detected after the aspiration of the dominant follicle, and would presumably have occurred after aspiration of all follicles on both ovaries. Such alterations in the pattern of the hormone secretion, in synergy with withdrawal of the putative luteinisation inhibitor of the follicular fluid might have triggered luteinisation of the punctured follicle.

Histological examination of the accessory structure revealed morphological features typical of the cyclic corpus luteum. The presence of the dark secretory granules in both large and small luteal cells is in agreement with some researchers (Chegini et al., 1984) but some (Hansel et al., 1991) have reported that secretory granules are present only in the large luteal cells. Tissue levels of progesterone were within the normal range for corpora lutea, though progesterone content of the accessory structure was half that of the natural corpus luteum, and oestradiol content was 45% higher, perhaps indicating incomplete luteinisation. Preovulatory follicles produce mainly oestradiol and androstenedione before the surge of gonadotrophins, while shortly after the surge, production of these steroids declines and progesterone synthesis increases (Fortune & Hansel, 1985). This shift of steroid production by the preovulatory follicle is accompanied by a decrease in

P450-17α mRNA and $P450_{arom}$ after the LH surge (Voss & Fortune, 1993). These mRNA species are normally expressed only in theca and granulosa cells respectively and their expression is down regulated as granulosa and theca cells differentiate into luteal cells (Rodgers et al., 1986; Voss & Fortune, 1993). The high oestradiol concentration in the accessory structure can be explained by the detection of mRNA encoding $P450_{arom}$. Although it is well documented that the gonadotrophin surge initiates the changes in steroidogenesis in theca and granulosa cells, very little information is available about the molecular mechanisms that regulate this shift.

As described above, the presence of a high oestradiol concentration in the accessory structure together with detection of aromatase activity, probably indicates incomplete luteinisation, suggesting that there was a luteinising signal that was not strong enough to stimulate luteinisation of the entire cellular population of the punctured follicle. Thus, some granulosa and theca cells luteinised as apparent from the histological studies, but others did not and continued producing appreciable quantities of oestradiol. The detection of the FSH receptor is further support for the concept of incomplete luteinisation. Although it is well documented that LH is the main luteotrophic factor in the cow, formation and development of the corpus luteum it seems to be independent of FSH support as progesterone secretion by bovine luteal cells cultured with purified FSH did not increase (Chopineau et al., 1993). Although Manns et al. (1984) detected FSH receptors in the bovine corpus luteum, recent attempts using techniques of high sensitivity (RT-PCR) have failed to detect mRNA for the bovine FSH receptor in the corpus luteum while they despite confirming the existence of FSH receptor in granulosa cells (Houde et al., 1994; Rajapaksha et al., 1996). Furthermore, in the latter study it was reported that a low level of full length FSH receptor mRNA was detectable on day one of the cycle but by day three, the receptor was undetectable

and remained so until the end of dioestrus (Houde et al., 1994). It seems probable that factors other than the surge of gonadotrophins down-regulate the FSH receptor from granulosa cells, since the receptor is not expressed during the luteinisation of granulosa cells *in vitro* (Rajapaksha et al., 1996).

Interestingly $P450_{17\alpha}$ was detected in both the natural corpus luteum and the accessory structure formed after follicle aspiration. In the normal cycle levels of $P450_{17\alpha}$ decrease by almost 94% approximately 20 hours after the surge of LH (Vos & Fortune, 1993). $P450_{17\alpha}$ mRNA is abundant in antral follicles but virtually undetectable in the corpora lutea except for weak expression in young corpora lutea (Rodgers et al., 1987). In the present study it was expected to detect $P450_{17\alpha}$ in the accessory structure since there was detectable aromatase activity there indicating that some follicular cells remained undifferentiated. The fact that $P450_{17\alpha}$ was found in the natural corpus luteum probably indicates that PCR amplification enables very small amounts to be detected.

The observed incidence of accessory luteal structure formation is low, 4 observations made after aspiration of 45 cows with an average of 4.2 aspirations per cow. In a separate trial five structures were recognised after aspiration of approximately 96 cows (Salaheddine, personal communication), the experimental a protocol in this case requiring aspiration of all ultrasonically visible follicles (>5mm) during the early luteal phase (days3-5). However no examination of these structures was performed.

In the experiment in which only the dominant follicle was aspirated in early, mid and late stages of the luteal phase, no accessory luteal structures were produced. Probably the aspiration of only the dominant follicle is not sufficient to induce the favourable hormonal environment for the luteinisation of the punctured follicle.

These results suggest that the LH surge which occurs prior to ovulation is essential for the rupture of the preovulatory follicle and for down-regulation of some but not

all mRNA species expressed in the granulosa and theca cells. However it appears that the LH surge is not the only factor required for the differentiation of the corpus luteum. Under certain circumstances, the process of follicular aspiration might be sufficient to induce development of a normal corpus luteum.

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