INVESTIGATIONS OF FORMATION AND FUNCTION OF NORMAL AND ABNORMAL LUTEAL TISSUE IN THE BOVINE

By

Mourad Salaheddine

D.M.V.

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UNIVERSITY OF GLASGOW

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
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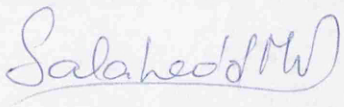
DECLARATION

I hereby declare that the work presented in this thesis was conducted by the author under supervision except for the following:

The ultrasound scanning described in chapter two was carried out by Dr. D.N. Logue of the Scottish Agricultural College, Auchincruive.

The processing of tissue samples for histology and electron microscopy described in chapters three and four was undertaken by the staff technicians of the Department of Surgery and Reproduction.

I certify that the work included in this thesis is original and no part of it has been submitted previously for the award of a degree to any university.



Mourad Salaheddine

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotrophic Hormone
AI	artificial insemination
ATP	adenosine triphosphate
bTSH	bovine Thyroid Stimulating Hormone
Ca^{2+}	calcium
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophosphate
CL	corpus luteum
CLs	corpora lutea
cpm	count per minutes
cytochrome $P450_{scc}$	side-chain cleavage cytochrome P450
°C	degree Celsius
D.C.S	donor calf serum
DG	diacylglycerol
DHA	5-androsten-3ß-ol-17-one
DNAse	deoxyribonuclease
EDTA	ethylenediaminetetracetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
FGF	fibroblast growth factor
FSH	Follicle Stimulating Hormone
g	relative centrifugal force
GER	granular endoplasmic reticulum
GnRH	Gonadotrophin Releasing Hormone
H & E	Haematoxylin and Eosin
hCG	human Chorionic Gonadotrophin
HCl	hydrochloric acid

5-HETE	5-hydroxyeicosatetraenoic acid
20α-HSD	20α-hydroxysteroid dehydrogenase
3ß-HSD	δ^5-3ß-hydroxysteroid dehydrogenase
IP_3	inositol-1,4,5-triphosphate
iu	international unit
LH	Luteinizing Hormone
M	mole
MBq	megabequerel
MHz	megahertz
mM	millimole
mRNA	messenger ribonucleic acid
MSB	Martius Scarlet & Blue
MW	molar weight
$Na_2HPO_4.12H_2O$	*di*-sodium hydrogen orthophosphate dodecahydrate
NAD	ß-nicotinamide adenosine dinucleotide
$NADH_2$	reduced nicotinamide adenosine dinucleotide
$NaH_2PO_4.2H_2O$	sodium dihydrogen orthophosphate dihydrate
NaOH	sodium hydroxide
No(s).	number(s)
NSB	non specific binding
oFSH	ovine Follicle Stimulating Hormone
oGH	ovine Growth Hormone
oLH	ovine Luteinizing Hormone
oPRL	ovine Prolactin
P	significance level
P_4	plasma progesterone
PGE-1	prostaglandin E-1
PGE-2	prostaglandin E-2
PGF2α	prostaglandin F2α
PGI-2	prostaglandin I-2

PIP_2	phosphatidylinositol-4,5-bisphosphate
PRID	progesterone-releasing intravaginal device
PVP	polyvinyl pyrrolidone
s.d. (S.D.)	standard deviation
s.e.m.	standard error to the mean
SER	smooth endoplasmic reticulum
TCs	total counts
TEM	transmission electron microscopy
vs	versus
w/v	weight per volume

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SUMMARY

The work included in this thesis describes studies of the cyclic bovine corpus luteum. Both normal and abnormal luteal structures were investigated using various approaches.

Initially, the formation, maintenance and lysis of the corpus luteum were investigated in a field trial. Heifers were subjected to two different treatments for the synchronization of oestrus and their response monitored using ultrasonic examination of the ovaries and plasma progesterone concentration. Although no difference was found in the pregnancy rate between the progestogen and the prostaglandin treatments, close examination of the results indicated that in a proportion of animals the response to both treatments was not as expected. In particular it was found that the use of a double injection of prostaglandins, given ten days apart, results in a significantly better pregnancy rate to the synchronized oestrus when the first injection is administered during a non-luteal phase.

The results obtained led to detailed studies on small groups of cows in which both the follicular aspect and the Luteinizing Hormone surge were given special attention during and following similar treatments. The variation in the timing and amplitude of the surge of Luteinizing Hormone did not hinder conception. However there were indications that follicular status at the end of progestogen treatments may be involved in delaying the surge of Luteinizing Hormone.

Subsequently investigations of the structure and function of the corpus luteum were undertaken using samples collected at a local abbatoir. Gross macroscopic morphology of the corpus luteum was used as a means of evaluating the stage of the cycle of the animals. The methods utilized included histology, histochemistry, electron microscopy and cell culture. The information obtained was compared to results of studies of corpora lutea formed following an injection of a prostaglandin analogue during either early or late dioestrus. In addition the injection of a GnRH analogue during mid-dioestrus was used in two cows to monitor the formation and function of accessory corpora lutea.

Although no difference in response and formation of luteal tissue could be found between groups treated differently, there was indications that corpora lutea induced by an injection of either a prostaglandin F2α analogue or a GnRH analogue have a shorter life-span than

spontaneous corpora lutea. Regressive changes were observed in both histology and electron microscopy sections from all induced corpora lutea. This was further supported by the functional insufficiency which was recognized in these structures when challenged *in vitro* with hormonal agents. Histochemistry did not prove very useful in determining changes in steroidogenic enzyme activity. However it did show the presence of these enzymes in both the normal and the abnormal corpus luteum.

It was concluded that further work was needed in this area in order to unveil the molecular mechanisms controlling formation, function and regression in both the normal and the abnormal corpus luteum.

CHAPTER ONE

GENERAL INTRODUCTION

I. INTRODUCTION

In 1897 Beard, the Edinburgh embryologist, ascribed maintenance of pregnancy and inhibition of ovulation to the corpus luteum a theory which was supported by Prenant 1898 who suggested that the product of the corpus luteum were carried in the blood stream. Early in the twentieth century the relationship between the corpus luteum and the pregnant and non-pregnant uterus was referred to by Bouin and Ancel (1910) and a year later Loeb (1911) published evidence showing that enucleation of a corpus luteum hastened the onset of the next oestrus. In 1923 Loeb observed that hysterectomy of the guinea pig prolonged the life-span of the corpus luteum (CL) but it was only in the seventies that Prostaglandin F2α (PGF2α) was identified as the luteolytic agent (McCracken et al., 1972; Pharriss and Wyngarden, 1969; Thornburn and Nicol, 1971; Douglas and Ginther, 1972 and Rowson et al., 1972).

In the cycling bovine the CL dictates the length of the oestrous cycle. Maturation and ovulation of a follicle cannot occur while the CL is producing progesterone. As this important ovarian structure develops from the cells of the follicular wall after ovulation, the mechanisms which contribute to the formation of the mature follicle have a profound effect on the subsequent composition and function of the resulting CL. Therefore an understanding of the events involved in follicular development is necessary in extending our knowledge of the development of normal and abnormal CL.

II. THE OESTROUS CYCLE OF THE COW

The oestrous cycle in the bovine varies in length from 18 to 24 days with cows having a slightly longer interoestrous interval than heifers (Arthur et al., 1989). The cycle consists of four different periods, viz oestrus lasting 6 to 30 hours, followed by ovulation; metoestrus the period of CL formation assumed to last four days; dioestrus, the period when the CL is fully functional, 13-15 days in length and prooestrus, when plasma oestrogens are increasing and progesterone is basal (Peters and Ball, 1987a).

Prooestrus is characterized by male-like behaviour ie. the cow attempts to mount other cows (Webster, 1987). The increase in circulating plasma oestrogens causes tumefaction and pinkening of the mucosa of the vaginal vestibule. The lips of the vulva are swollen and can be easily parted (Peters and Ball, 1987b). As oestrus approaches the vaginal secretions become increasingly fluid and the vaginal mucosa appears shiny pink in colour. Close observations reveal a slight cloudy discharge often voided from the vulva at this time, indicating that oestrus is nearing.

The main ovarian structures identified by rectal examination of a cow during prooestrus are follicles of varying size but dominated by one large follicle of more than 1.5 cm. Often the protrusion of the ovulatory papillum is all that remains of the CL of the previous cycle which at this stage is no more than a small block of connective tissue easily confused with the remainder of the ovarian stroma. The most striking palpable feature of the reproductive tract of a bovine approaching oestrus is the tone of the uterus. This increases markedly as oestrus nears. During overt oestrus the uterus is at its most turgid status. Towards the end of prooestrus and during overt oestrus, the horns of the uterus

are more difficult to separate than at any other period of the cycle. Even attempts at separating the two horns by inserting a finger between them becomes difficult. The behaviour of the cow during prooestrus includes vocal restlessness and seeking interest from companions. These signs of prooestrus are obvious if the cow is being watched continually whilst free roaming in a group of cattle. In general the restlessness is accompanied by repeated butting, sniffing of other cows, and attempts at resting the chin over the tail end of others (Kilgour and Dalton, 1984). The most accurate signs of prooestrous behaviour occur at 6 to 48 hours before standing oestrus. These include the cow seeking to mount other cows that may or may not be in season. The cow may become interested in a single partner if the latter is also approaching or in oestrus, otherwise she attempts to mount several cows. Moreover and surprisingly the cow approaching oestrus will not allow other cows to mount her albeit she has been desperately trying to get their interest.

Overt *oestrus* is characterized by the cow standing to be mounted. This will continue for as long as 6 to 15 hours and in some extreme cases for up to 30 hours. The end of overt oestrus is not as abrupt as its beginning. The animal if teased frequently during overt oestrus (e.g. every 2 hours) will show a gradual decrease in desire to be mounted and starts to take a few steps when mounted. Finally she will escape any further attempts to be mounted. One important clinical sign is a vaginal discharge called "bulling string" which differs from the discharge voided during prooestrus in that it is sparkling clear, copious and so fluid that it is almost impossible to collect. Twelve to fifteen hours after the end of standing overt oestrus ovulation occurs spontaneously (Hansel, 1959).

Following release of the oocyte and the follicular fluid the follicular walls contribute to the formation of the new CL. This phase is called *metoestrus*. During the first two days of metoestrus a blood stained vaginal discharge is

present in a large proportion of cows (Peters and Ball, 1987b) and is thought to represent capillary rupture as a result of the high pressure of the blood flow in the endometrium triggered by the increased amounts of oestrogens present prior to ovulation. Formation of the CL requires between 3 to 6 days, and no behavioural changes are obvious during this phase.

During *dioestrus* the CL is fully functional and can be palpated in the ovary as by now it is the size of a large olive with tissue protruding at its apex which corresponds to the follicular point of rupture ie. the ovulatory papillum.

III. ENDOCRINOLOGY OF THE OESTROUS CYCLE

In cattle follicles grow and regress continuously throughout the oestrous cycle. However, normally final maturation and subsequent ovulation of a dominant follicle occurs only after the decrease in circulating plasma progesterone during late dioestrus (Savio et al., 1988; Sirois and Fortune, 1988). The preovulatory size of the mature follicle in the bovine varies from 14 to 16.5 mm (Ginther et al., 1989). The changes which take place between lysis of the CL and overt oestrus involve the cellular components of the wall of the follicle in increased activity and the secretion of increasing amounts of oestrogens mainly oestradiol-17β (Chenault et al., 1975). The cellular components of the follicle include the outer two layers of theca cells separated from the internal granulosa cells by a basement membrane.

During prooestrus and oestrus the pattern of plasma luteinizing hormone (LH) changes from that of continuous pulses controlled by the tonic centre in the hypothalamus to pulses of increased amplitude and frequency culminating in a peak of LH brought about by activation of the surge centre in the hypothalamus (Halasz, 1966; Jackson et al., 1978; Rahe et al., 1980). The increase in the

pattern of gonadotrophin secretion (LH and FSH) acts on both cellular elements of the follicle (granulosa and theca cells) and brings about a complete change in the distribution of receptor-sites on the surface of the cells causing their accelerated differentiation into highly active steroidogenic cells (Hsueh et al., 1984; Ireland and Roche, 1983; Staigmiller et al., 1982). The androgens synthesized in the theca layers are converted to oestrogens through aromatization by granulosa cells and secreted into the follicular fluid and hence into the blood stream (Armstrong and Dorrington, 1977; Fortune and Hansel, 1979). The increasing levels of oestrogens enhance the amplitude and frequency of the LH pulses which culminate in a surge of LH of more than 30 ng/ml (Chenault et al., 1975; Martin et al., 1978).

Following ovulation the follicular walls fold inwards so that both theca and granulosa cells contribute to the formation of the new CL which is completed at the cellular level by day seven of the oestrous cycle when the cells stop dividing. However both increase in size of cells and organization of the tissue is not definitive until about day nine (Donaldson and Hansel, 1965). During the first three to five days of metoestrus when progesterone is increasing slowly, LH secretion is controlled by the tonic centre of the hypothalamus and is characterized by its "low amplitude-high frequency" pattern (Rahe et al., 1980). At this time the new CL is refractory to exogenous pharmacological doses of PGF2α (Rowson et al., 1972; Louis et al., 1973) and the first wave of follicles occurs (Sirois and Fortune, 1988; Savio et al., 1988; Ginther et al., 1989). Two small peaks of oestrogens appear during this phase of the cycle (Hansel et al., 1973). Circulating plasma progesterone is usually between 3 and 6 ng/ml during the mid-luteal phase (Lukaszewska and Hansel, 1980) and was shown to fluctuate according to FSH changes (Walters et al., 1984).

In the cow as in the ewe PGF2α produced by the endometrium is thought to

induce luteolysis of the CL. However neither the mechanisms involved nor the factors which prompt its release during late dioestrus are fully understood. This is particularly true in the cow (Hansel and Dowd, 1986) in which other luteolytic factors have been isolated such as products of the lipoxygenase pathway of arachidonic acid metabolism, in particular 5-hydroxyeicosatetraenoic acid (5-HETE) (Milvae et al., 1986). However, no evidence is available yet to demonstrate a role of these substances in natural luteolysis.

Some other hormones and factors have been described as playing roles in the cyclic events in the cow. These include oxytocin which is thought to be involved in both luteotrophy and luteolysis in this species (Hansel and Dowd, 1986). Recently inhibin and similar follicular polypeptides have also been shown to play a major role in follicular turnover in synchrony with FSH (Ireland, 1987; Findlay and Clarke, 1987). Mechanisms governing this relationship are under recent investigations but appear promising in revealing some aspects of endocrine and paracrine interaction in the bovine ovary in particular between follicles and the CL. Prostaglandin E-2 (PGE-2) and prostaglandin I-2 (PGI-2) as well as several other products of arachidonic acid metabolism are also involved in normal luteal function in the cow (Reynolds et al., 1983; Hansel and Dowd, 1986) and the ewe (Schwall et al., 1986).

IV. THE CORPUS LUTEUM: ORIGIN, STRUCTURE AND FUNCTION

1. Macroscopic morphology

Following ovulation the follicle shrinks in size from a diameter of 16-19 mm to one of 5-8 mm. At the point of ovulation, a slight hemorrhage is present. The protrusion at the point of rupture is reddish in colour. In the bovine the

irregular central cavity formed after ovulation is not normally filled with blood clot as it is in the case of other species (MacNutt, 1924). Instead the walls of the follicle collapse under the pressure caused by the contractions of the theca externa (Gier and Marion, 1961) and form a small central cavity which may be obliterated later on (Okuda et al., 1988). However in about a third of CLs this cavity remains after it is walled off by a connective tissue capsule thus producing a small fluid filled cyst or lacuna (MacNutt, 1924). Usually at the apex of the CL a protuberance is formed as the result of eversion of the luteal tissue through the point of rupture before it is covered by the epithelium. However in many cases this protuberance is absent. A central depression is usually present on the protuberance indicating the point of rupture of the follicle (MacNutt, 1924).

Ireland et al. (1980) described four readily identifiable changes in the gross appearance of cyclic CLs at different stages of the cycle, viz. from day one of the cycle to day four, the point of rupture remains uncovered by epithelium (stage one). After the point of rupture closes the apex of the CL remains red or brown for an additional five days (stage two), the rest of the luteal tissue is orange or tan. CLs of stage three when dissected are entirely orange or tan and appear as such from day 11 through day 17 of the cycle. Stage four includes CLs which have a yellow to orange internal colour and light yellow to white external appearance, found during the remaining three to four days of the cycle, ie. from late dioestrus to prooestrus.

The size of the CL and its consistency are other indications as to the stage of its life-span. Thus while a forming CL appears as a very soft structure of less than 1.5 cm, a mature functional CL of mid-dioestrus is more compact and is of more than 1.8 cm in size. The regressing CL is firm and less than 1.5 cm in size. Poor vasculature is another characteristic of the CL during involution (Ireland, 1980). Whilst functional CLs bleed when sectioned as a result of abundant vasculature,

regressing ones do not (MacNutt, 1924). The CL also varies in weight according to the stage of the cycle. (Foley et al., 1964) reported that CLs of two to five, six to eight, twelve and 18 to 19 days weighed 1.05, 3.58, 5.30 and 3.84 g respectively.

2. Histology and ultrastructure

2.1. The preovulatory follicle

During prooestrus there is little indication as to which of the large follicles present in the ovaries will ovulate and size alone is thought to be an unreliable predictor of the ovulatory follicle until the day of oestrus (Quirk et al., 1986). Histological changes only become identifiable after the surge of LH when signs of cellular luteinization become apparent. Six hours after the onset of oestrus histological examination of the preovulatory follicles show early cellular luteinization of both the theca and the granulosa layers (Donaldson and Hansel, 1965). At this stage cells undergo cytoplasmic enlargement as well as division (Dieleman et al., 1983; Loos et al., 1991). Examination of these cells by transmission electron microscopy shows a preponderance of smooth reticulum endoplasmic (Enders, 1973). When granulosa cells are incubated in the presence of added LH, the mitochondria in these cells acquire an increasingly complex cristae, often with lamelliform-villiform organization. Mitochondria as well as other organelles such as lysosomes can be observed in clusters around the nucleus. In addition the high nuclear/cytoplasmic ratio and the active proliferation which are a characteristic of granulosa cells are also lost following exposure to LH. The nuclear heterochromatin is transformed to a lighter staining euchromatin pattern. Moreover an increase in the number of lipid droplets is known to occur following both *in vitro* and *in vivo* luteinization (Amsterdam and Rotmunsch, 1987).

Prior to ovulation at a particular area of the follicle both the thecal cells and the cortical component of the follicle (tunica albuginea) become thin and this portion of the follicle protrudes from the surface of the ovary. This protrusion, the stigma, is the site of rupture. Deterioration of the follicular wall caused by enzymatic hydrolysis of connective tissue components by LH-induced collagenase, protease or plasmins is thought to play a role in ovulation. Contrary to what used to be suggested an increased intrafollicular fluid pressure is not associated with the ovulatory process (Banks, 1986).

2.2. The forming corpus luteum

Following rupture and expulsion of the oocyte with its intimately associated granulosa cells (corona radiata), the remainder of the granulosa layers become deeply folded and each fold contains a core of cells from the theca interna. The cores of theca interna also contain connective tissue and blood capillaries (Gier et al., 1961). Fibroblasts and endothelial cells rapidly invade the granulosa stratum following the disappearance of the basement membrane which is no longer visible 24 to 48 hours after ovulation. Therefore these changes occur during the period corresponding to very early metoestrus. Division occurs in granulosa cells up to day four of metoestrus and in theca interna cells up to day seven (Donaldson and Hansel, 1965; MacNutt, 1924). Alila and Hansel (1984) characterized the origin of large and small luteal cells using specific antibodies raised against granulosa and theca cells. These authors found that early in the oestrous cycle large luteal cells are derived from the granulosa cells of the follicle; whereas small luteal cells derive from theca interna cells. The granulosa derived cells then contribute to the obliteration of the cavity left by the follicle by enlarging, reaching their maximum size by day seven (Donaldson and

Hansel, 1965). Mitotic activity is also marked in the cells of the connective tissue and vascular endothelium, so that by day four of metoestrus vaso-factive cells have formed capillaries which are surrounded by fibroblasts of connective tissue (Gier et al., 1961; MacNutt, 1924). The small theca derived cells migrate from the peripheral areas and the thecal cores towards the large granulosa derived cells by moving along the trabeculae of connective and vascular tissue, thus intermixing with large luteal cells (Gier et al., 1961). The dividing small luteal cells are generally associated with trabecular areas of connective tissue whereas small luteal cells mixed with large luteal cells do not show division and appear as mature-sized cells with a foamy cytoplasm (Donaldson and Hansel, 1965).

2.3. The mature corpus luteum

Seven days after ovulation the cellular composition of the new CL appears more homogeneous as no more mitotic activity is taking place. The trabeculae of connective tissue are not as numerous as in the first few days but are still distinguishable. The homogeneity of the luteal tissue reaches culmination about day nine when small cells are found concentrated around connective and vascular trabeculae (Donaldson and Hansel, 1965). In their studies Alila and Hansel (1984) obtained strong evidence that by day nine of the cycle some of the large luteal cells appear to derive from the theca layers (see figure 1.1). Indeed dynamic changes in the proportions of small and large cells in the CL occur throughout the luteal phase suggesting that these cells undergo continuous differentiation (Hansel et al., 1987). Observations were made on enzymically dissociated luteal tissue, a method which involves inevitable and perhaps selective loss of cells, indicate that few large cells are found in the CL early in the cycle but their numbers increase with the age of the CL.

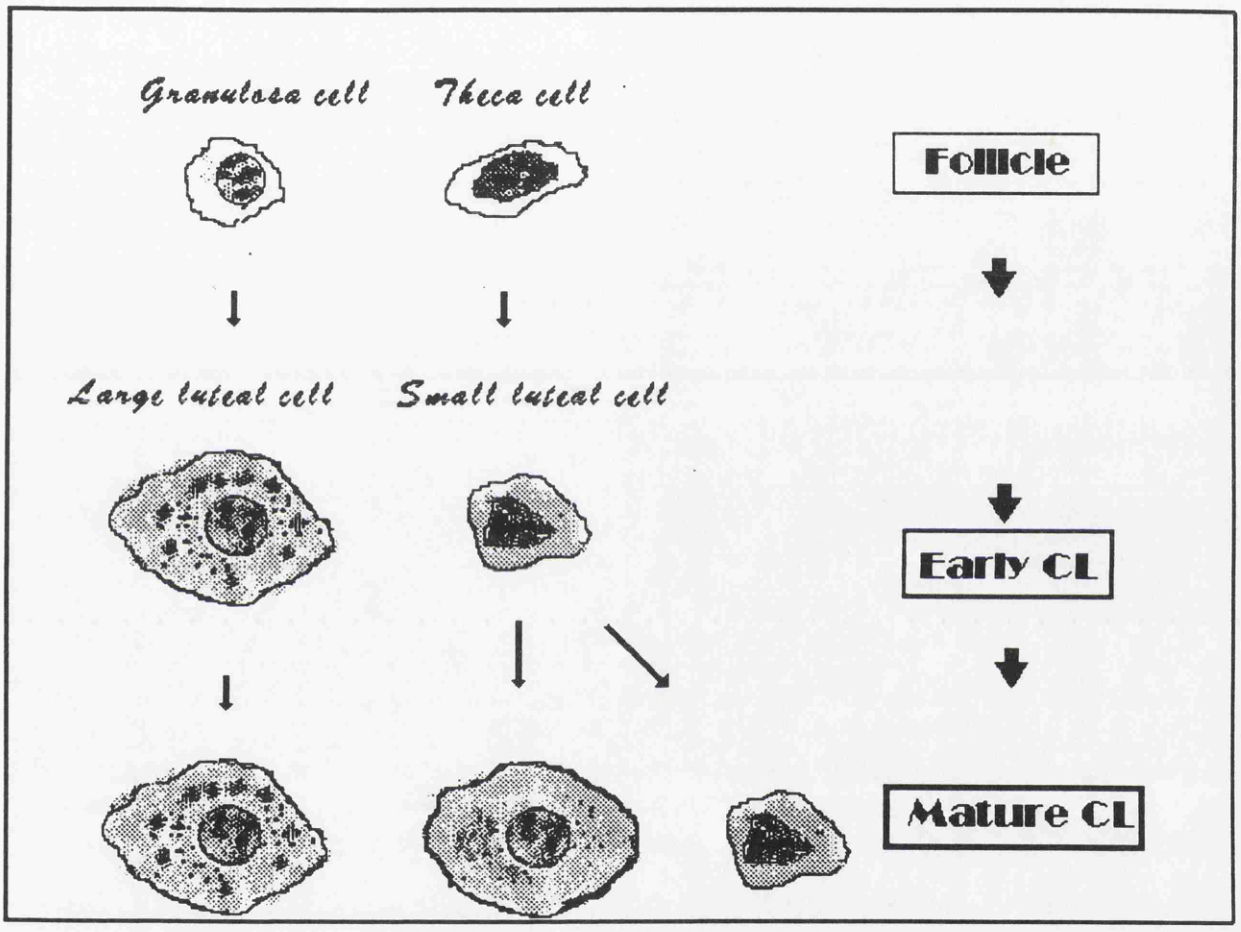


Fig. 1.1 Origin of bovine luteal cell-types

Generally there are fewer large cells per gramme of tissue during metoestrus and dioestrus than during gestation. Conversely there are fewer small cells during pregnancy than in the luteal phase of the cycle (Hansel et al., 1987).

Using morphometric analyses of luteal sections O'Shea et al. (1989) found significantly different numbers of large and small luteal cells despite accepting the fact that small luteal cells outnumber the large ones. They argued that enzymatic dissociation of cells may involve selective losses resulting in an erroneous estimation of cell numbers. However these authors used CLs from animals synchronized with a double injection of prostaglandins eleven days apart, a treatment reported to result in abnormal cell type proportions (Hansen et al., 1987). It is also unfortunate that O'Shea et al. (1989) carried their work only on CLs obtained on day twelve of the oestrous cycle. Whatever the case, there seems to be a general agreement as to the fact that the small cells outnumber the large ones whereas the latter occupy most of the volume of the luteal tissue.

At the ultrastructural level both small and large steroidogenic cells of the bovine CL have abundant smooth and occasional rough endoplasmic reticulum, large and polymorphic mitochondria with tubular cristae, Golgi apparatus and varying number of lipid droplets (Parry et al., 1980; Hansel et al., 1987). While Parry et al. (1980) treated large and small luteal cells as a single type and recognized no ultrastructural differences, Koos and Hansel (1981) enumerated a few criteria for each cell type. The small cells usually occur in clumps held together by interdigitating microvilli or junctional complexes and have peripherally located and deeply lobulated nuclei with densely staining nucleoplasm and heterochromatin lining the nuclear envelope. Their mitochondria are arranged in an arc opposite to the nucleus. The large cells of the bovine CL of the cycle have large central nuclei with a distinct nucleolus

and dispersed chromatin. Mitochondria more abundant than in the small cells, surround the nucleus and are almost absent from the cell periphery. Their membrane surfaces are highly convoluted and contain extensive microvillous projections. Some of the large luteal cells exhibit electron dense granules of various sizes in the cytoplasm which were shown to contain oxytocin (Fields et al., 1992). The cytoplasm of small cells does not contain such granules (Koos and Hansel, 1981; Hansel et al., 1987). These granules also allow distinction between the two population of large luteal cells, ie. those originating from granulosa cells and those originating from the theca-derived small cells. Even at the level of light microscopy many of the granulosa-derived large cells appear darker and more granular. On examination by electron microscopy the darker cells clearly contain more mitochondria and electron-dense granules in the cytoplasm. The mitochondria in these dark cells are small, elongated and diffusely distributed throughout the cytoplasm. The lighter staining cells have larger rounded mitochondria and fewer granules. Large cells derived from granulosa cells seem to disappear during pregnancy. This disappearance coincides with a drop in oxytocin secretion suggesting that these cells are responsible for this (Hansel et al., 1987).

2.4. The regressing corpus luteum

Signs of degenerative changes in the CL can be seen using histological means. As the CL ages a thickening of the walls of arteries occurs. This process continues until, at 21 days after ovulation the lumina of many arteries are obliterated (Donaldson and Hansel, 1965). In the luteal cells, the first degenerative signs are a decrease in the stippling of the cytoplasm and rounding of the cell outline. Vacuolation occurs around the periphery whereas in actively secreting cells vacuoles are observed evenly distributed in central areas. The

first nuclear change noted is a lighter staining and a loss of a prominent nucleoli. The nuclear membrane becomes less distinct. Together with the condensation of the cytoplasm the nucleus shrinks, stains darkly and appears pyknotic. At day 21 only a few large cells are distinguishable and no small cells can be seen (Donaldson and Hansel, 1965).

3. Corpus luteum function and regression

Under certain circumstances, the presence of a CL thought to be functional after manual appreciation of its size coupled to oestrus detection, is not reflected in circulating plasma progesterone which is below the functional levels. Such structures are either CLs which have a normal life-span but secrete lower levels of progesterone or CLs which have a shorter life-span than normal (see review by Hunter, 1991). Therefore it is essential to understand the mechanisms controlling both progesterone secretion and its regulation as well as structural formation of the CL.

At the cellular level the structural changes which appear during luteinization can be described as a mere accentuation of the changes that are induced by gonadotrophins in both granulosa and theca cells during follicular maturation. These include acquiring organelles which are common to steroid secreting cells. On the other hand functional luteinization ie. progesterone synthesis, implies blocking of aromatization in granulosa cells followed by inhibition of androgen synthesis in theca cells so that the pathway of steroidogenesis is interrupted at the level of progesterone synthesis (Henderson and Moon, 1979). These changes occur almost immediately after the LH surge. Moreover *in vitro* luteinization has been induced following continual exposure of granulosa cells to LH indicating that this hormone is the main factor responsible for CL formation (Amsterdam and Rotmensch, 1987).

3.1. Quantitative aspects of progesterone synthesis

Although studies on separated small and large luteal cells *in vitro* suggest that progesterone synthesis is a function of both types of cells, the fact that the CL is made up of two steroidogenic cell types of disparate origin and distinct ultrastructures raises the question as to the existence of functional differences between these types. *In vitro* the small luteal cells produce less basal progesterone (without added LH) than the large cells. However the small cells are at least six times more responsive to added LH than the large cells (Koos and Hansel, 1981; Ursely and Leymarie, 1979).

Fitz et al. (1982) showed that the small luteal cells in the sheep CL contain the majority of LH receptors (33260 per small cell compared with 3074 per large cell), while the large cells contain the majority of the PGF2α receptors (68143 per large cell and 2115 per small cell).

Large cells do respond to LH but only when high doses are used. Hansel et al. (1987) claimed that although relatively unresponsive to added LH, the large luteal cell still produce 35 times as much progesterone per cell as the small luteal cells. However these workers admit that such calculations, being based on results from enriched fractions of large and small cells, may bear little relationship to production *in vivo*, because of possible interactions between these two cell types and also other non-steroidogenic cells in the CL *in vivo*. Using incubation of large luteal and small luteal cells separately and after recombination, Lemon and Mauléon (1982) demonstrated an interaction between pig large and small luteal cells in the synthesis of progesterone. Their results suggested that some product(s) of the small luteal cells was able to enhance progesterone production by the large luteal cells. Rodgers et al. (1985) were not rewarded in their attempts to obtain similar results in the ewe.

In the cow attempts to demonstrate such interaction between large and small luteal cells have also been unsuccessful (Ursely and Leymarie, 1979), although preliminary data with highly purified large and small cells suggest that these two cell types synergize during mid-dioestrus but do not during CL regression (Hansel et al., 1991).

Owing to the continuous change in the proportions of these two types of cells during the life-span of the CL the question as to which of the large or small luteal cells produces most of the progesterone secreted by the CL remains controversial. The fact that large luteal cells synthesis 35 times as much progesterone as do the small ones, suggests that the large cells produce most of the progesterone from the CL; and that small luteal cells which are six times more responsive to LH than large luteal cells and largely outnumber them suggests that they are the main source of progesterone.

3.2. Cell signalling and control of progesterone synthesis

Recent reports confirm that albeit both cells secrete progesterone *in vitro*, it appears that the way this function is controlled is indeed specific to each type (Hansel et al., 1991; Wiltbank et al., 1991). The mechanism by which gonadotrophin hormones such as LH are known to induce their action involve binding of the hormone to an external receptor-site or first messenger present in the target cell (attached to the surface of the cytoplasmic membrane). This binding brings about the activation of a catalytic unit of a specific protein -the **G protein**- present in the cytoplasmic membrane which would then bind to an enzyme known as adenylate cyclase. As a result the latter is induced to convert adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP) which in turn causes the activation of cAMP-dependent protein kinase (Kinase A). The latter is an enzyme which phosphorylates proteins present in the cytosol

(mostly enzymes) and in so doing controls their activation (Schramm and Selinger, 1984; Alberts et al., 1989). The activation of protein kinase A by adenylate cyclase is usually the starting point for a cascade of enzyme phosphorylation the end of which would be, in the case of steroidogenic cells, the conversion of cholesteryl diesterase into its active form. This enzyme is known to convert cholesterol ester which is stored in lipid droplets into free cholesterol which on entering the mitochondria is metabolized into pregnenolone (Henderson and McNatty, 1975). The rate-limiting step in the synthesis of steroid hormone from cholesterol is considered to be the cholesterol side-chain cleavage reaction catalyzed by cytochrome P450 ($P450_{scc}$) of the inner mitochondrial membrane and the product of which is pregnenolone (Rodgers et al., 1986). In the rat granulosa cells, once the $P450_{scc}$ mRNA is induced by the LH surge, it is constantly maintained by the luteinized cells in the absence of gonadotrophins and is no longer regulated by cAMP (Oonk et al., 1989). If this is the case in the bovine, it would explain the relative insensitiveness of the large luteal cell to LH *in vitro*.

In the luteal cells the pregnenolone is transformed in a final step into progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD). In the rat the decrease of progesterone secretion during parturition is thought to be owing to a PGF2α-dependent blockade of gonadotrophic stimulus which in turn determines (1) the decrease in progesterone synthesis and (2) the induction of luteal 20α-hydroxysteroid dehydrogenase (20α-HSD) which converts progesterone into its inactive metabolite, 20α-hydroxyprogesterone (Bussmann, 1989).

This biochemical pathway is therefore first controlled by the activation of protein kinase A via cAMP mobilization in the cellular cytoplasm following binding of LH to its receptor. However in 1984, Shemesh et al. found that

bovine placental cells secrete progesterone by a calcium-dependent mechanism. In the same year Nishizuka (1984) summarized information concerning a new second messenger system by which hormones pass information from the surface to the interior of the cell. This system appears to be present in a wide range of tissue and is thought to be involved in almost all types of cell responses including synthesis and release of various hormones and neuro-transmitters (Nishizuka, 1984). The system involves two pathways: (1) intracellular calcium mobilization and (2) protein kinase C activation. These two steps are activated following binding of the first messenger, eg. a hormone, to its receptor which results in hydrolysis of the membrane-bound phosphatidylinositol-4,5-bisphosphate (PIP_2) yielding two compounds, diacylglycerol (DG) and inositol-1,4,5-triphosphate (IP_3). The former compound (DG) activates protein kinase C the latter (IP_3) mobilizes intracellular calcium from its sequestering compartment. Both calcium and DG act synergistically in evoking the cellular response. During this process DG is converted into arachidonic acid which is the precursor of all of the prostanoids. These compounds are known to have both luteolytic and luteotrophic effects on the bovine CL and it is now apparent that a such mechanism exists in the luteal cells in the bovine (Hansel and Dowd, 1986; Brunswig et al., 1986). Recently separation of the two main cell types of the CL using the more efficient flow cytometry technique and their subsequent culture in presence of a protein kinase C activator (phorbol ester), and calcium ionophore resulted in increased progesterone production by the small luteal cells only, suggesting that this newly described mechanism was confined to this cell-type (Alila et al., 1988). The response of small luteal cells as measured by progesterone synthesis was known to be primarily controlled by cAMP, intracellular concentrations of which are elevated in response to LH, (Hoyer et al., 1984). However the new second messenger system (Ca^{2+}-phosphoinositol-

protein kinase C second messenger) described by Nishizuka (1984) does exist in the bovine small luteal cell perhaps as a second alternative pathway to the cAMP-system (Hansel et al., 1987). The fact that the stimulatory effect of phorbol esters on progesterone production is rapidly desensitized with chronic exposure while their effect on prostanoid production is not desensitized within a 24-hour period is an advocate for the possibility of involvement of this system in luteolysis (Hansel et al., 1991). Further support to this concept is provided by the results of Plate and Condon (1984) who showed that while PGF2α has no effect on progesterone production by luteal cells during the first 24 hours of incubation, it significantly inhibited LH-stimulated progesterone synthesis during subsequent days of culture. The receptor through which this new pathway is stimulated is suspected to be a PGF2α receptor. Davis et al. (1987a, 1987b) showed that in addition to LH, PGF2α also stimulates PIP_2 hydrolysis in the small luteal cells, a process that produces DG which activates protein kinase C but also supplies prostanoids metabolism with arachidonic acid.

These prostanoids which include PGF2α are thought to target the large but not the small luteal cells for two main reasons. First PGF2α but not protein kinase C-activators enhance LH-stimulated progesterone synthesis in the small luteal cells. Secondly, although PGF2α has no effects on basal progesterone synthesis in the large luteal cells, it inhibits LH-stimulated progesterone (Alila et al., 1988). However the information available is far from complete and so the interaction between the two steroidogenic cell types of the CL will not be fully understood until accurate enzyme and receptor quantifications are achievable in both cells incubated with various treatments.

The mobilization of Ca^{2+} from either its sequestration compartment in the cytosol by IP_3 or the opening of Ca^{2+} channel in the cytoplasmic membrane which allows penetration of extracellular Ca^{2+} and raises intracellular

concentration of this cation are both critical to both progesterone synthesis and cell viability (Wiltbank et al., 1991). Again differential responses between the large and the small luteal cell are shown regarding intracellular Ca^{2+} levels. Small cells which contain relatively low resting intracellular Ca^{2+} levels, are extremely sensitive to LH, and are able to produce basal levels of progesterone in the absence of Ca^{2+} in the medium. However progesterone synthesis stimulated by LH, PGE-2, 8-bromo-cAMP or PGF2α requires calcium ions and the effects of calcium ion removal appear to be exerted distal to cAMP generation in the small cells. In contrast, large cells, which contain high levels of resting Ca^{2+} and are relatively insensitive to LH, require calcium ions for basal and LH- and forskolin-stimulated progesterone production and the effect of calcium ion removal is exerted proximal to the generation of cAMP in these cells (Hansel et al., 1991). Thus the differences between the large and the small luteal cells appear in almost every aspect of their function.

Early studies established the fact that, in the cow as in several other species, LH is not the only luteotrophic factor *in vitro*. Progesterone synthesis by small luteal cells in response to human chorionic gonadotrophin (hCG) stimulation has been reported by Bourdage et al. (1984). Studies *in vitro* also suggest the possibility that other hormones including prostaglandins, oestradiol, serotonin and oxytocin, may have physiological stimulatory actions on the secretion of progesterone by luteal cells. Both PGE-1 and PGE-2 were shown to have a stimulatory effect on basal progesterone synthesis (i.e. without added LH) by the small luteal cells of the ewe but not by the large (Fitz et al., 1984). In the cow PGE-2 was shown to have a stimulatory effect on progesterone synthesis by dispersed luteal cells (Shelton et al., 1990). However these authors did not exclude possibilities of PGE-2 having also some luteolytic effect. Prostacyclin (PGI-2) has been shown to play a luteotrophic role (Hansel and Dowd, 1986).

Both oxytocin (Battista and Condon, 1986) and serotonin (Tan et al., 1982) were shown to enhance progesterone synthesis in bovine luteal cells. However Milvae and Hansel (1983) did describe the effect of oxytocin on progesterone synthesis by luteal cells as insignificant.

3.3. Regression

While LH is essential for normal luteal function in ruminants, the decline in progesterone preceding oestrus cannot be explained by any change in circulating concentrations of LH. On the other hand one may question whether spontaneous luteolysis results from the inability of LH to bind to the luteal cell. It has been shown that as luteolysis approaches there is a decrease in both serum progesterone and LH-receptors. Even so, the decrease in progesterone precedes the loss of LH receptors and therefore receptor loss is probably not the initial steps in luteolysis (Auletta and Flint, 1988).

In sheep and cows luteolysis is attributed to the uterine synthesis and secretion of PGF2α and its subsequent transport to the ovary via a local counter-current mechanism which although is well established in the ewe, remains questionable in the cow in the light of the conflicting results obtained so far (Hixon and Hansel 1974; Milvae and Hansel 1980).

PGF2α receptors have been identified in large and small luteal cells of sheep (Fitz, 1982) and cows (Rao, 1975). The mechanisms controlling PGF2α synthesis and release from the endometrium are as yet unknown. Systemically administered oestradiol, given during the mid luteal phase causes premature luteolysis in both sheep (Genbenbach et al., 1977) and cows (Greenstein et al., 1958). This effect is actually used when combining oestradiol to progestogens for the purpose of administration to sheep and cattle as a means of grouping oestrus. Destruction of ovarian follicles, the prime source of oestrogens by X-

ray irradiation prolongs luteal function (Villa-Godoy et al., 1981). Oestradiol given systemically or locally causes uterine secretion of PGF2α with an ensuing decrease in progesterone and luteal regression (Caldwell et al., 1972). The latter observation suggests an oestrogen-PGF2α interaction during luteolytic process in ruminants.

Oxytocin injections given during a critical period early in the oestrous cycle (day 2-6) result in inhibition of CL development and shortening of the oestrous cycle. This effect requires the presence of the uterus but is not accompanied by any change in LH pulsatile secretion suggesting the possibility of an oxytocin-PGF2α combined mechanism to cause this anti-luteotrophic action on the early CL (Hansel and Dowd, 1986). There is also a widely held view that oxytocin released from the CL reaches the uterus where it causes the release of PGF2α which in turn causes luteal regression. However oxytocin is secreted by granulosa derived large cells of the bovine CL and its synthesis was shown to begin as early as the critical period when exogenous oxytocin would hamper luteal development. Moreover exogenous PGF2α is incapable of causing premature luteal regression during early luteal phase and exogenous oxytocin does not have any effect on the CL at other stages than the early critical period (Hansel and Dowd, 1986).

To complicate matters, exogenous oxytocin causes the release of PGF2α into the uterine vein in the cow and Milvae & Hansel (1980) were unable to show that this release of PGF2α was reflected in concurrently collected ovarian arterial blood samples. Thus it would appear that PGF2α in the cow may have a rather systemic action. Unlike the ewe in which 99% of injected PGF2α is metabolized in a single passage through the lungs, substantial amounts of injected PGF2α pass through the lungs of the cow without metabolic breakdown (Davis et al., 1984). It is postulated (Auletta and Flint, 1988) that an initial

release of small quantities of PGF2α from the uterus results in release of oxytocin and reduction in progesterone secretion by large luteal cells. The decreased circulating progesterone allows an increase in oestrogens which in turn causes an increase in uterine oxytocin receptors. The latter interact with oxytocin secreted by the large luteal cells to stimulate further release of PGF2α sufficient to inhibit local ovarian blood flow and cause regression of the small cells (Auletta and Flint, 1988). Such a mechanism would provide an explanation for the observation that major release of PGF2α follows rather than precedes the commencement of luteolysis and for the coincident fall in progesterone and rise in uterine oxytocin receptor-sites. Two hypothesis have been proposed to explain the action of PGF2α:

(1) one or more direct actions on the luteal cells and/or

(2) a decrease or redistributed blood flow away from the CL at the appropriate time in the nonfertile cycle.

In the cow, PGF2α stimulates progesterone in small cells but has no effect on basal (without added LH) progesterone synthesis in large cells even though it inhibits LH or cAMP-stimulated progesterone synthesis in these cells. The ability of PGF2α to inhibit forskolin or 8-Bromo-cAMP-stimulated progesterone in large luteal cells suggests that its effect occurs at some step distal to the intracellular generation of cAMP (Plate and Condon, 1984). It has been suggested that PGF2α has a direct inhibitory effect on ovine luteal 3β-hydroxysteroid dehydrogenase (Hoppen and Findlay, 1976).

The current view of the possible mechanism of the direct effect of PGF2α on the luteal cell was summarized by Hansel et al., (1991). These authors suggested that the chronic activation of Ca^{2+}-protein kinase C system results in desensitization of LH-induced progesterone synthesis and release of luteolytic prostaglandins and Ca^{2+}.

V. AIM OF THE STUDY

This thesis describes initial investigations using clinical ultrasound and endocrinological methods, of the role of the CL in the outcome for synchronization of oestrus in dairy cattle in the field. This resulted in detailed studies of the formation of CLs in cycling cows by monitoring behavioural endocrinological and clinical changes in a small group of cattle. These preliminary studies led to the detailed investigations of CLs of different stages of the cycle by histological, histochemical, electron microscopy and cell culture means. Subsequently similar studies were carried out on CLs obtained from cattle following induction of oestrus by an injection of a PGF2α-analogue during two different phases of the cycle. Finally the response to a GnRH analogue given during mid-dioestrus was assessed in terms of luteal formation and function.

CHAPTER TWO

FIELD TRIAL

I. INTRODUCTION

The development of intensive dairy farming systems has imposed new commitments on farmers, including aiming for the production of a calf per cow per year. Our increasing understanding of the endocrinological control of the oestrous cycle in domestic animals now allows manipulation of this aspect of bovine reproduction.

The major applications for synchronization of oestrus in the bovine has been in the management of dairy heifers and has usually involved PGF2α analogues as until recently, at least in the United Kingdom, synchronization using progesterone or progestogens has only been commercially available by means of the intravaginal releasing device. This method presents technical difficulties in heifers. Recently a product utilizing a prolonged progestogen-releasing device not involving the vaginal route and combined with an injection of oestradiol, "Crestar" (Intervet UK Ltd) has been developed (Aguer et al., 1987).

The use of both PGF2α and progestogens for synchronization of oestrus are associated with manipulation of the luteal phase of the cycle. Different mechanisms are involved; either lysis of the luteal structure in the case of PGF2α and its analogues resulting in the return of the animal to oestrus, or administration of progestogens inducing a period of artificial progesterone dominance (Scanlon et al., 1972; Lauderdale et al., 1973; Cooper, 1974; Sreenan and Mulvihill, 1975).

Each of these approaches must have an effect on the integrated factors associated with formation and function of the CL (reviewed by Odde, 1990). There is now evidence that treatments such as cloprostenol (a PGF2α analogue) can result in the formation of abnormal luteal structures in the bovine although this effect has been shown so far to occur mainly in *Bos Indicus* breeds (Hansen

et al., 1987; Hardin and Randel, 1982).

During a recent field trial, the ovarian response to a progestogen/oestradiol combination (Crestar) and a PGF2α analogue (Prosolvin) as well as the subsequent pregnancy rates following a fixed-time single artificial insemination (AI) were compared. Parameters including the relationship between plasma progesterone concentration and ovarian structures as determined by ultrasound were recorded. Growth as indicated by weight gain was also taken into consideration. In these preliminary studies, it was hoped to identify the changes in the various forms of luteal tissue present in the ovaries prior to and during the treatment period.

II. MATERIALS AND METHODS

1. Animals used

Four separate groups of Friesian cross Holstein dairy heifers on two Scottish Agricultural College farms were allocated in pairs to one of two treatments such that within each group there were pairs of animals of comparable weight and age (table 1.1).

All the heifers were at least 13 months of age, with a range in weight of 280 to 380 kg at the beginning of the experiment and they were weighed at regular intervals, from the onset of treatment to approximately 80 days after AI.
Each group was fed an ad libitum diet of grass or grass/clover silage supplemented with a small quantity (0.5-1 kg) of concentrate daily.

2. Treatments

2.1. Prosolvin regime

7.5 mg of luprostiol (a synthetic PGF2α analogue) in 1 ml of Prosolvin (Intervet UK Ltd.) was injected intramuscularly on two separate occasions ten days apart.

2.2. Crestar regime

A 2.5 cm silastic implant containing 3 mg of norgestomet (a progestogen) was inserted subcutaneously into the dorsal surface of the ear using an implant applicator (Intervet France Ltd). In addition a 2 ml injection containing 3 mg norgestomet and 5 mg oestradiol valerate was administered intramuscularly, at the time of implantation (Crestar, Intervet UK Ltd). Nine days later, after prior cleansing with an antiseptic solution, the implant was removed by incising the skin at one of its extremities and pressing it out.

Table 1.1 Grouping of cattle, number per treatment and AI dates.

Farm	Group	Number/treatment group	Date of AI
Crichton	*1*	11	Nov. 23, 1989
	2	8	Dec. 30, 1989
	3	9	Dec. 30, 1989
College	*4*	9	Nov. 17, 1989

3. Insemination

Semen from two proven Friesian cross Holstein bulls was used. One bull was used on each farm. Each pair of heifers was inseminated by the same inseminator. Fixed-time AI was carried out 48 hours after removal of the implant for Crestar-treated animals and 72 hours after the second injection for Prosolvin-treated animals. Subsequently any returns were rebred by either AI or natural service.

4. Ultrasound examination

An Aloka 210DX11 ultrasound machine incorporating a 7.5 MHz linear array probe was used for examination of ovarian structures by the method of Logue (1990). Prior to every scanning session the rectum was emptied and ovaries were located and held between the middle fingers of the operator's left hand. The transducer was then introduced taped to a plastic rod and guided from outside with the right hand. At least one picture of each ovary was taken using an instantaneous Sony printer irrespective of whether or not a CL was identified. Several views of it were taken when a CL was present. The criteria used to identify follicles, CLs or ovarian stroma are shown in table 1.2. CL size was determined by recording two dimensions, ie. height and width, using callipers. Only the largest dimension is presented as an estimation of the size of the CL. Each CL was then given a score for the echogenicity and the uniformity of its appearance. Table 1.3 describes the features considered for each score. See also plate 1.1.

Table 1.2 Differentiating features of ovarian structures on ultrasound.

STRUCTURE	APPEARANCE ON ULTRASOUND
Corpus luteum	Echogenic area with grey to grey-black image and diameter of 1.6-3.0 cm. Some with a non-echogenic (dark) area within the luteal tissue indicating the presence of a fluid-filled lacuna.
Follicle	Circumscribed non-echogenic areas with defined thin borders.
Ovarian stroma	Echogenic and non-homogenous areas of distinct white and grey pin-points.

Table 1.3 Scoring and differentiating features of the various types of luteal structures on ultrasound.

SCORE	FUNCTIONAL STATUS	APPEARANCE OF THE LUTEAL STRUCTURE ON ULTRASOUND
+++	Fully functional CL.	Structure with defined outlines containing diffuse distribution of black and grey pinpoints with predominance of the grey ones. The overall appearance is homogenous. Size >2.0 cm.
++	Indefinite functional status.	More accentuated predominance of grey pinpoints than in (+++), arranged in dense cores so that the whole section appears less homogenous. A few white pinpoints present increase the echogenicity. Outlines of the section are less defined than in (+++). Can be associated with either a large or a small size.
+	Non-functional CL.	As in (++) but more white pinpoints resulting in more echogenicity than in (++). Size <2.0 cm.

Plate 1.1.

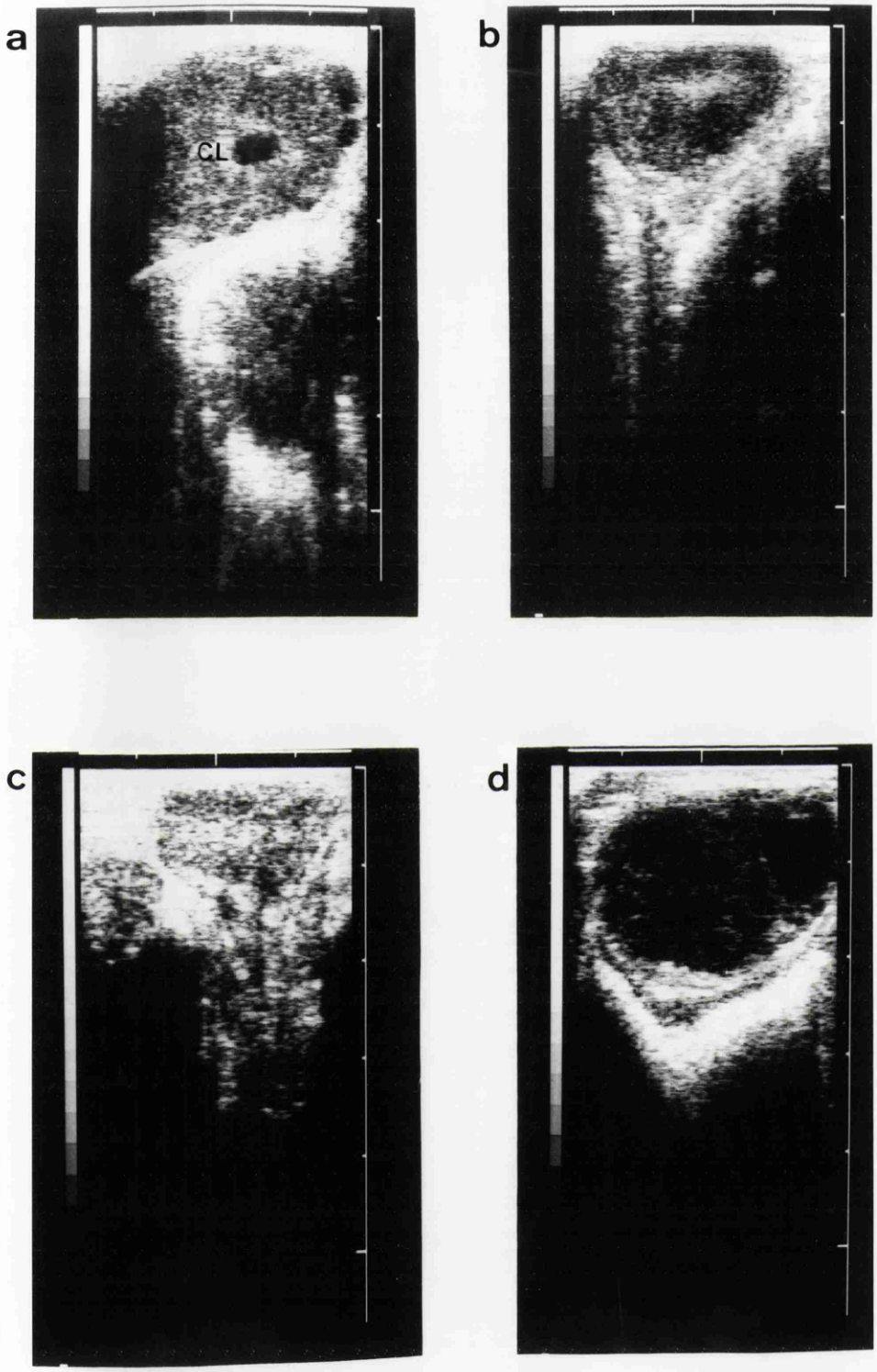
An example of each category of luteal tissue as they appeared on ultrasound is shown.

a) Typical appearance on ultrasound of a CL scored as (+ + +). Most of the time this appearance was associated with a high circulating plasma progesterone profile.

b) Appearance of a CL scored as (+ +). Some of these structures were not functional, others were.

c) Ultrasound appearance of luteal structure scored as (+). This structure could be either regressing or forming and was hardly ever associated with a raised peripheral progesterone concentration.

d) Unusual ultrasonic appearance of luteal structures scored as (?). The dark appearance of the tissue associated with a large size could be due to lack of one of the tissue components, eg. connective tissue.


a
CL
b
c
d

5. Pregnancy diagnosis

Animals were scanned twice for pregnancy using a sector scanner (Vet Scan 2 BCF Technology Ltd). In those animals in which unobserved mating occurred, a service date was deduced from calculations of ultrasound measurements of foetal crown-rump length and head and trunk diameter (White et al., 1985).

6. Blood sampling

Blood samples were collected by jugular venepuncture using heparinized Vacutainer glass tubes and 20-gauge Vacutainer needles (Becton Dickinson, England). Plasma samples were separated by centrifugation (10 minutes, 1000 x g) and stored at -20 °C in labelled plastic tubes until assayed.

7. Progesterone estimation

Plasma progesterone concentration was determined using a progesterone enzyme-linked immunosorbent assay kit (Ovucheck Bovine Plasma, Cambridge Life Sciences PLC.) as described by Eckersall and Harvey (1987). All samples were assayed in duplicate by adding 10 μl of standard or sample to wells precoated with an antibody followed by the addition of 200 μl of progesterone labelled with phosphatase alkaline. After 30 minutes incubation at room temperature the wells were washed 3 times with distilled water and 200 μl of a solution (*pH* 9.8) containing 1 M diethylanolamine and 0.5 mM magnesium chloride acting as the enzyme substrate was added and incubation continued for a further 30 minutes in the same conditions. The reaction was stopped by adding 100 μl of a solution containing 0.5 M dipotassium hydrogen orthophosphate and 5 mM ethylenediaminetetracetic acid (EDTA) (*pH* 10.0). A spectrophotometer (Titertek Multiscan Plus) was utilized to read the absorbance at 405 nm. The curve was plotted and results calculated using a

personal computer connected to the spectrophotometer and programmed for calibration of data from ELISA assays (Titersoft, Flow Laboratories). Cross-reactivity with steroids other than progesterone has been calculated by the manufacturer and was found to equal 66, 16, 4.5, 3.3 and 3.0% with 11-α-hydroxyprogesterone, 5-pregnan-3-β-ol-20-one, 5-β-pregnan-3,20-dione, 5-α-pregnane-3,20-dione and deoxycorticosterone acetate respectively and was insignificant (< 1%) with other steroids. Sensitivity was 0.5 ng/ml. The interassay coefficient of variation calculated after assaying the same control sample in 16 assays was 12.34% at 4 ng/ml. The intra-assay coefficient of variation estimated by assaying the same sample in 10 replicates was 11.3% at 4 ng/ml. A concentration greater than or equal to 2 ng/ml was taken as indicative of a functional CL.

Table 1.4 gives a summary of the protocol for each treatment.

8. Response to Prosolvin injections

A raised concentration of plasma progesterone (> 2 ng/ml) on the day of the first Prosolvin injection followed by a drop to less than 2 ng/ml two days afterwards was considered as a positive response by the luteal structure to the first injection. Responsiveness of the CL to the second injection was related to whether or not the animal became pregnant to the first service, or if not pregnant, when the circulating plasma progesterone concentration was low three weeks after AI. However it was not possible to determine whether a CL responded to the second injection when the animal was not pregnant and plasma concentration of progesterone were found to be high three weeks after AI. This was because of the possibility that embryonic death could occur around or after the third week following AI. In addition it was not certain whether a CL, identified by ultrasound, responded to the second injection when the animal

did not conceive, and had a low progesterone both on the day of second injection and three weeks after AI.

9. Statistical analysis

Data were analysed using a computer program (Minitab Release 7.1, Minitab Inc, State college, PA 16801, USA). Frequency distribution of conception rate to the synchronized oestrus between groups was tested by computation of *squared-qui*. Means were compared by *Student's t-Test*. A *P*-value less than 0.05 was taken as a significant result.

Table 1.4 Experimental design for both Crestar- and Prosolvin-treated heifers.

DAYS	CRESTAR GROUP	PROSOLVIN GROUP
-23	Bled Scanned Weighed	
-13	Bled Scanned Weighed	Bled Scanned Weighed **First injection**
-11	Bled Scanned **Crestar In**	Bled Scanned
-3	Bled Scanned	Bled Scanned **Second injection**
-2	**Removal of Crestar implant**	
0	AI (48 *hours*)	AI (72 *hours*)
20, 21 or 22	Bled Weighed	
35, 36 or 44	Scanned for pregnancy diagnosis	
70, 71 or 72	Scanned for pregnancy diagnosis Weighed	

Group 4 was first bled and scanned on day -13.

III. RESULTS

Table 1.5a.

Data are not available for some of the animals. Based on the progesterone results available 15 out of 23 heifers had a functional CL (> 2 ng/ml) and so were cycling. The remaining eight had a low plasma progesterone concentration. Despite this ultrasound indicated that in these animals luteal tissue was present and so they were cycling. There was good correlation between ultrasound scoring and progesterone concentrations in the majority that had results (19 out of 23). In the four in which there was not, animals with both raised and baseline levels of plasma progesterone did not relate to the ultrasound findings (Nos. 468, 575, 577 and 549).

Table 1.5a Ultrasound findings and plasma progesterone concentration in heifers prior to treatment with Prosolvin.

ID. No.	Day -10		
	CL on ULSD		P_4 (*ng/ml*)
	Size (*cm*)	Score	
458	2.9	?	NA
437	1.3	+	1.6
438	2.5	+++	8.5
459	2.4	+	NA
461	1.3	++	<0.5
574	2.1	++	<0.5
14	NA	NA	NA
25	NA	NA	NA
19	NA	NA	NA
21	NA	NA	NA
28	NA	NA	NA
439	1.9	++	<0.5
468	2.2	?	2.8
557	1.6	+++	2.8
573	1.4	+	<0.5
432	2.5	+++	5.2
446	2.3	++	4.9
464	2.6	+++	3.7
20	NA	NA	NA
15	NA	NA	NA
575	2.2	+++	1.7
577	1.7	+	3.1
434	2.9	+++	3.8
442	2.7	+++	5.5
447	2.3	+++	9.6
450	2.7	+++	NA
456	2.7	+++	NA
474	2.5	+++	5.9
457	2.8	+++	NA
24	NA	NA	NA
33	NA	NA	NA
546	2.6	+++	2.4
549	2.0	+++	1.7
567	2.1	++	2.4
552	2.5	+++	3.8
561	2.4	+++	>10
572	2.2	++	0.8

ID. No. = heifers' identification number. P_4 = plasma progesterone concentration. ULSD = ultrasound. NA = data not available. Day 0 = day of first Prosolvin injection. Those CLs scored as "?" were structures which did not correspond to the criteria described in table 1.3 as they had little echogenicity even though they were of a size equal to that of a functional CL.

Table 1.5b.

Heifers were examined on two occasions prior to the insertion of the implant. In some animals data are available on one occasion only. There was good correlation in the majority of the animals between ultrasound scoring and progesterone findings (50 out of 63). In three of the animals with results (Nos. 431, 449, 462) plasma progesterone concentrations were less than 2 ng/ml at the two samples taken at a ten day interval prior to treatment (the highest values obtained ranging between 1.3 and 1.8 ng/ml). Another heifer (No. 470) appeared to be in anoestrus as plasma progesterone was low throughout this period. Added to these four, is heifer No. 587 for which no data are available on day -2. On the day of implantation (day 0) this animal had still no functional CL present (see table 1.7b).

Table 1.5b Ultrasound findings and plasma progesterone concentration in heifers prior to treatment with Crestar.

ID. No.	Day -12			Day -2		
	CL on ULSD		P_4	CL on ULSD		P_4
	Size (*cm*)	Score	(*ng/ml*)	Size (*cm*)	Score	(*ng/ml*)
12	NA	NA	NA	3.5	+++	2.5
13	NA	NA	NA	2.6	+++	>10
16	NA	NA	NA	2.0	+++	2.6
27	NA	NA	NA	2.2	+++	2.5
551	2.0	++	<0.5	2.4	+++	3.2
555	2.5	+++	1.5	2.7	+++	2.9
560	1.7	+	<0.5	2.5	+++	3.1
563	no CL	-	<0.5	2.7	?	3.1
566	1.8	+	<0.5	2.7	+++	3.0
569	1.8	++	<0.5	3.5	+++	2.9
570	NA	NA	NA	2.6	+++	3.2
586	2.5	+++	3.0	2.3	++	<0.5
431	2.3	+++	1.8	1.5	++	<0.5
435	2.2	++	9.2	2.3	+++	3.3
440	2.7	+++	3.0	2.1	++	<0.5
441	1.4	?	<0.5	2.8	+++	5.2
443	2.9	+++	1.7	2.1	+++	6.0
444	2.3	++	5.3	2.0	++	>10
448	2.1	++	<0.5	2.5	+++	9.7
449	2.6	+++	1.6	2.2	?	0.9
451	2.3	+++	5.1	2.1	++	<0.5
455	2.3	+++	4.8	2.7	+++	4.3
460	2.2	++	NA	2.7	++	7.3
445	2.9	?	5.2	2.8	+++	7.1
466	2.2	++	<0.5	3.2	+++	>10
17	NA	NA	NA	1.9	?	<0.5
22	NA	NA	NA	2.4	++	3.1
29	NA	NA	NA	2.1	++	<0.5
26	NA	NA	NA	no CL	-	<0.5
23	NA	NA	NA	1.5	+	2.9
559	1.9	++	3.2	2.5	++	3.1
568	2.1	+++	2.8	1.7	++	<0.5
587	2.8	++	1.6	2.6	?	NA
430	no CL	-	2.6	no CL	-	<0.5
436	2.3	++	5.9	1.9	++	<0.5
462	2.7	+++	1.3	2.1	++	<0.5
470	no CL	-	<0.5	no CL	-	<0.5

ID. No. = heifers' identification number. P_4 = plasma progesterone concentration. ULSD = ultrasound. NA = data not available. Day 0 = day of Crestar insertion. Those CLs scored as "?" were structures which did not correspond to the criteria described in table 1.3 as they had little echogenicity even though they were of a size equal to that of a functional CL.

Part (i): Prosolvin group

Based on circulating plasma progesterone profiles at the beginning of the treatment (first Prosolvin injection), the heifers were divided into those with functional CLs and those without.

Table 1.6a.

Following the first injection plasma progesterone concentration remained above 2 ng/ml in four (Nos. 438, 461, 574 and 432) out of the 18 heifers. In two of these non-responsive heifers (Nos. 461 and 574), some decrease in both progesterone and CL scores and sizes on ultrasound occured following the first Prosolvin injection (day 2). In the remaining two, a slight increase in these parameters was noted. Note that ten of the heifers (Nos. 14, 25, 19, 21, 28, 439, 468, 461, 574 and 557) did not have a functional CL on the day of the second Prosolvin injection. Three of the heifers with a low progesterone concentration on the day of second Prosolvin injection conceived to the fixed-time AI. Of the eight heifers which had a high progesterone profile on the day of second Prosolvin injection five failed to conceive (Nos. 458, 573, 432, 446 and 464). One of these heifers (No. 458) had a low progesterone concentration on day 20-22 following AI.

Table 1.6a Details of circulating plasma progesterone concentration and ultrasound findings in Prosolvin-treated heifers with **a functional CL** present at the commencement of the treatment and subsequent pregnancy rate after fixed-time AI.

ID. No.	Day 0			Day 2			Day 10			Day 33-35*	Day 48-57**
	CL on ULSD		P_4 (ng/ml)	CL on ULSD		P_4 (ng/ml)	CL on ULSD		P_4 (ng/ml)	P_4 (ng/ml)	PD by ULSD
	Size (cm)	Score		Size (cm)	Score		Size (cm)	Score			
458	2.3	+++	6.9	no CL	-	<0.5	2.4	+++	3.4	<0.5	-
437	2.7	+++	6.0	1.9	++	<0.5	2.8	+++	3.2	4.9	+
438	2.4	++	3.0	2.5	+++	6.7	2.6	+++	>10	6.0	+
459	2.7	+++	6.2	1.7	+++	<0.5	2.9	+++	4.3	3.7	+
461	2.3	+++	3.5	1.9	++	2.6	1.7	++	0.7	3.3	-
574	2.5	+++	>10	2.0	++	2.9	1.9	++	1.1	1.3	-
14	2.9	+++	6.0	2.1	++	<0.5	2.2	?	1.2	2.8	-
25	2.4	++	2.9	2.0	++	<0.5	2.2	+	<0.5	<0.5	-
19	2.3	+++	4.4	2.2	++	<0.5	3.0	++	<0.5	0.7	-
21	2.5	+++	4.5	2.3	++	<0.5	2.5	++	0.8	6.8	+
28	2.4	+++	6.0	no CL	-	<0.5	2.7	?	0.6	7.0	-
439	2.7	+++	4.5	2.3	+++	<0.5	2.9	+++	1.6	<0.5	-
468	3.3	+++	4.3	1.8	+++	<0.5	2.1	+	0.9	5.3	+
557	2.7	+++	>10	2.5	++	<0.5	2.1	+++	<0.5	>10	+
573	2.7	+++	2.7	1.9	++	<0.5	2.6	++	2.1	3.1	-
432	2.0	++	2.3	2.4	++	4.2	2.6	+++	5.0	8.5	-
446	2.5	+++	7.7	2.3	++	<0.5	2.7	+++	2.9	6.4	-
464	2.3	?	7.0	2.1	+++	<0.5	2.6	+++	2.0	8.7	-

ID. No. = heifers' identification number. P_4 = plasma progesterone concentration. ULSD = ultrasound. PD = pregnancy diagnosis. Day 0 = day of first Prosolvin injection. Day 10 = day of second Prosolvin injection. (*) = day 20-22 after AI and (**) = day 35-44 after AI. Those CLs scored as "?" were structures which did not correspond to the criteria described in table 1.3 as they had little echogenicity even though they were of a size equal to that of a functional CL.

Table 1.6b.

This table describes the response to the Prosolvin treatment in heifers with no functional CL at the beginning of the treatment. It is worthy of note that eight of the 19 heifers comprising this group never had a progesterone concentration above 2 ng/ml during the treatment period (Nos. 24, 33, 546, 549, 567, 552, 561 and 572). In six of these heifers ultrasound indicated the presence of luteal tissue on the day of the first Prosolvin injection and two of them were scored functional according to their appearance. On the day of the second injection a CL was detected by ultrasound in one more heifer and only heifer No. 33 had no CL detected by ultrasound during the whole treatment period. Thirteen of this group of heifers conceived to fixed time AI, five of which were animals which did not have a raised progesterone on the day of the second Prosolvin injection. Note that three heifers (Nos. 575, 549 and 572) had no functional CL present on either day -10 (table 1.5a) or day 0.

Table 1.6b Details of circulating plasma progesterone concentration and ultrasound findings in Prosolvin-treated heifers with **no functional CL** present at the commencement of the treatment and subsequent pregnancy rate after fixed-time AI.

ID. No.	Day 0			Day 2			Day 10			Day 33-35*	Day 48-57**
	CL on ULSD		P_4 (ng/ml)	CL on ULSD		P_4 (ng/ml)	CL on ULSD		P_4 (ng/ml)	P_4 (ng/ml)	PD by ULSD
	Size (cm)	Score		Size (cm)	Score		Size (cm)	Score			
20	2.3	+++	0.6	1.7	++	1.2	3.0	?	2.4	<0.5	-
15	2.1	++	<0.5	1.7	++	<0.5	2.7	?	2.5	>10	+
575	no CL	-	<0.5	1.9	++	<0.5	2.6	++	2.6	3.1	+
577	1.7	++	<0.5	no CL	-	<0.5	2.7	++	3.6	>10	+
434	2.3	++	<0.5	2.3	+	<0.5	2.2	+++	3.2	1.0	-
442	2.3	++	<0.5	1.8	++	1.7	2.8	+++	6.7	NA	+
447	1.1	+	<0.5	no CL	-	1.3	2.8	+++	3.3	4.0	+
450	2.5	+	1.8	1.9	+	3.1	2.6	+++	2.1	5.3	+
456	2.1	++	<0.5	2.0	++	1.3	2.8	+++	4.0	5.2	+
474	2.6	++	1.1	2.0	+++	4.1	2.4	+++	3.3	5.7	+
457	1.9	++	<0.5	2.0	++	1.2	2.7	+++	>10	6.9	-
24	no CL	-	<0.5	no CL	-	<0.5	2.4	?	<0.5	>10	+
33	no CL	-	<0.5	no CL	-	<0.5	no CL	-	<0.5	1.3	-
546	2.4	++	<0.5	1.1	+	<0.5	2.3	++	0.7	>10	+
549	2.3	?	<0.5	2.1	++	<0.5	2.0	?	<0.5	>10	-
567	0.7	+	<0.5	1.8	++	<0.5	1.9	?	<0.5	8.5	+
552	2.1	++	1.4	2.5	++	<0.5	2.8	?	1.7	5.8	-
561	2.7	+++	1.5	2.2	++	<0.5	2.7	?	<0.5	>10	+
572	2.7	+++	1.4	1.9	++	<0.5	1.8	+	0.9	8.7	+

ID. No. = heifers' identification number. P_4 = plasma progesterone concentration. ULSD = ultrasound. NA = data not available. PD = pregnancy diagnosis. Day 0 = day of first Prosolvin injection. Day 10 = day of second Prosolvin injection. (*) = day 20-22 after AI and (**) = day 35-44 after AI. Those CLs scored as "?" were structures which did not correspond to the criteria described in table 2.3 as they had little echogenicity even though they were of a size equal to that of a functional CL.

Part (ii): Crestar group

Based on circulating plasma progesterone profiles at the beginning of the treatment (Crestar insertion), the heifers were divided into those with functional CLs and those without.

Table 1.7a.

This table describes progesterone and ultrasound findings during the experimental period for animals with a functional CL on the day of Crestar implantation. Out of the 25 animals in this group, four had a functional luteal structure present one day prior to the removal of the implant (Nos. 555, 444, 449 and 451). In heifer No. 555 ultrasound did not detect the presence of a CL whilst in the other three a CL of more than 2.0 cm diameter was shown. Of these four, one conceived to fixed time AI (No. 451). Note that two of the other three animals (Nos. 444 and 449) had a low progesterone profile around day 21 after AI. In this group 17 of the 25 conceived to the fixed time AI. Of the 21 animals which responded normally to the treatment only five did not conceive to fixed-time AI.

Table 1.7a Details of circulating plasma progesterone concentration and ultrasound findings in Crestar-treated heifers with **a functional CL** present at the commencement of the treatment and subsequent pregnancy rate after fixed-time AI.

ID. No.	Day 0			Day 8			Day 31-33*	Day 46-55**
	CL on ULSD		P_4 (*ng/ml*)	CL on ULSD		P_4 (*ng/ml*)	P_4 (*ng/ml*)	PD by ULSD
	Size (*cm*)	Score		Size (*cm*)	Score			
12	3.1	+++	3.9	no CL	-	<0.5	8.3	+
13	2.6	++	5.7	1.8	++	<0.5	>10	+
16	2.4	+++	3.9	1.4	++	<0.5	>10	+
27	2.1	+++	2.0	no CL	-	<0.5	5.4	+
551	2.6	+++	6.3	no CL	-	1.7	>10	+
555	3.0	++	5.9	no CL	-	2.7	7.8	-
560	2.8	+++	3.5	no CL	-	0.6	>10	+
563	2.4	+++	7.1	no CL	-	<0.5	>10	+
566	2.3	++	4.9	1.8	++	<0.5	>10	+
569	3.3	+++	4.0	no CL	-	<0.5	<0.5	-
570	2.4	+++	>10	no CL	-	0.6	>10	+
586	2.2	++	2.7	2.0	++	<0.5	<0.5	-
431	2.3	+++	3.3	2.4	+	<0.5	4.5	+
435	2.3	+++	4.9	2.0	+	<0.5	5.4	+
440	1.8	++	2.5	2.1	++	0.9	5.2	+
441	2.8	+++	4.8	1.4	++	<0.5	4.6	+
443	2.5	++	5.7	1.7	+	<0.5	<0.5	-
444	2.6	+++	7.8	2.2	++	5.3	<0.5	-
448	2.3	+++	6.0	no CL	-	<0.5	5.2	-
449	1.9	?	4.3	2.8	+++	4.1	1.1	-
451	2.8	++	3.8	2.3	+++	3.5	2.4	+
455	2.8	+++	5.2	1.8	++	<0.5	4.9	+
460	2.5	++	4.7	no CL	-	<0.5	2.6	+
445	2.5	+++	6.1	no CL	-	<0.5	9.2	+
466	3.2	+++	>10	no CL	-	NA	3.5	-

ID. No. = heifers' identification number. P_4 = plasma progesterone concentration. ULSD = ultrasound. NA = data not available. PD = pregnancy diagnosis. Day 0 = day of Crestar insertion. Day 8 = one day prior to removal of Crestar implant. (*) = day 20-22 after AI and (**) = day 35-44 after AI. Those CLs scored as "?" were structures which did not correspond to the criteria described in table 1.3 as they had little echogenicity even though they were of a size equal to that of a functional CL.

Table 1.7b.

This table describes the progesterone and ultrasound findings during the experimental period for animals with no functional CL on the day of Crestar implantation. In all heifers in this group, one day prior to implant removal, circulating plasma progesterone was less than 2 ng/ml. On the day of implant insertion ultrasound showed the presence of a CL in seven animals. Eight days later, in six of these heifers the CL was not found. In heifer No. 587 a CL was still present one day prior to implant removal and appeared larger and was scored higher than on day 0. In this animal progesterone was slightly raised on day eight as well (1.0 ng/ml). This animal did not conceive. In another three heifers (Nos. 26, 568 and 430) ultrasound showed no CL on day of implant insertion. Eight days later a CL was detected. On this occasion circulating plasma progesterone was undetectable in two of these three heifers but was raised to 1.0 ng/ml in the other one (No. 26). This heifer did not conceive the other two did.

Table 1.7b Details of circulating plasma progesterone concentration and ultrasound findings in Crestar-treated heifers with **no functional CL** present at the commencement of the treatment and subsequent pregnancy rate after fixed-time AI.

ID. No.	Day 0			Day 8			Day 31-33*	Day 46-55**
	CL on ULSD		P_4 (*ng/ml*)	CL on ULSD		P_4 (*ng/ml*)	P_4 (*ng/ml*)	PD by ULSD
	Size (*cm*)	Score		Size (*cm*)	Score			
17	2.1	++	<0.5	no CL	-	<0.5	<0.5	-
22	1.8	++	0.8	no CL	-	<0.5	>10	+
29	2.0	++	<0.5	no CL	-	<0.5	>10	+
26	no CL	-	<0.5	2.3	++	1.0	7.4	-
23	no CL	-	<0.5	no CL	-	<0.5	>10	+
559	2.0	++	0.5	no CL	-	<0.5	>10	+
568	no CL	-	<0.5	2.3	++	<0.5	>10	+
587	1.7	++	0.5	2.8	+++	1.0	0.7	-
430	no CL	-	<0.5	1.9	++	<0.5	4.7	+
436	2.2	++	1.7	no CL	-	<0.5	6.7	+
462	1.9	+	<0.5	no CL	-	<0.5	>10	+
470	no CL	-	<0.5	no CL	-	<0.5	5.6	+

ID. No. = heifers' identification number. P_4 = plasma progesterone concentration. ULSD = ultrasound. PD = pregnancy diagnosis. Day 0 = day of implant insertion. Day 8 = one day prior to removal of Crestar implant. (*) = day 20-22 after AI and (**) = day 35-44 after AI.

Table 1.8.

This table compares the fertility of the two treatment groups at the synchronized oestrus. Crestar treatment resulted in a higher proportion of pregnancies to the fixed-time AI than Prosolvin but the difference was not significant. Pregnancy rate to the fixed-time AI in animals with functional luteal tissue when treated with Prosolvin was significantly lower than in animals with no functional luteal tissue treated in the same way (33.3% *vs* 68.4% respectively; $P < 0.025$). This difference was not found significant ($P < 0.2$) when heifers with less than 2 ng/ml but more than 1 ng/ml of plasma progesterone concentration were considered as having a functional CL. No difference was found in pregnancy rate between the two groups of animals (68% *vs* 75% respectively) for Crestar-treated animals grouped in the same way with reference to luteal tissue.

Table 1.9.

This table presents the mean number of days open in both Prosolvin- and Crestar-treated animals. The mean number of "days open" (the interval between the initial treatment and either conception or, if the heifer did not become pregnant, the last mating in that group) was significantly less in the Crestar-treated group ($P = 0.025$). However animals treated with Prosolvin had a longer interval from the commencement of the treatment to the day of AI than animals treated with Crestar (13 *vs* 11) and so the results between the two groups are not significant.

Table 1.8 Pregnancy rate at the synchronized oestrus in both Prosolvin- and Crestar-treated heifers related to grouping of cattle according to their luteal status at the commencement of treatment.

Group	Number heifers per treatment		Number (and percentage) pregnant to synchronized oestrus	
	Crestar	Prosolvin	Crestar	Prosolvin
A	25	18	17(68)	6(33.3)*
B	12	19	9(75)	13(68.4)*
TOTAL	37	37	26	19
%	100	100	70.3	51.4

Group A = animals with a functional CL at the commencement of treatment
*Group B = animals with no functional CL at the commencement of treatment .*P<0.025.*

Table 1.9 Mean numbers of days open in groups of heifers treated with either Prosolvin or Crestar.

Group	Means (± SD) days open*		Corrected means (± SD) days open**	
	Crestar	Prosolvin	Crestar	Prosolvin
A	21.2 ± 17.4	40.9 ± 26.9	22.2 ± 17.4	39.9 ± 26.9
B	23.7 ± 25.7	28.4 ± 28.4	24.7 ± 25.4	28.3 ± 28.4
Total	22.0 ± 20.1***	34.9 ± 27.9***	23.0 ± 20.1	33.9 ± 27.9

**Heifers not pregnant at the end of breeding period were included as days open.*
***Note that the interval from initiation of the treatment to the day of AI is longer for Prosolvin- than Crestar-treated heifers (13 Vs 11 respectively). When this difference was corrected by assuming a similar interval for all animals (12 days) and number of open days were changed accordingly no significant difference was found between treatment groups.*
****Means computed without correction differed, P=0.025.*

Table 1.10.

This table compares the initial and the final pregnancy rates and presents the mean daily weight gain of the cattle grouped according to location. There was no significant difference in pregnancy rate over the eventual breeding period. Although there was no significant difference in mean daily weight gain between the treatments there was a highly significant difference *($P < 0.05$)* between heifers' groups. There was no significant difference between the live weight gains of those animals which became pregnant to first service and those which did not.

Table 1.10 Comparison of initial and final pregnancy rate and mean weight gains (kg/day) as related to farm groups in heifers synchronized with either Prosolvin or Crestar.

Group (location)	Number of heifers per treatment	Number pregnant to synchronized oestrus		Eventual number pregnant at the end of breeding season		Mean weight gain (*kg/day*)	
		Crestar	Prosolvin	Crestar	Prosolvin	Crestar	Prosolvin
1 (Crighton)	*11*	7	7	11	9	0.596	0.694
2 *(Crighton)*	*8*	5	5	8	8	0.962	1.034
3 *(Crighton)*	*9*	7	4	9	8	0.977	0.887
4 *(College)*	*9*	7	3	8	9	0.960	0.881
Total *(%)*	*37 (100)*	26 (70.3)	19 (51.4)	36 (97.3)	34 (91.9)		

The mean of standard deviations for weight gains was ± 0.1807

IV. DISCUSSION

It is well recognized that around puberty and for a short period afterwards the cycles in the bovine may be abnormally short. This is thought to be because the necessary integration of the mechanisms of the hypothalamo-pituitary-gonadal axis has not been achieved (Kinder et al., 1987; Moran et al., 1989). Energy requirements obviously enter into this as puberty which culminates in cyclicity is not attained until the growth and development of the animal has reached its final target. If this is not achieved then the animal remains in anoestrus (Moran et al., 1989). The importance of the role of energy in normal reproduction is also demonstrated in cows after parturition where high yielders or animals who have had problems during the periparturient period take longer to return to oestrus (reviewed by Short et al., 1990). Each of the treatments used in the field trial described in this thesis require that the animals are cycling. This is especially so with regard to treatment with prostaglandins which in the manner of their administration for synchronization of oestrus require firstly that luteal tissue is present and secondly that this tissue is maintained for the period associated with normal cyclicity. PGF2α analogues cause lysis of the luteal tissue and return to oestrus within a reasonably specific period of time. As two injections of the synthetic prostaglandin Prosolvin were administered ten days apart this required that the cycle lengths in the heifers treated were within the normal range of say 18 to 20 days. Crestar, the other method used in the trial, acts by suppressing the release of gonadotrophins by the pituitary axis so that on removal of this progestogen the released FSH and LH bring the animal into overt oestrus again within a recognized period of time. In situations where there is lack of gonadotrophins, or their releasing factor, the administration of a progestogen like Crestar, by acting by negative feed-back on the pituitary and

the hypothalamus, may allow a build up of FSH, LH and GnRH and this can sometimes have the desired effect of bringing the animal into oestrus and in these circumstances synchronization is achieved. However progestogens can be expected to work best in normal cycling animals (Brown et al., 1988). Based on the ultrasound examinations and the associated circulating plasma progesterone concentrations determined during the ten to twelve days prior to the beginning of this field trial the majority of the heifers used were cycling. This assumption is based mainly on their circulating plasma progesterone concentrations, indicating the presence of functional luteal tissue. However a few of the heifers (Nos. 575, 549, 572, 462, 431, 470, 587, and 449) were either in anoestrus or had abnormal cycles. Obviously under a normal field situation this would not have been recognized but does indicate that where a rapid cheap method of determining circulating plasma progesterone concentration in cattle is available more acceptable results could be achieved especially from an economic point of view. Obviously the use of the ELISA method applied in the study described here would be economically unsound at its present price.

Part (i): Prosolvin group

If we split the animals treated with Prosolvin into two categories viz those which had a functional CL (plasma progesterone concentration above 2 ng/ml) on the day of the first injection and those which had not, it is obvious that subsequent fertility amongst the first group is significantly lower than the second. This difference was not found significant when the minimum value of plasma progesterone corresponding to a functional CL was lowered to 1 ng/ml. Therefore any further investigations into this aspect should take into account the level of plasma progesterone concentration used to discern animals with a

functional CL from those without.

In the animals with functional luteal tissue (table 1.6a) at the beginning of the treatment period, one would have expected that within two days of administration of Prosolvin their circulating plasma progesterone would have dropped to baseline (Louis et al., 1973). However in four (Nos. 438, 461, 574 and 432) of the eighteen animals comprising this group, this did not happen. In two of these four animals (Nos. 438 and 432) ultrasound findings on two separate occasions tend to suggest that the CLs present when the first Prosolvin injection was given were still forming. However the circulating plasma progesterone concentrations recorded in these two animals, on the day of the first Prosolvin injection, were higher than usually associated with a CL of less than 5 days. It may be that progesterone levels do not always reflect CL maturity and responsiveness to exogenous PGF2α. Momont et al. (1984) reported that only 41% of heifers injected with cloprostenol (a PGF2α analogue) on day five of the cycle responded. An explanation for this could be that in some bovine, the CLs may acquire the capability for secreting more than 2 ng/ml of plasma progesterone by day five to seven of the oestrous cycle but only develop receptor-sites for PGF2α at a later stage and so even though they may appear in an advanced stage of maturity according to the circulating plasma progesterone concentrations, they are not capable of being lysed by prostaglandins. If the CLs in these two particular animals did fall into this category it could explain the raised circulating plasma progesterone concentrations and the fully formed CL on ultrasound on the day of second injection suggesting that the structure found earlier had been maintained.

The other two heifers with the unexpected response to the first injection (Nos. 461 and 574) had apparently fully formed structures at the commencement of the treatment according to both ultrasound and increased circulating plasma

progesterone concentration. Although both showed a decrease in progesterone and ultrasound parameters two days later, the decline in progesterone was not as expected. In one of these two heifers the decrease was such that although regression took place, it was much more slowly than expected (No. 574). Louis et al. (1973) reported that circulating plasma progesterone levels had returned to 1.5 ng/ml within 12 hours of the administration of PGF2α. However in the other animal (No. 461) the ultrasound findings and circulating plasma progesterone concentrations indicated little if any regression by two days after injecting Prosolvin. At this time the progesterone concentrations in this heifer were indistinguishable from those found in animals with a fully functional CL. However, both animals had a low progesterone profile on the day of second injection and on ultrasound evidence of a newly forming CL (present in a different ovary) indicated that ovulation had occurred between the two injections. Therefore in both animals the original CL did eventually regress. Aside from the possibility that the first injection of Prosolvin somehow did not reach its target there is no obvious explanation why in those two heifers the CLs did not respond. Furthermore had they been less than five days old at the first injection, they should have been responsive structures on the day of the second injection (Cooper, 1974). Neither had a fully mature CL at the time of the second Prosolvin injection and this explains why pregnancy was not achieved by the fixed time AI. Delayed return to oestrus following cloprostenol injection on days 6 through 17 has been reported previously in lactating cows (Macmillan, 1978; Momont, 1984). Although this is said to happen less frequently in heifers the results in those two animals indicate that it may occur. In their trial (Refsal and Seguin, 1980) also reported the occurrence of incomplete luteolysis and absence of signs of oestrus in heifers treated with cloprostenol on day five to eight. The ultrasound findings available in these particular animals do not allow

firm conclusions to be drawn on this aspect but there is one possibility which would explain the events which occurred. Such a response could be related to the lack of receptor-sites on the particular CLs so that they were not able to react to the Prosolvin injections.

With regard to the other 14 animals in this group, although the first injection resulted in baseline circulating plasma progesterone concentrations two days afterwards, eight of these 14 heifers (Nos. 14, 25, 19, 21, 28, 439, 468 and 557) did not have a functional CL when injected ten days later. Ultrasound and progesterone records indicated that although in three of these eight animals (Nos. 557, 439 and 28), ovulation took place between the two injections, most of these animals were either in metoestrus or early dioestrus on the day of the second Prosolvin injection. A delay in ovulation and/or subsequent formation of the CL could explain these findings. Jackson et al., (1979a) reported the occurrence of 'long low progesterone' associated with the administration of prostaglandins to normally cycling cows rather than heifers. Jackson et al. (1979a) could give no explanation for their findings but suggested that nutrition might play a part. No evidence of such an association was found in the present studies.

In another two heifers in this group (Nos. 19 and 25) in which no evidence of a functional CL was present on the day of second Prosolvin injection, follicles of a larger than preovulatory size (2.4 and 2.7 cm diameter respectively) were present. These structures had a thicker wall than usually encountered in follicles and so could be follicles which failed to ovulate. If this were the case the CLs recorded on ultrasound examination in these two heifers on the day of the second injection could have been the remnants of the CL lysed by the first injection. Unfortunately ultrasound alone did not allow for reliable differentiation between the forming and the regressing structure and only when

two successive sessions were carried out two days apart, was there a possibility to speculate on such findings. With some expertise ultrasound can be usefully applied in the diagnosis of cystic ovaries. In particular, it could be used to determine whether a cyst is luteinized or otherwise and so either prostaglandins or GnRH can be used accordingly to treat the condition saving time and efforts to the practitioner and money to the farmer. Indeed luteinized cysts appear as large (> 2.5 cm diameter) follicles with thick walls, whereas non-luteinized cysts are thinner-walled. However it is important to differentiate between luteinized cysts and CLs containing a lacuna as the latter are normal structures which cannot be described as a pathological condition (Okuda et al., 1988). Usually the relative size of the fluid-filled cavity compared to the size of the whole structure is a reliable tool.

Anovulation or delayed ovulation are not the only possible interpretations of the low progesterone profile on the day of second injection. It is also possible that in these eight heifers ovulation took place after the first injection with subsequent CL formation but this structure was not maintained for the normal length of time and the CL seen on ultrasound on the day of the second injection was a structure undergoing an earlier demise than usual. Short-lived CL do occur in heifers following the first ovulation at puberty (Gonzales-Padilla et al., 1975) and in post partum cows (Short et al., 1990; Lamming et al., 1981). Such structures also occur in heifers following experimental induction by GnRH (Rusbridge and Webb, 1991) injections during the period of early dioestrus, ie. days four to seven of the luteal phase.

Both ovulation and CL formation require all the components of the hormonal axis of reproduction ie. the hypothalamo-pituitary-gonadal axis to respond normally. When the first injection of Prosolvin was given, these heifers were in luteal phase which implies that the subsequent ovulation was induced within a

shorter interval than normal after the previous ovulation. It could be that in such circumstances the hypothalamo-pituitary axis is incapable of generating sufficient gonadotrophins, eg. LH, necessary for ovulation and maintenance of a CL for the normal period.

Delayed ovulation can result from delay in the maturation of the selected follicle so that insufficient oestrogens are secreted at the appropriate time resulting in a delayed LH surge. This sequence of events could result in the findings described above.

The manipulation of the cycle by lysis of the functional luteal tissue shortens the interoestrous interval which will result in interruption of the mechanisms required for normal maturation culminating in ovulation and subsequent formation of a new CL. It is therefore possible that the follicle which is required to ovulate under these conditions has not completed normal development and that the subsequent CL does not have the necessary cellular constituents required for its maintenance or the ability to respond in the proper pattern to the luteotrophic stimuli it receives. However the fact that these unexpected results were not found in all heifers suggests that the mechanisms required for follicular maturation, ovulation and CL formation can be forthcoming even in these altered circumstances. Although several possible explanations for ovulation and CL formation and function not being achieved can be postulated the molecular aspects of these failures are as yet not fully understood.

In some instances the progesterone results did not agree with the ultrasound findings. The use of a commercial ELISA kit only allows routine checking of the precision with quality control samples and so closer control of the assay is limited and one has to rely on the manufacturer's assessment. In order to determine the accuracy of this system, a large batch of random samples were assayed by an independent party using a routine radioimmunoassay. The results

from this were highly correlated to the ELISA's. Consequently progesterone results were considered to be the primary indicator as to whether a CL was functional or not.

Of the eight animals which had a low circulating plasma progesterone concentration on the day of the second injection, three became pregnant. Ultrasound findings in these eight animals suggested that these CLs were either forming or regressing. In the three heifers which became pregnant, if the CLs were formed during the interval between the two injections, they must have responded to the second Prosolvin dose. The finding of a CL which although secreting low levels of progesterone and yet responds to Prosolvin is certainly worthy of further investigations. On the other hand if on the day of second injection the CLs in these animals were those of late dioestrus then in these three particular heifers this resulted in them coming into oestrus coincidentally with fixed-time AI. Ultrasonography is an excellent technique for the identification of luteal tissue. However under certain circumstances there seem to be occasions when the presence or absence of luteal structure on ultrasound does not fit with the circulating plasma progesterone.

Among the eight heifers which had a high progesterone profile on the day of second injection five failed to conceive (Nos. 458, 573, 432, 446 and 464).
Thus failure to conceive was not a characteristic feature only associated with the animals which had a low progesterone on the day of second injection. In one of the animals with high circulating plasma progesterone (No. 458) there was evidence that the CL present on the day of second injection regressed suggesting that the failure in conception was either due to anovulation, lack of fertilization or to the next CL not being maintained resulting in early embryonic

loss. In another of the heifers (No. 446) ultrasound did show a degenerating embryo indicating here that this animal had conceived after the fixed-time AI and that synchronization had been achieved. However in the other three it cannot be stated whether the CL did not respond to the second injection or whether embryonic loss occurred around or after the third week following mating. In all five cases the CL could have been directly involved in the poor outcome associated with this category of response to the treatment. Embryonic loss is often considered as a result of CL failing to be maintained (Shelton et al., 1990). Certain factors which emerged from the early investigations on the use of prostaglandins for synchronization of oestrus in dairy heifers tend to suggest that these powerful luteolytic agents give better results in terms of synchrony and pregnancy rates if the animals are in dioestrus with a fully functional CL than if the luteal tissue is either forming or regressing. Hence the use of the double injection regimen spaced by an interval of ten to eleven days to overcome the problem related to the non responsive phase of dioestrus ie. before day five (Cooper, 1974). In the study described in this chapter it appears that the outcome of such treatments may not be so favourable with regard to achieving and maintaining pregnancy. Furthermore the action of Prosolvin even in animals in dioestrus emanated with either an apparent lack of response or in a structure that is not fully formed within an appropriate period of time or not maintained for the necessary duration. This results in asynchrony followed by disappointing pregnancy rates.

In the other group of animals treated with Prosolvin (table 1.6b) ie. animals with no evidence of functional luteal tissue at the commencement of treatment the results are more acceptable in that 13 of the 19 animals treated (68.42%) became pregnant. However some of the results warrant discussion. The eight

animals in this group which had a baseline progesterone level throughout the treatment period are of interest. In theory, had they been cycling normally at the start of the treatment they should have had a raised progesterone at some point during the trial. In four out of eight this was indeed the case (raised progesterone ten days before the start). Five out of the eight animals including these four heifers conceived to the fixed-time insemination. It does seem somewhat too much of a coincidence to suggest that five out of eight just happened to come into fertile oestrus at the time of insemination. Ultrasound indicated that some ovarian activity was present during the trial period; in particular there was evidence on ultrasound of ovulation having occurred in six of the eight animals shortly before the first injection or during the ten days between the two injections. Some of these CL which resulted in low circulating plasma progesterone concentrations apparently did respond to the second injection as they conceived to the fixed time AI. These results of low progesterone yet a responding CL are intriguing and raise doubts about the accuracy of predicting CL response to exogenous Prosolvin using either progesterone levels or size of the structure.

The incidence of low progesterone on the day of the second injection was found in a higher percentage in two of the farm groups of heifers (groups 1 and 4; table 1.1) but could not be explained by any relationship to initial weight or weight gain over the period of trial. The heifers in group 1 and 4 were inseminated in November rather than in late December suggesting that there may be a seasonal factor.

In general the findings recorded in this study in the use of Prosolvin as a means of synchronizing heifers tend to suggest that the stage of the cycle at which treatment is initiated has a bearing on the fertility of the oestrus produced.

Administering of the initial PGF2α injection whilst the heifers are in a period other than dioestrus gave better results than when animals were treated in dioestrus when a fully functional CL was *in situ*. This could be due to the presence of the luteal structure and/or the follicular state of the gonad at this time or to the capability of the hypothalamo-pituitary axis in heifers to respond to successive stimuli at a shorter interval than usual. Obviously this warrants further investigation. In heifers the priorities of the body are such that growth is to be favoured before reproduction (Moran et al., 1989). In fact even after the first ovulation heifers may be incapable of reproducing themselves and so some time is needed before short cycles and irregular and silent ovulations are replaced by normal cycles. Weight, body size, nutrition, season and social environment are all factors which influence the age at which puberty is reached. It appears that the absence of antecedent progesterone priming is responsible for the frequent CL inadequacy in anoestrus ewes (Legan et al., 1985). If this is the case with heifers around puberty it would mean that the use of progestogen releasing devices could be more suitable for oestrus synchronization in heifers than prostaglandins. The effect of progesterone priming seems to be exerted in the ovaries rather than the pituitary. Hunter et al. 1987 found that follicles from progesterone-primed ewes had greater oestradiol output, testosterone concentration and granulosa cell-binding capacity than follicles from non-primed ewes. This implies that progestogen treatment may be increasing the sensibility of granulosa cells to gonadotrophin enabling them to take better advantage of the preovulatory surge of LH which induces maturation and ovulation of the follicle and triggers luteinization of its remnants.

Part (ii): Crestar group

Comparing the results of those animals with raised progesterone at initiation of the Crestar treatment with those with a baseline progesterone (< 2 ng/ml), there appeared to be no difference in pregnancy rate between these two groups of animals. However in some animals the response to the treatment was not entirely as expected. Crestar treatment is expected to result in no functional CL being present by the day of removal. It is claimed by the manufacturer that the oestradiol valerate injection induces regression of an early CL, if present and the progestogen priming period should be long enough for a mid-luteal CL present at the time of introduction of the progestogen to regress spontaneously by the time of implant removal. However several authors (Lemon, 1975; Seguin, 1979) reported that oestradiol induces a slow decline of progesterone concentration when given during the mid-luteal phase and does not prevent luteal formation and function when administered during the early luteal phase of the cycle. Therefore animals implanted and injected during early dioestrus are unlikely to be synchronized as the early CL will certainly remain viable during the period when the implant is *in situ*. The incorporation of the progestogen norgestomet with oestradiol valerate as a single dose is thought to prevent continuation of CL formation following a recent ovulation (Wishart and Young, 1974). Despite this antiluteotrophic effect of the norgestomet, data published more recently indicated that luteal function is not hampered by the oestradiol/norgestomet injection when given between day three and five of the oestrous cycle (Peters, 1984).

In these studies, out of 25 animals with a functional CL at implantation with Crestar (table 1.6a) four showed raised progesterone on day eight of the treatment i.e. one day prior to removal and four days prior to insemination

(Nos. 555, 444, 449 and 451). One of these animals (No. 451) became pregnant to the fixed-time AI suggesting that in this animal, subsequent regression of the CL and coincidental return to oestrus around the time of AI occurred.

The failure to conceive by the other three heifers could be explained by them being out of phase at the time of insemination. However if low progesterone concentration three weeks after AI is considered as evidence that two of these three did come into season at a time close to the day of insemination, it would suggest otherwise. One of the following possibilities could explain this response: (a) the animals were in season on the day of AI but did not hold, (b) they were in season around the time of AI but did not ovulate, (c) were not in oestrus around the time of AI despite the evidence mentioned above or (d) they conceived but lost the embryo before maternal recognition.

Oestrogens have been reported to be luteolytic in cattle (Wiltbank et al., 1961; Brunner et al., 1969 and Lemon, 1975). However a difference in response to exogenous oestrogens by the differing types of luteal tissue has also been reported. Lemon (1975) observed that when oestradiol benzoate or valerate (5 mg IM) was given during dioestrus (day 9-15) it only resulted in a hastening of CL regression, as indicated by plasma progesterone and shortening of the oestrous cycle by only two to three days. It was clear from the study conducted by Seguin (1979) that oestradiol should not be considered luteolytic in the same sense as is PGF2α for it induced no consistent or rapid luteolysis. Thus it is possible that some of the animals reported here were still in the luteal phase one day before removal of the progestogen implant. Combining progestogen implants or devices with a prostaglandin injection at the end of the treatment period seems to give better results both in dairy (Logue, personal communication) and beef cattle (Peters, 1984) regarding lysis of any remaining luteal tissue.

The animals with low circulating plasma progesterone concentrations at the start of the treatment (table 1.7b) had all low circulating plasma progesterone concentrations one day before removal. However close examination of the progesterone and ultrasound data shows that in two heifers (No. 26 and 587) the progesterone was slightly raised on day eight of treatment with evidence from ultrasound that ovulation occurred sometime between one to two days before implantation of Crestar and day eight of treatment. The size of these CLs on the day prior to removal of the implant were 2.3 and 2.8 cm in heifers Nos. 26 and 587 respectively. The plasma progesterone concentration (1.0 ng/ml) indicates that these structures where not fully functional but could be developing CLs. If this was the case it would be a further evidence of failure of the oestradiol valerate injection in causing CL regression, in particular of those still forming. Intriguing results are also given for another two animals (No. 430 and 568) in which ultrasound showed the presence of a CL one day before removal. No such structure was seen in the previous scanning occasions and progesterone was still low. Even so the animal conceived. For one heifer (No. 430) it is hard to believe that a luteal structure was present and not detected by ultrasound during the preceding three sessions (see table 1.5b). It is also difficult to imagine that these structure arose from an ovulation which occurred before the treatment began. They would have otherwise been fully functional on day eight of the treatment. One explanation for this finding is the possibility of ovulation taking place during the treatment another that these structures were inadequate CLs of more than eight days old secreting subnormal amounts of progesterone. In another heifer (No. 470) both progesterone and ultrasound results indicated no cyclical activity prior to and during treatment, yet ovulation occurred and conception was achieved. In this case the treatment which suppressed release of gonadotrophins and their releasing factors during the time the implant was *in*

situ may have allowed the hypothalamus and pituitary to accumulate the necessary amounts of gonadotrophins to allow an LH surge. On the other hand as discussed earlier it is also possible that the progestogen acted by increasing the sensitivity of the follicular cells to LH resulting in a better response such that the removal of the progestogen coincided with the availability of a mature follicle capable of responding to the subsequent LH surge.

Although the difference in pregnancy rate to the synchronized oestrus between the types of treatment was not statistically significant the Crestar-treated heifers had fewer "days open". This result was primarily caused by the fact that heifers treated with Prosolvin had a longer interval from the initiation of the treatment to the day of AI. When this difference was corrected and the treatment period made uniform between groups (12 days for all animals) the means of days open between the two treatment groups was not found to be significant. Therefore one cannot attribute the apparent difference in days open to synchronization problems caused by prostaglandin.

However the fact that the effect of the stage of the cycle apparently plays a part in the outcome of the Prosolvin-treated heifers suggests that the use of a progestogen in field conditions would result in a better oestrus synchronization in cattle especially if the oestradiol/norgestomet injection of the Crestar treatment is replaced by an injection of a prostaglandin analogue given prior to implant removal. In this study, it was not possible to compare statistically heifers treated with Crestar during different periods of the cycle as most heifers had a functional CL at the beginning of the treatment and only a few had not.

In summing up the results of this field study of the synchronization of cattle several interesting findings are brought to light. Of importance is the great

variation in the capabilities and responsiveness of the luteal tissue demonstrated in this range of cattle. Obviously a better understanding of the integrated mechanisms controlling and the variation in the composition of this important gonadal structure would be of great benefit.

CHAPTER THREE

DETAILED STUDIES OF OVARIAN AND HORMONAL CHANGES IN COWS UNDERGOING TREATMENT FOR OESTROUS SYNCHRONIZATION

I. INTRODUCTION

The results of the field study where synchronization of oestrus in heifers was attempted by using either Crestar or Prosolvin agreed with those of previous workers in that in some heifers the response of the luteal tissue was not as expected irrespective of the treatment used. The results presented in this thesis also suggested that the stage of the cycle when the treatment is initiated may well influence its outcome. This could be related to the follicular population present at that time. There is little information in the literature regarding the contribution of this aspect of ovarian contents to the successful synchronizing of oestrus in cattle or to the subsequent development and maintenance of the CL.

In an attempt to increase our understanding of this aspect of oestrous synchronization in the bovine detailed investigations were undertaken in small groups of cows synchronized by progestins. These animals were monitored by behavioural, clinical and endocrinological methods to determine the follicular and luteal changes during treatment and overt oestrus, the timing of the LH surge, the demonstration of overt oestrus and ovulation and the achievement of conception. In addition the timing and amplitude of the LH peak was studied in cows synchronized by a double injection of a prostaglandin analogue given ten days apart.

II. MATERIALS AND METHODS

1. Animals used and treatments

1.1. Progestogen/oestradiol synchronization

The experimental protocol for the synchronization of the cows is shown in table 2.1a. Six multiparous non-lactating Friesian cows were selected at random. Before allocation to groups all cows were examined for ovarian status by ultrasound examination and determination of circulating plasma progesterone concentration. Animals were fed good hay *ad libitum* and concentrates.

The six cows were injected intramuscularly with 15 mg in 2 ml of luprostiol (Prosolvin, Intervet UK Ltd) and grouped in pairs as follows:- Group 1: fourteen (14) days afterwards an implant containing 3 mg of norgestomet was inserted into one ear of each cow. At the same time the animals were injected with 2 ml containing 3 mg norgestomet and 5 mg oestradiol valerate (Crestar, Intervet UK Ltd), - Group 2: two days after luprostiol injection cows were treated as above (Crestar) and - Group 3: two days after luprostiol injection, cows received an intravaginal coil containing 1.55 g progesterone and in a capsule attached to it 10 mg oestradiol benzoate (Prid Ceva Ltd).

Both PRIDs and implants were removed ten days later. Fixed-time AI was carried out approximately 48 hours afterwards.

1.2. Prostaglandin synchronization

The experimental protocol for this group is summarized in table 2.1b. Four adult Friesian cows were injected intramuscularly with 15 mg in 2 ml luprostiol (Prosolvin Intervet UK ltd) on two occasions ten days apart. The first injection was administered when a palpable CL was present on rectal examination.

Table 2.1a Protocol of experiment for progestin-treated animals

		Days of Trial						
Group	*Stage of the cycle at insertion of PRID/ implant*	*-14*	*-2*	*0*	*10*	*12*	*33* (21d)*	*46** (34d)*
1	Luteal Phase	Prosolvin		Crestar in	Crestar out	AI	PD	PD
2	Non-luteal phase		Prosolvin	Crestar in	Crestar out	AI	PD	PD
3	Non-luteal phase		Prosolvin	PRID in	PRID out	AI	PD	PD

AI = artificial insemination. Pregnancy diagnosis (PD) was performed by ultrasound and progesterone assay on day 21 after AI (*) and by ultrasound on day 34 after AI (**).

Table 2.1b Protocol of experiment for prostaglandin-treated cows.

Days of trial	*0*	*10*	*13*	*14*	*33 (21d)*	*46 (34d)*
Event	First Prosolvin injection	Second Prosolvin injection	Artificial insemination	Artificial insemination	Pregnancy diagnosis	Pregnancy diagnosis

21d and 34d = 21 and 34 days after artificial insemination.

Animals were artificially inseminated twice approximately 72 and 96 hours after the second prostaglandin injection.

2. Behavioural studies

The animals were observed running free in a courtyard for approximately 20 minutes, twice daily for signs of approaching oestrus. When any indications of oestrus, no matter how slight, were seen observations were increased to a maximum of four times daily (every six hours). The interval from termination of treatment or administration of second Prosolvin injection to first observed overt standing oestrus was recorded.

3. Blood sampling

3.1. Sampling for plasma progesterone

Blood samples for progesterone estimation were collected from the tail by venepuncture either into heparinized Vacutainer tubes or by Monovette syringes (Sarstedt Ltd. Leicester, UK). Plasma was separated by centrifugation (1000 x g, 10 minutes) and stored at -20 °C until assayed.

In the progestogen-synchronized animals, bleeding for progesterone was carried out once daily on the day of Prosolvin injection, the subsequent two days following Prosolvin injection, the day of Crestar administration, the second and the sixth days following Crestar administration, the day of implant removal and the following days until the cows were observed in oestrus. Cows synchronized with prostaglandin were bled for progesterone once daily during the induced oestrus periods and on day six or seven following the synchronized oestrus.

3.2. Sampling for plasma Luteinizing Hormone

Method 1: Blood samples were collected via an 80 mm long 14-gauge trocar catheter with an internal diameter of 1.6 mm (Intraflon 2, Vygon France), inserted into the jugular vein. The skin was shaved, cleaned with an antiseptic and anaesthetized by injection of 2 to 3 ml of 2% w/v lignocaine hydrochloride (Lignol, Arnolds Veterinary Products Ltd, Chesham House Romford, Essex UK). The trocar and needle were then inserted into the vein and the needle withdrawn. Two sutures were inserted to maintain the cannula in the vein.

Method 2: was as described by Jeffcoate (personal communication). After cleaning the skin a 14-gauge needle was inserted into the jugular vein. A catheter with an internal diameter of 1.0 mm (Portex Ltd.; Hythe, Kent UK) was then inserted through the needle into the vein. The needle was then withdrawn and the free end of the catheter taped onto the surface of the neck using two pieces of elastic adhesive bandage (Treatplast, Veterinary Drug Co. PLC, Dunnington, York UK). The catheter was tested for patency by withdrawing a small quantity of blood. A suitable stopper attached to a 20-gauge needle whose sharp end had been removed was used to seal the cannula. After each sampling the cannula was flushed with heparinized saline to prevent blood clotting.

Plasma separation was carried out as described for plasma progesterone samples.

Blood samples were collected every six hours during the 24 hours preceding the day of expected oestrus and every two hours when signs of approaching oestrus were detected and for 12 to 15 hours after onset of overt standing oestrus.

In all animals bleeding for the LH surge was only undertaken around the time of induced oestrus i.e. following removal of progestogen-releasing device or following the second prostaglandin injection.

The time when the LH started to peak, was estimated as the point midway between the first LH value more than two standard deviations above mean basal levels and the previous sample.

4. Hormonal studies

4.1. Plasma progesterone

Plasma progesterone was measured in triplicate as described in chapter two.

4.2. Luteinizing Hormone

Plasma LH concentration was determined using the double antibody radioimmunoassay as described by Boyns et al. (1972) with the following modifications:

4.2.1. Buffers

General purpose buffer: 0.5 M phosphate buffer (*pH* 7.4) was prepared and stored at room temperature. The following two solutions were made up first:

(i) 358.15 g *di*-sodium hydrogen orthophosphate dodecahydrate ($Na_2HPO_4.12H_2O$, MW = 358.15; Fison Lab. Supplies, Loughborough, England) was dissolved in 2 litres distilled water.

(ii) 39 g sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_2O$, MW = 156.01; Formachem Ltd., Strathaven, Scotland) was dissolved in 500 ml distilled water.

When both solutions were homogeneous and clear 475 ml of solution (ii) was added to 2 litres of solution (i) and *pH* was checked.

LH assay buffer: fresh assay buffer was made weekly by dissolving 5 g bovine serum albumin (Sigma Chemical Co., St. Louis, U.S.A) and 2 g sodium azyde

(BDH Chemicals Ltd, Poole, England) into 200 ml 0.5 M phosphate buffer followed by the addition of 1 ml *iso*-octylphenoxy-polyethoxyethanol (triton X-100, BDH Chemicals Ltd, Poole, England).

Iodination buffers: elution buffer was prepared on the day of iodination by dissolving 9 g sodium chloride (Sigma Chemical Co., St. Louis, U.S.A), 1 g sodium azyde and 5 g bovine serum albumin into 100 ml of 0.5 M phosphate buffer and making the solution up to 1 litre with distilled water. Column cleaning buffer was prepared as elution buffer except that bovine serum albumin was omitted.

4.2.2. Iodination and label

The iodination procedure was that described by Renton et al. (1991) and is as follows:

100 μg purified ovine LH (LER 1374A, kindly donated by Dr. L. Reichert, Albany Medical College) was reconstituted with 500 μl 0.5 M phosphate buffer and aliquots of 10 μl (2 μg oLH) each were put into conical iodination tubes (Sarstedt Ltd, Leicester, England) and stored at -80 °C.

For iodination 2 μg oLH was thawed and 37 MBq of ^{125}I (Amersham, UK) was added into the conical tube without allowing it to mix with oLH. The reaction was initiated following the addition of a freshly prepared solution of chloramine T (BDH Chemicals Ltd; 10 mg in 10 ml 0.05 M phosphate buffer *pH* 7.4). The three reagents were mixed and the reaction was stopped after 30 seconds by the addition of a freshly prepared solution of sodium metabisulphite (BDH Chemicals Ltd; 20 mg in 10 ml 0.05 M phosphate buffer *pH* 7.4). The mixture was then applied to a G-150 sephadex column (100 x 1.6 cm) and eluted with elution buffer. Fractions of 2 ml each were collected and 10 second-counts were

carried out in an auto-gamma scintillation spectrometer. The column was prewashed with elution buffer prior to adding the iodinated LH. Following the elution process the column was washed with cleaning buffer.

Initially maximum binding tests were carried out for this ligand by Renton et al. (personal communication) to detect the optimal fractions to be used. The three fractions corresponding to the upper part of the second peak were consistently found to give an acceptable maximum binding and so in all subsequent iodinations were chosen without further testing (figure 2.1). The chosen fractions were divided into 100 μl aliquots and stored at -20 °C until used.

For every assay the working solution was prepared by further diluting the label with assay buffer to give 12000 to 17000 counts per minute per 100 μl.

4.2.3. Antiserum

The first antibody used was a rabbit anti-sheep LH serum (NIDDK-Anti-oLH-1, AFP-192279) supplied by Dr. Reichert at a 1:100 dilution in 2% normal rabbit serum in phosphosaline buffer containing 0.01% merthiolate. Further dilution to 1:1000 was made with assay buffer and solution was divided into aliquots and stored at -20 °C. The antiserum was used in the assay at a final dilution of 1:320000. This concentration was chosen after carrying out a test to establish the antibody dilution curves in presence of three different concentrations of standard, ie. 0, 4 and 16 ng/ml bLH, (figure 2.2).

4.2.4. Standards

Purified bovine LH (USDA\bLH\1\1), supplied by the National Institute of Health - USA, was used for the standard curve. Aliquots of 1 ml at a concentration of 125 ng/ml were prepared and stored at -20 °C. The working range of the standard curve was 0, 0.24, 0.48, 0.97, 1.95, 3.9, 7.8 and 15.63 ng/ml

and was set up at the beginning of every assay by diluting the 125 ng/ml aliquot with assay buffer to obtain a solution of 15.63 ng/ml. Of this, serial double dilutions were made to cover the range down to the 0.24 ng/ml standard.

Fig. 2.1 Elution profile of iodinated LH

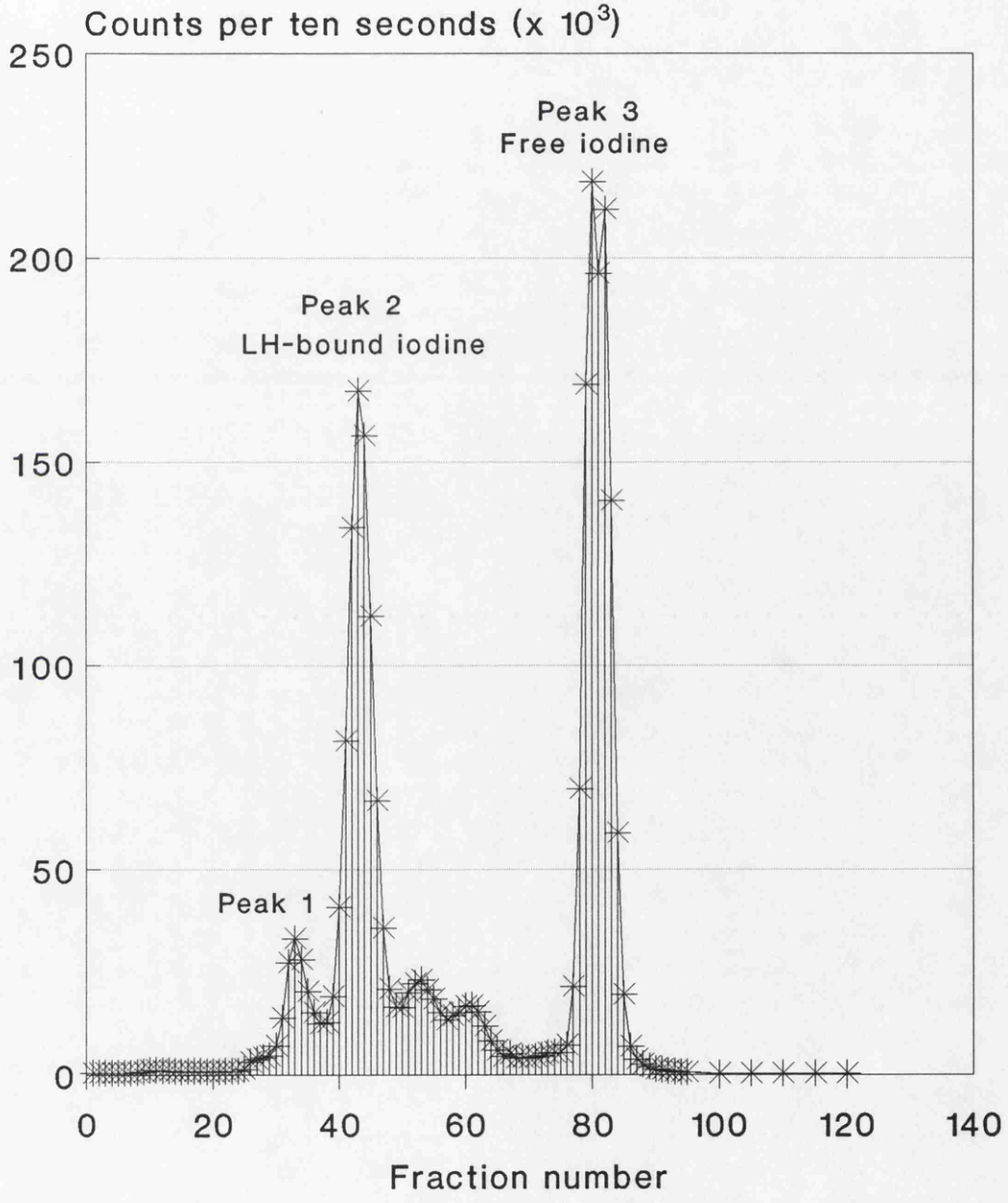
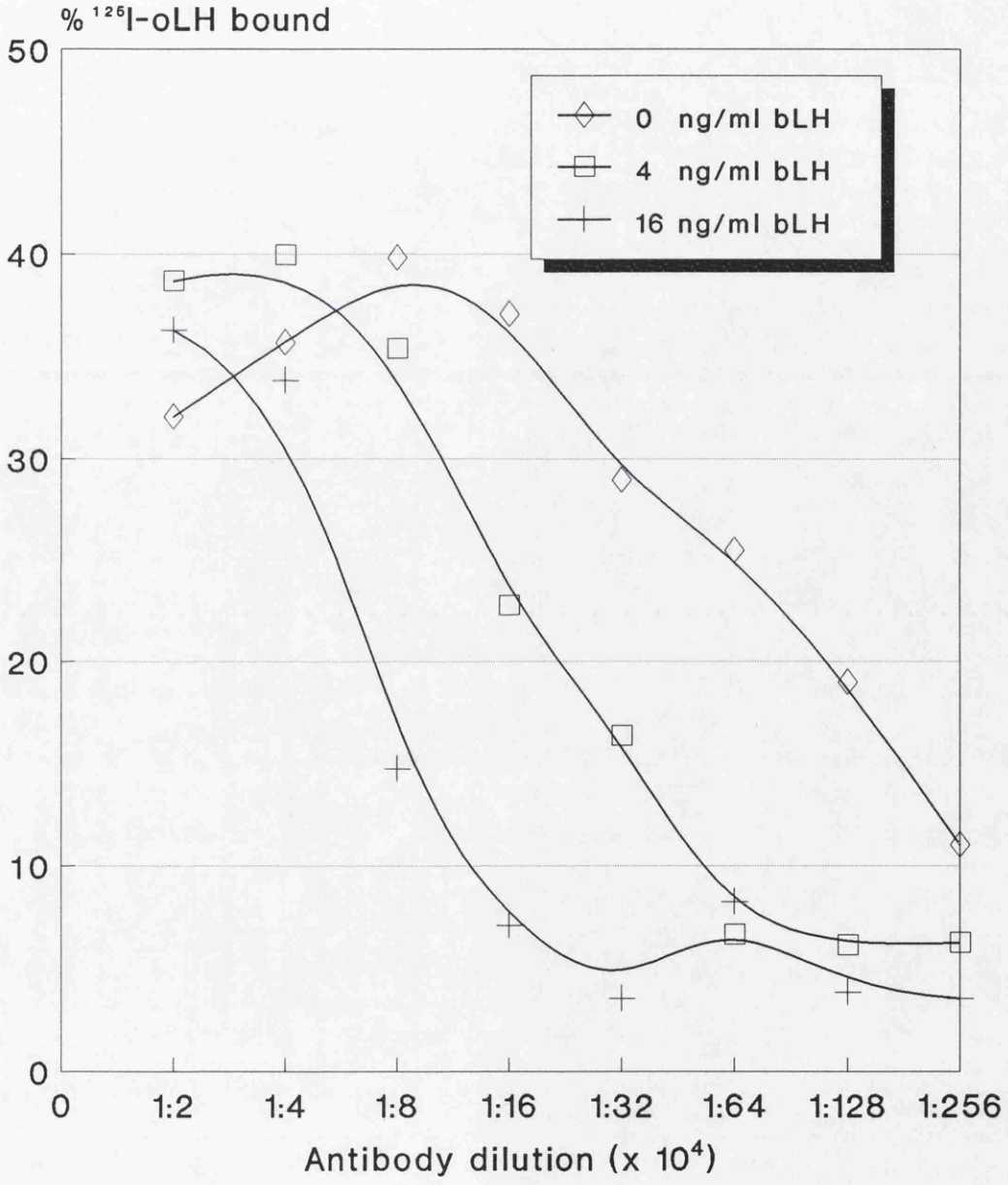


Fig. 2.2 Antibody dilution curves



4.2.5. Second antibody

The second antibody used was Dynospheres^{R} donkey anti-rabbit provided by the Royal Infirmary, Glasgow (McConway et al., 1986).

4.2.6. Assay protocol

The assay was set up to include four tubes for total counts (TCs), two tubes for non-specific binding (NSB), and two tubes for each of the standards and samples. In all assays 100 μl of assay buffer was added to the (0) standard tubes and 100 μl of every appropriate standard or plasma sample was added to the other corresponding tubes. The first antibody was then added (100 μl) to all tubes except the TCs and NSB tubes. Instead assay buffer (100 μl) was added to the NSB tubes. The reagents were mixed by gentle shaking of all tubes and incubation was carried out overnight at 4 °C. The following day 100 μl of the radioactive label was added to all tubes, reagents were mixed and incubation continued for an additional night at 4 °C.

Separation of the bound and free fractions was achieved by addition of 100 μl of second antibody to all but the TCs tubes and a further incubation at room temperature for at least one hour. Physiological saline (1 ml) was added to all tubes but the TCs, centrifugation at 2000 x g, at 4 °C for 25 minutes was carried out and supernatant aspirated and decanted. The precipitate was counted using an auto-gamma scintillation spectrometer (Packard 5230, Berks, England). The curve was plotted and results calculated using a computer program (S.A.S Immunoassay Program 632014).

Plasma samples of known high and low LH were included twice to three times in every assay to act as quality controls.

Samples with a higher value than the higher standard (> 15 ng/ml) were repeated in another batch after a two to three-fold dilution in fetal calf serum.

4.2.7. Assay validation

a) Specificity

Specificity of this antiserum in terms of its reactivity with pituitary hormones other than LH was challenged in the supplier's laboratory with highly purified preparations of oFSH, oGH, bTSH, oPRL and ACTH and was equal to:

oFSH	6.2%,
oGH	0.61%,
bTSH	0.16%,
oPRL and ACTH	0% each.

b) Repeatability of standard curve

A composite standard curve from ten assays is presented in figure 2.3. Repeatability was poor at low LH concentrations (first four standards). Other parts of the curve were highly repeatable.

c) Precision

The intra-assay coefficients of variation derived by twelve times assaying two plasma pools containing high and low LH concentrations were 8.24 and 6.09% at 15 and 8 ng/ml respectively.

The inter-assay coefficients of variation calculated for the same plasma pools were 7.58 and 5.9% at 15 and 8 ng/ml respectively (n = 6).

d) Sensitivity

The sensitivity of the assay defined as twice the standard deviation of the blank values (i.e. maximum binding), was 2.09 ± 1.62 ng/ml (n = 10).

e) Accuracy

For assessment of accuracy known amounts of bLH standards at a concentration of 7.8, 3.9, and 1.9 ng/ml were added to a pool of low LH samples and assayed. The mean ± s.d. recovery value of 93.32% (± 7.73) was obtained. This being done once.

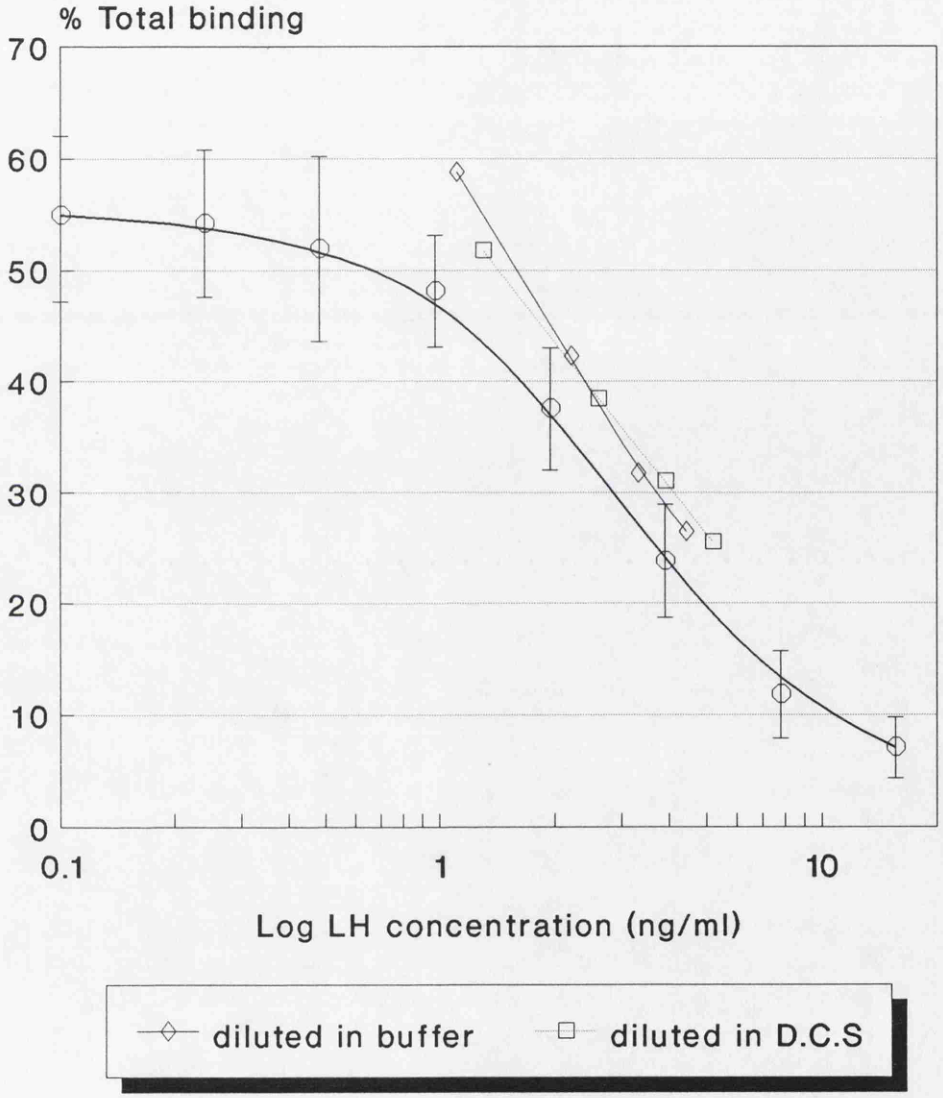
f) Parallelism

Samples of known LH concentration (≈5 ng/ml) were diluted serially 4/5, 3/5, 2/5 and 1/5 with either assay buffer or donor calf serum and assayed. The dose-response curves produced with either donor calf serum or assay buffer appeared to be parallel to the standard curve (figure 2.3).

g) Validity of the LH radioimmunoassay results

This assay was primarily intended to identify the LH surge and so was designed to measure high values with acceptable accuracy and precision. As this sort of 'biological targeting' is usually more important than maximisation of sensitivity, the latter had to be sacrificed in this assay and therefore variations occurring at baseline values were ignored. The choice of an anti-ovine serum was because the labelled ligand was ovine LH. The latter was readily available in our laboratory because it was used in canine LH assay. To allow for the reaction antibody-bovine standard (sample) to reach equilibrium without major displacement by the labelled ovine LH, incubation was carried out in two stages with the latter being added last.

Fig. 2.3 composite standard curve from ten assays and parallelism



5. Ultrasound examination and rectal palpation of reproductive tract

Ovarian structures were monitored by both manual palpation and by real-time ultrasound using a real time B-mode scanner equipped with a 7.5 MHz probe (Concept I, Dynamic Imaging Ltd) as described in chapter one. Table 2.2 shows the frequency of ultrasound examinations of progestin-treated animals during the experiment. Follicles were characterized as small (< 0.8 cm of diameter), medium (0.8 to 1.5 cm diameter), and large (> 1.5 cm diameter).

A follicular wave was defined as the appearance of a group of small to medium-sized follicles dominated by a follicle of more than 1 cm which increased in size between at least two sessions of scanning. The criteria used to describe a CL on ultrasound were as described in chapter two. Size of luteal and follicular structures was measured using the in-built scanner callipers. Scanning sessions were recorded on VHS video tapes and these subsequently reviewed for collection of data.

Animals synchronized with prostaglandin were not scanned during treatment. Instead rectal palpation for the presence or otherwise of a CL and large follicles was carried out once prior to the first injection, twice during the interval between the two injections and once after the second injection.

Ultrasound was used in all animals for pregnancy diagnosis on days 21 and 34 after AI using the method described in chapter two.

Table 2.2 Frequency of scanning during the trial for progestin-treated cows.

Days of trial when scanning was carried out	*-14*	*-2*	*0*	*2*	*6*	*10*	*12*	*33*	*46*
Associated events	Prosolvin to group 1	Prosolvin to groups 2 & 3	**Implant or PRID insertion**			**Implant or PRID removal**	AI	Pregnancy diagnosis (*21d*)	Pregnancy diagnosis (*34d*)

AI = artificial insemination. 21d and 34d = 21 and 34 days after insemination.

III. RESULTS

Part (i): Progestogen/oestradiol groups

Table 2.3.

Details of ultrasound examination and circulating plasma progesterone concentrations prior to progestogen treatment indicate that five of the six cows were cycling at the start of the experiment (CL seen on ultrasound and raised progesterone). One cow in Group 2 (No. 56) had several cystic structures on the right ovary and one "cystic" follicle on the left ovary.

Table 2.3 Details of circulating plasma progesterone concentration and ovarian structures prior to the initiation of progestin treatment (day of Prosolvin injection).

Group	Cow's identity	Day of trial	Plasma progesterone (*ng/ml*)	CL	Number of large follicles (*>1.5cm*)
1	24	(-14)	3.2	present	-
	7		2.1	present	one (left ovary)
2	22	(-2)	8.7	present	one (left ovary)
	56		<0.5	absent	cysts (both ovaries)
3	23	(-2)	3.2	present	one (right ovary)
	19		10	present	one (left ovary)

Plate 2.1.

The image of a scan of the right ovary of cow No. 56 is shown. Two of the cysts present are visible. Note that from the thinness of the walls it cannot be said that they are luteinized.

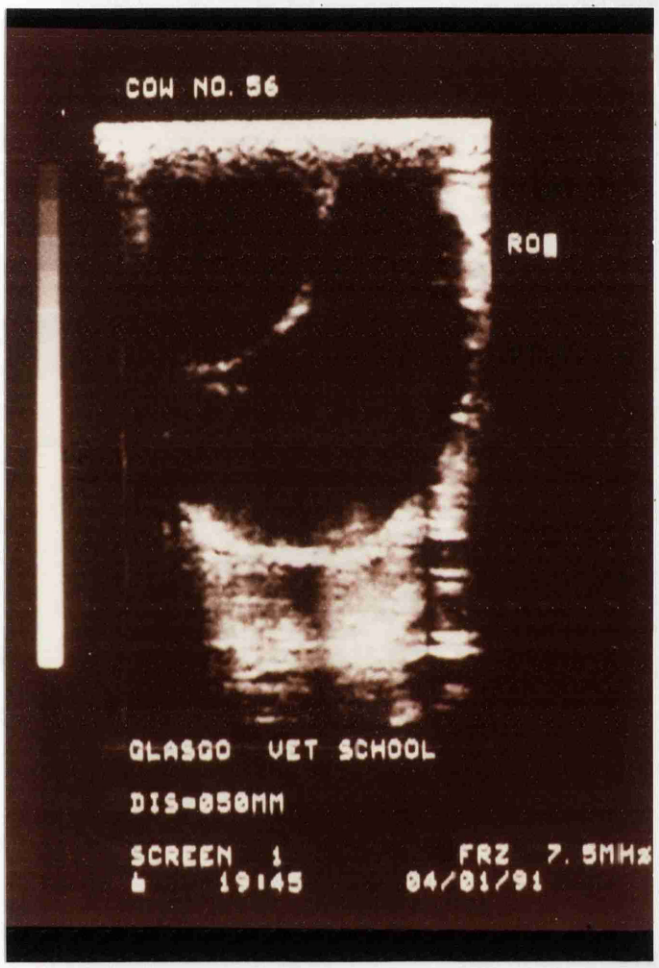

COW NO. 56
RO
GLASGO VET SCHOOL
DIS=050MM
SCREEN 1
19145
FRZ 7.5MHz
04/01/91

Table 2.4.

This table summarizes both the ovarian status and the associated circulating plasma progesterone concentration on the day of Crestar/PRID insertion and during the treatment period. At the start of the treatment, progesterone estimation indicated that only in the cows of group 1 was there fully functional luteal tissue (> 2 ng/ml). Note that in the animals in group 3 the progesterone was 1.6 and 1.8 ng/ml. Ultrasound indicated that a CL was present in both cows of group 1 and in one cow of group 2 (cow No. 24). No CL was recorded by ultrasound in the remaining three cows (No. 56, group 2; No. 23 and 19, group 3).

No functional CL was identified during the treatment period in groups 2 and 3. Raised progesterone was recorded in the circulating plasma of both cows in group 1 until two days after Crestar insertion. As determined by ultrasonography CLs were still present in the ovaries of these cows until day six.

Table 2.4 Summary of ovarian status and circulating plasma progesterone at insertion of progestin-releasing devices (day 0) and during treatment.

Group	Cow's identity	*Day 0*			*Day 2*			*Day 6*		
		P_4 *(ng/ml)*	CL	Number of large follicles	P_4 *(ng/ml)*	CL	Number of large follicles	P_4 *(ng/ml)*	CL	Number of large follicles
1	24	>10	+	one	2.8	+	two	<0.5	+	-
	7	9.9	+	one	8.9	+	-	<0.5	+	one
2	22	<0.5	+	two	<0.5	-	one	<0.5	-	-
	56	<0.5	-	cysts	<0.5	-	cysts	<0.5	-	cysts
3	23	1.6	-	one	6.1*	-	-	5.8*	-	-
	19	1.3	-	one	7.7*	-	two	5.9*	-	one

**Progesterone released by Prid. P_4 = circulating plasma progesterone concentration.*
Large follicles = follicles of >1.5 cm in size. (+) = structure present. (-) = structure absent.

Details of the various changes in follicular dynamics observed during treatment are given in figures 2.4a, 2.4b and 2.4c. Only medium and large follicles are shown. However small follicles were observed on most occasions.

Figure 2.4a.

In cow No. 24 (group 1), at the start of the treatment the morphologically dominant follicle was in the right ovary. By the end of the treatment a follicle in the left ovary developed and ovulated following removal of the progestogen releasing implant.

In cow No. 7 (group 1) most of the medium-sized and large follicles were in the right ovary at the beginning of the treatment. These were apparently replaced by a fresh follicle from the left ovary which was in turn replaced by another dominant in the right ovary. The latter was the one which ovulated.

Fig. 2.4a Changes of follicular pattern during Crestar treatment in group 1

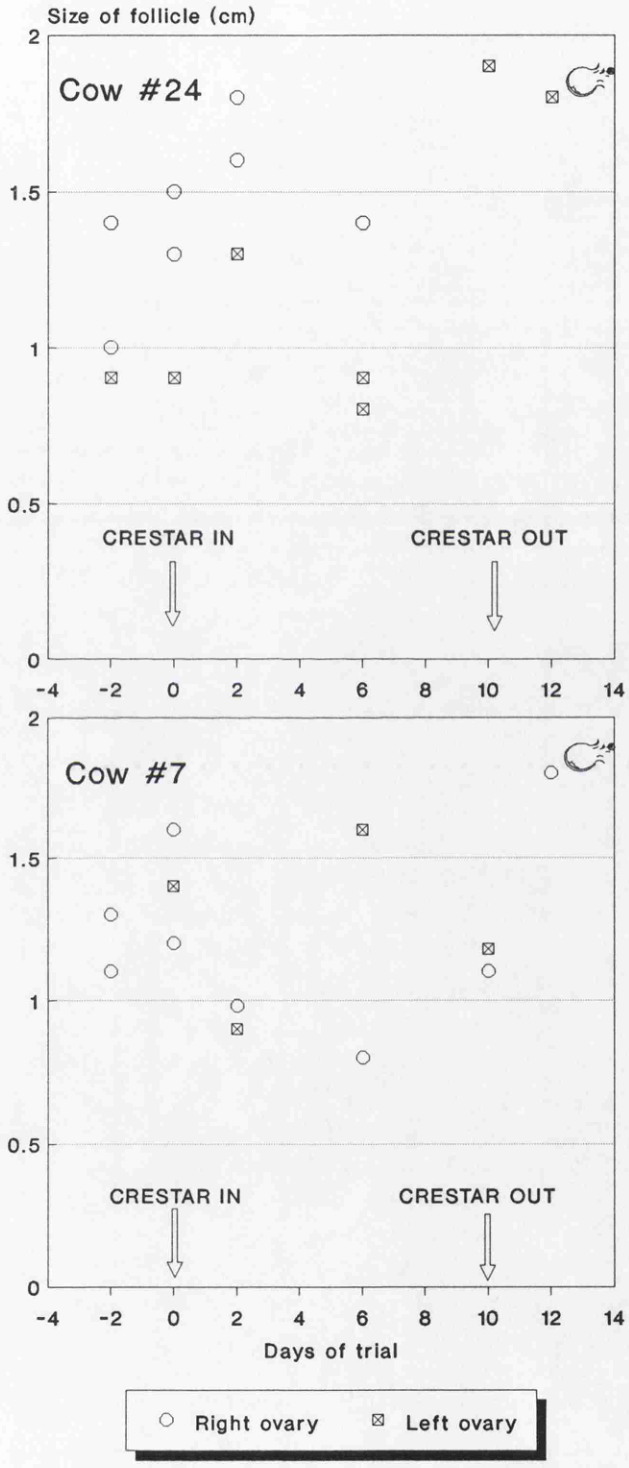


Figure 2.4b.

In cow No. 22 (group 2) both ovaries had medium to large follicles at the start of the treatment and the largest was in the left ovary. Only one large follicle was observed on day two of the treatment when no medium-sized ones were present. By the end of the treatment medium-sized follicles had developed in the left ovary and one of these became dominant and ovulated.

In cow No. 56 (group 2) the size of the cysts in the right ovary did not appear to change during the treatment period. In the left ovary one cyst was observed and no medium or large follicles were present. This cystic follicle decreased in size during treatment and was replaced by two medium-sized follicles one of which ovulated following removal of the progestogen implant.

Fig. 2.4b Changes of follicular pattern during Crestar treatment in group 2

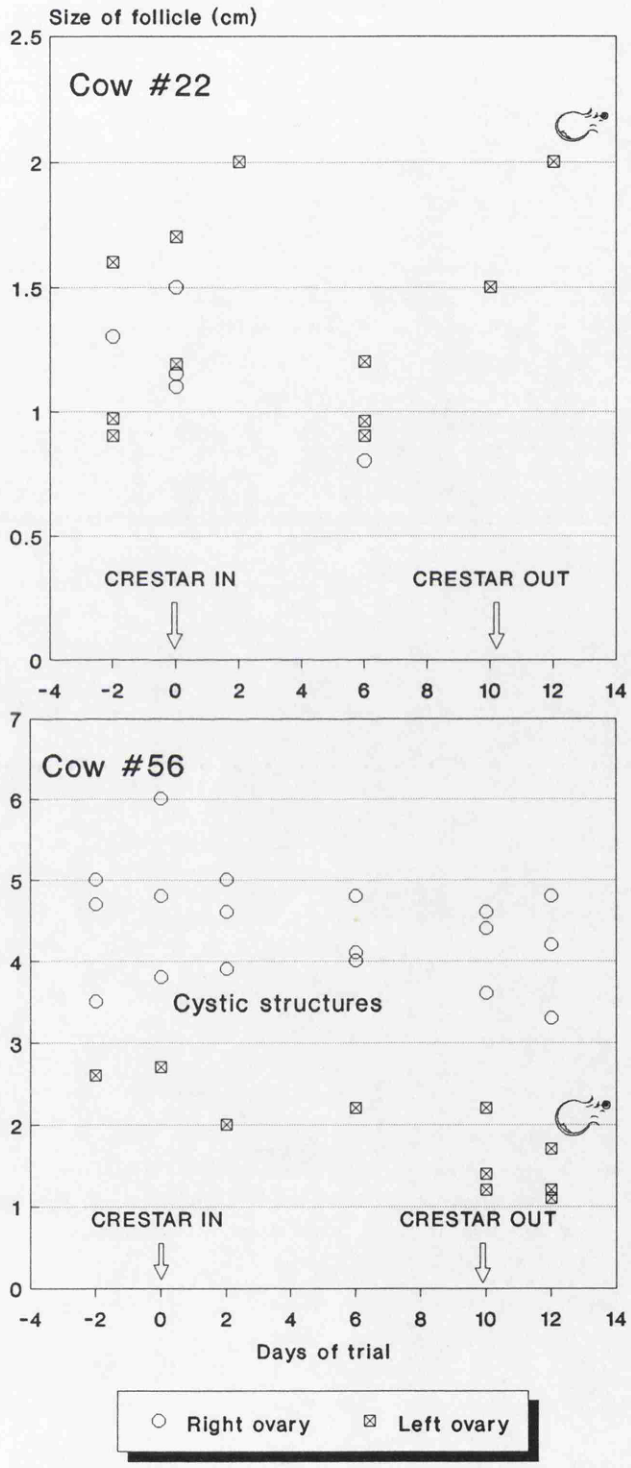


Figure 2.4c.

In cow No. 23 (group 3) the large follicles present in the right ovary at the beginning of the treatment were absent during subsequent scanning sessions and follicles in the left ovary were observed by the end of the treatment. Note also that three medium-sized follicles appeared on day 12 (day of AI). Ovulation occurred in the left ovary.

In cow No. 19 (group 3). Medium and large follicles were seen in both ovaries on most occasions. An unusually large follicle present in the left ovary at the beginning of the treatment persisted throughout the treatment period and showed a slight decrease in size. In the right ovary two waves of follicles apparently occurred, the last one being at the end of the treatment. However no other follicles other than the persistent one were observed in the left ovary and the latter ovulated following removal of the PRID.

Fig. 2.4c Changes of follicular pattern during PRID treatment in group 3

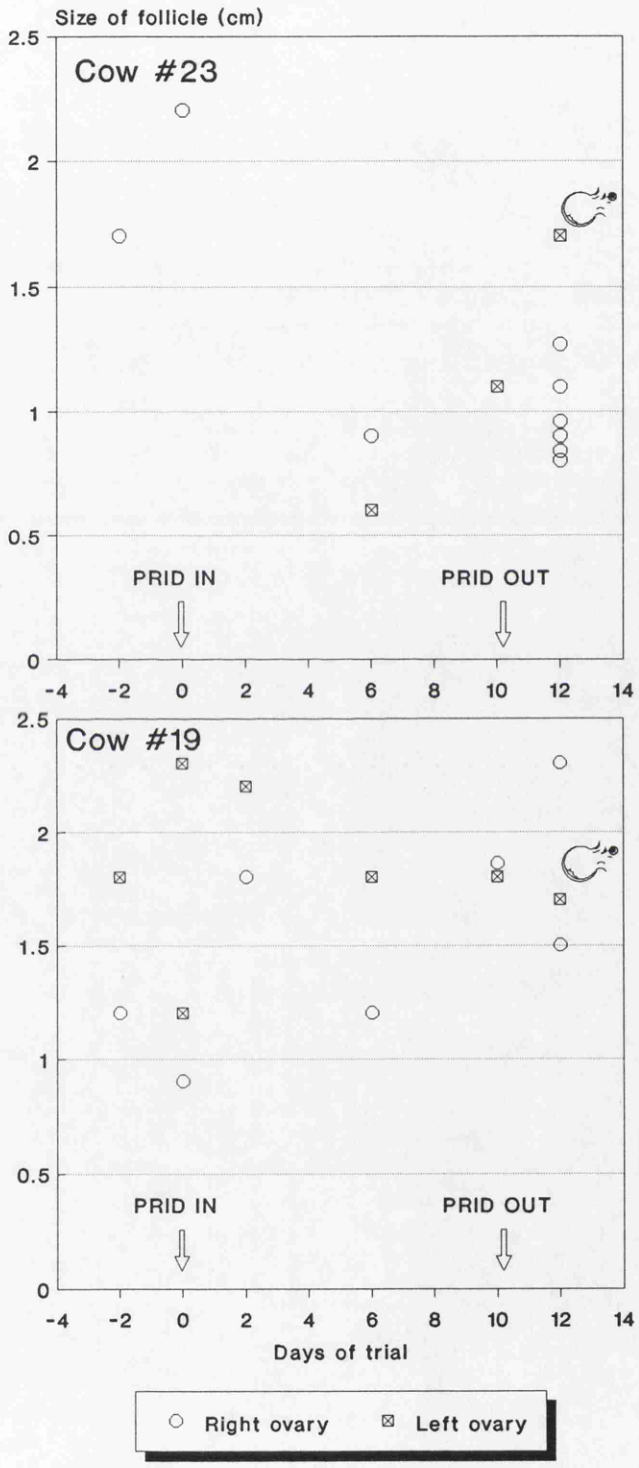


Table 2.5.

In this table the findings at the end of the Crestar / PRID treatment period are presented. In group 1, on the day of removal of the ear implant, the CL present in both cows at insertion had regressed. Two medium-sized follicles were present in the ovaries of cow No. 7 and one large follicle in one of the ovaries of the other cow (No. 24).

In group 2, where the cows had no functional CL at insertion of Crestar, by the end of treatment the normal cow (No. 22) had a large follicle whereas in cow No. 56 the cysts were still visible in the right ovary and a large follicle in addition to two medium-sized follicles (data shown in table 2.6) were present in the other ovary.

In group 3 in cow No. 19 a large follicle (1.8 cm in diameter) which was in the left ovary at the beginning persisted to the end of treatment. Another large follicle was present in the right ovary by the end of treatment. However the follicle in the left ovary apparently ovulated as the subsequent CL was in that ovary. The other cow in this group (No. 23) had only a medium sized follicle by the end of treatment.

Worthy of note is the fact that in one of the cows in group 3 treated with PRID, the circulating plasma progesterone concentration on the day of removal of the PRID was almost basal whereas in the other it was still raised.

Table 2.5 Details of circulating plasma progesterone and ovarian structures on the day of termination of the progestin treatment.

Group	Cow's identity	Progesterone *(ng/ml)*	CL	Number of follicles	
				Large	Medium
1	24	<0.5	-	1	-
	7	<0.5	-	-	2
2	22	<0.5	-	1	-
	56	<0.5	-	Cysts in right ovary One large follicle in the left one	
3	23	1.8*	-	-	1
	19	4.0*	-	2	-

**Progesterone released from PRID. Large follicle = follicle of >1.5 cm in size. Medium follicle = follicle of >0.8 cm but <1.5 cm in size.*

Table 2.6.

This table includes the events recorded after removal of the progestogen device. The majority of the cows were first noted in standing oestrus at approximately 44 hours after removal of the progestogen (either Crestar or PRID) and were inseminated 4 hours later. Cow No. 56 (the cow with cystic ovary) showed signs of oestrus approximately four hours earlier than the other cows. The interval from termination of treatment to the appearance of the LH surge ranged between 37 and 50 hours. The shortest interval occurred in the cow with cystic ovaries. The maximum amplitude of the LH peak varied between 10.6 and 33.2 ng/ml with the lowest amplitude occurring in one of the cows treated with PRID.

Twelve hours (sixteen for cow No. 56) after the first observed overt oestrus (approximately 56 hours after removal) all 6 cows had a follicle of between 1.5 to 2.0 cm present in one ovary.

Table 2.6 Details of events recorded at termination of progestogen treatment and on day of AI in six adult cows.

Group	Cow's identity	Day of treatment removal		Day of AI		Interval removal to onset of LH surge (*hours*)	Interval removal to first observed oestrus (*hours*)	Maximum amplitude of LH surge (*ng/ml*)
		Large foll.	Medium foll.	Large foll.	Medium foll.			
1	24	1	-	1	-	40	44	18.5
	7	-	2	1	-	50	44	33.2
2	22	1	-	1	-	43	44	18.2
	56	1 (Cysts in right ovary)	2	1 (Cysts in right ovary)	2	37	40	30.3
3	23	-	1	1	Several	45	44	10.6
	19	2	-	3	-	43	44	32.9
				MEAN ± S.D.*		44.2 ± 3.7	44.0 ± 00	22.6 ± 9.9

** Cow No.56 was not included in computation of the means. Large foll. = follicle of >1.5 cm in size. Medium foll. = follicle of >0.8 cm but <1.5 cm in size.*

Figure 2.5.

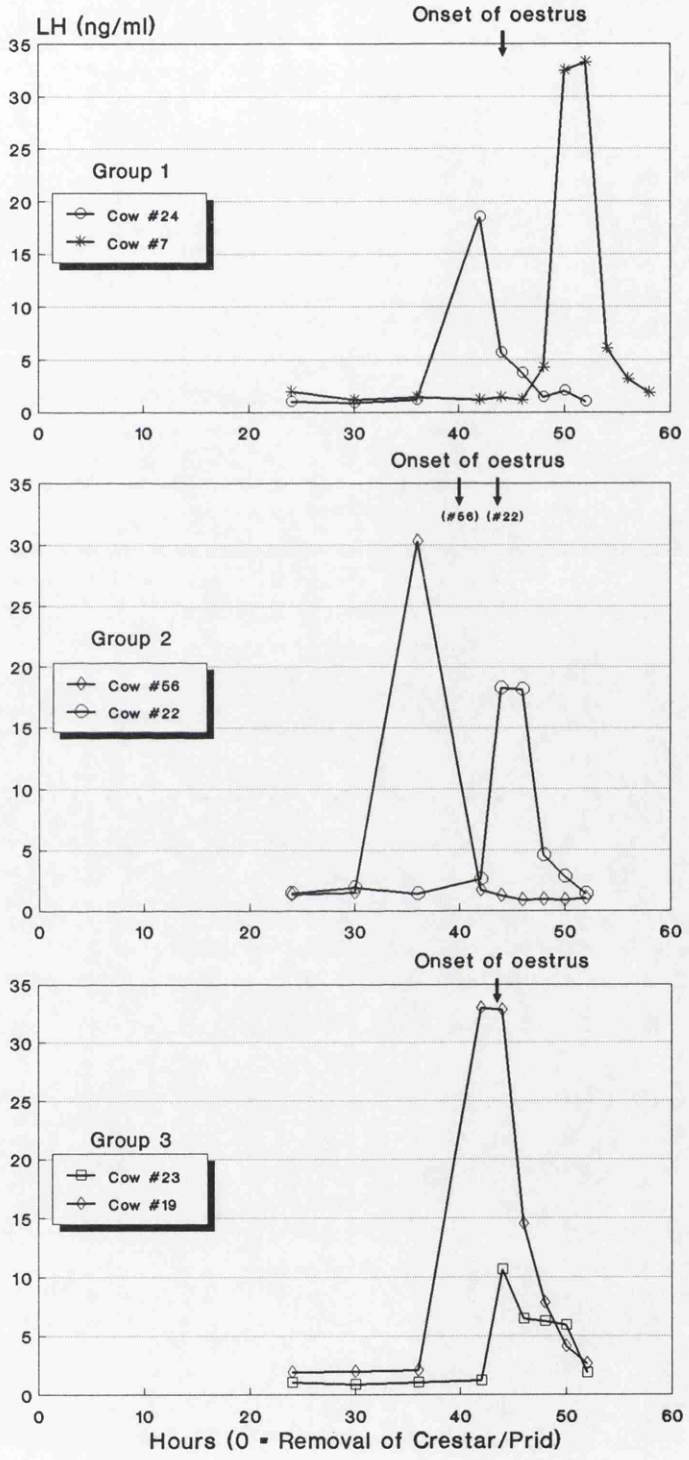
Pattern of the LH surge is presented. Note that the amplitude of the LH surge varies between individuals in the same group. The correlation between the timing of the onset of the LH surge and the onset of standing oestrus was most obvious in group 3.

In cows of group 1 the LH peak occurred about six hours after oestrus was first observed in cow No. 7 and four hours before oestrus was first observed in cow No. 24. Both cows were first observed in oestrus at the same time.

In cows of group 2 (including cow No. 56) the LH surge occurred, one and three hours before overt oestrus was first observed in cows No. 22 and 56 respectively. Cow No. 56 was first seen in standing oestrus four hours earlier than cow No. 22.

In cows of group 3 the LH surge occurred one hour after and one hour before the onset of overt oestrus in cows No. 23 and 19 respectively. In one of the cows (No.23) a relatively reduced amplitude of the LH surge was noted. Both cows were seen in oestrus at the same time.

Fig. 2.5 Pattern of LH surge following removal of progestin treatment



Five of the six cows treated with progestins became pregnant. The cystic cow did not conceive. However she did ovulate at the oestrus after removal of implant and a functional CL was formed (plasma progesterone concentration of more than 10 ng/ml).

Part (ii): Prostaglandin group

Table 2.7.

All cows had a CL determined by rectal examination on the day of first prostaglandin injection. Following the first prostaglandin injection only one cow (No. 94) apparently came into oestrus. This animal was in fact not seen in standing oestrus, but a clear vaginal discharge (bulling string) was observed and it was assumed that the cow was close to oestrus. Rectal palpation was carried out on all the cows on two separate occasions during the ten days between the prostaglandin injections. On both occasions a palpable CL of a size suggesting functional status was recorded in all the cows except cow No. 94 in which a large follicle and a small firm regressing CL were found.

Following the second injection, three cows were seen in standing oestrus. In Cow No. 94 no evidence of oestrus was observed after the second injection of prostaglandin, although she showed some vague signs of prooestrus ie. reddening of the vestibular mucosa, restlessness, etc...

Table 2.7 Oestrous detection and rectal examination findings in prostaglandin-treated cows.

Cow's identity	Rectal examination findings				Oestrus observed	
	Day of 1st PG	Two days after 1st PG	Day of 2nd PG	Six days after AI*	Following 1st PG	Following 2nd PG
156	CL (≈3.0 cm)	CL (≈2.5 cm)	CL (≈2.0 cm)	CL (≈2.5 cm)	–	+
17	CL (≈2.5 cm)	CL (≈3.0 cm)	CL (≈2.5 cm)	CL (≈3.0 cm)	–	+
94	CL (≈2.5 cm)	Hard structure (≈1.5 cm) Follicle (≈1.5 cm)	new CL? (≈2.0 cm)	CL (≈2.5 cm)	+	–
115	CL (≈3.0 cm)	CL (≈3.0 cm)	CL (≈2.5 cm)	CL (≈2.5 cm)	–	+

**Day of AI = three days after the second Prosolvin injection. PG = Prosolvin injection. (+) = oestrus observed. (–) = oestrus not observed.*

Table 2.8.

This table summarizes important hormonal parameters collected around the time of synchronized oestrus and six or seven days afterwards. On the day of synchronized oestrus, all the cows had a circulating plasma progesterone concentration of less than 0.5 ng/ml including cow No. 94 (day of AI for this cow). The LH surge was detected in all cows and its maximal amplitude varied between 8.9 and 18.3 ng/ml.

Table 2.8 Summary of hormonal parameters recorded from four cows following two injections of Prosolvin given 10 days apart.

Cow's identity	P_4 *(ng/ml)*		Maximum amplitude of LH surge *(ng/ml)*	Interval from 2nd PG injection to onset of LH surge *(hours)*	Interval from 2nd PG injection to overt estrus *(hours)*
	Day of AI*	Day 6 or 7 after AI*			
156	<0.5	>10	18.3	61	65
17	<0.5	6.6	13.7	45	46
94	<0.5	2.7	8.9	57	-
115	<0.5	6.2	13.3	91	94
MEAN ± S.D.			13.58 ± 3.84	63.5 ± 19.55	68.3 ± 24.17

**Day of AI = three days after the second injection of Prosolvin. PG = Prosolvin. P_4 = circulating plasma progesterone concentration.*

Table 2.9.

This table compares the timing and the amplitude of LH surge and the timing of onset of oestrus in both progestin- and prostaglandin-treated groups. The mean amplitude of the LH surge in the prostaglandin-treated group was lower but not found significantly different (*P = 0.12*) from that found in the cows synchronized with progestogens (13.58 ± 3.84 *vs* 22.6 ± 9.9 ng/ml; mean ± s.d.). Both the intervals from 'second prostaglandin injection' to (a) 'onset of LH surge' and (b) 'first observed oestrus' were longer in the prostaglandin-treated group than in the progestin-treated cows. Similarly this result could not be confidently qualified as significant (*P = 0.15*). Circulating plasma progesterone concentration and rectal palpation on day six or seven following synchronized oestrus confirmed the presence of a functional CL.

Out of the four cows treated, two (No. 17 and No. 115) became pregnant to the double AI carried out following synchronized oestrus.

Table 2.9 Comparison of timing and amplitude of LH and timing of overt oestrous behaviour in cows synchronized with progestin-releasing devices *vs* cows synchronized with two injections of a PGF2α analogue.

Treatment	n	Maximum amplitude of LH surge *(ng/ml)*	Interval from end of treatment to onset of LH surge *(hours)**	Interval from end of treatment to onset of overt oestrus *(hours)**
Progestin/oestradiol	6	22.68 ± 9.9	44.2 ± 3.7	44.0 ± 00
Prostaglandin analogue	4	13.58 ± 3.84	63.5 ± 19.55	68.3 ± 24.17

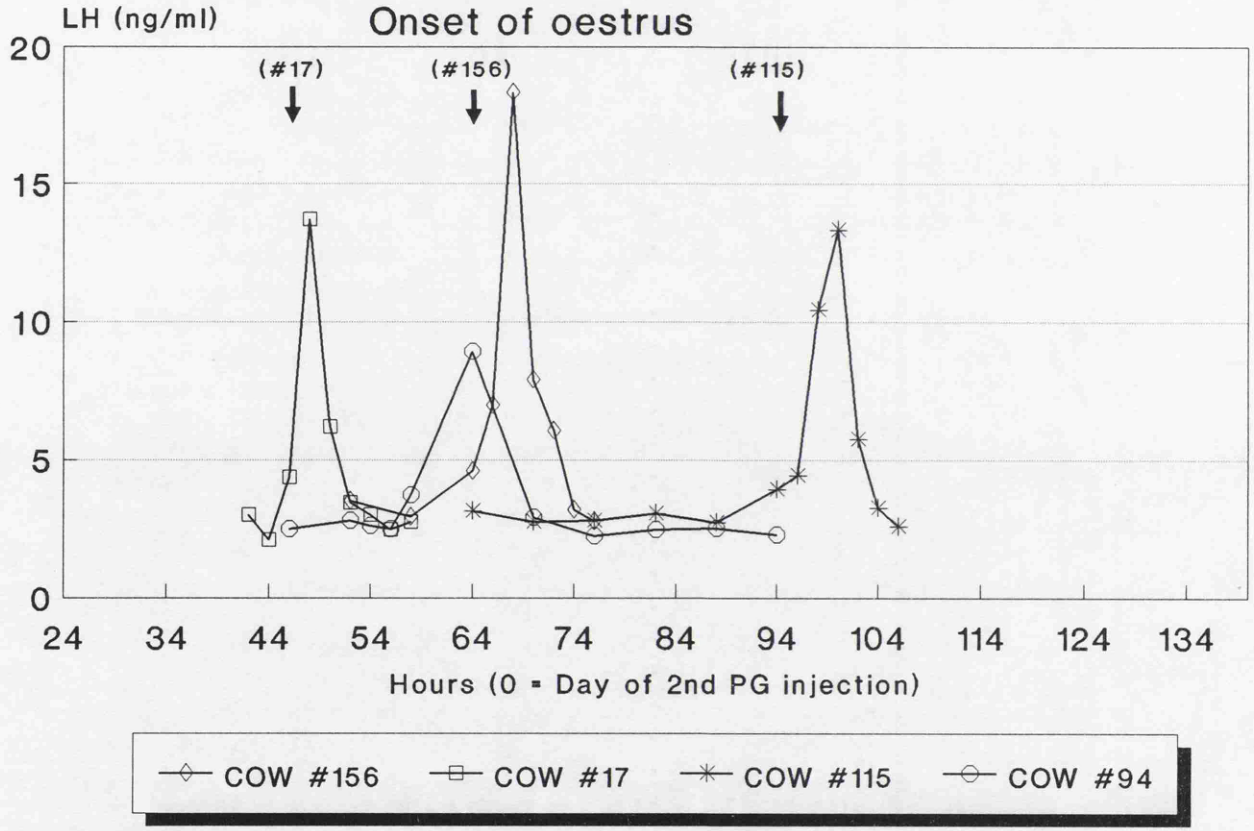
** End of treatment = either removal of progestin-releasing device or second injection of PGF2α analogue. Mean ± S.D.*

Figure 2.6.

In this figure the timing of the LH surge in relation to onset of oestrus for the four cows of this group is shown. Note that both the timing of the onset of LH surge and the timing of the first observed oestrus appeared to vary widely between individual cows.

Note that cow No. 94 was not observed in oestrus following the second Prosolvin injection.

Fig. 2.6 Pattern of LH surge at synchronized oestrus (PG group)



* The arrows indicate the onset of oestrus.

IV. DISCUSSION.

The studies described in chapter two were carried out using heifers which are known to present far less reproductive problems than cows. The use of culled dry cows, bought in the market, for the purpose of the present investigations appears unsound as these animals might have had reproductive problems. Consequently the cows were monitored for oestrous behaviour during two successive cycles prior to the beginning of the experiment, in order to determine the length of the oestrous cycle and eliminate those animals showing irregularities. Therefore the cows chosen for these studies have been cycling normally for a raisonnable length of time and this includes the cow with the cystic ovary. However this does not eliminate any variabilities in the results which may arise from the use of cows instead of heifers and so the possibility of observing differences in the response of these cows to treatments previously applied to heifers was expected.

Part (i): Progestogen/oestradiol groups

While recognizing the doubtful validity of comparing results from groups of just two animals the variation in hormonal and ovarian changes found amongst the groups treated by different regimes of progestogens are of interest. At the synchronized oestrus, five of the six cows treated conceived to fixed-time AI indicating that each treatment produced a follicle capable of responding to surges of LH which culminated in ovulation and release of an ova and surrounding cumulus cells at the appropriate time for fertilisation by semen inseminated artificially. In addition, and of paramount importance was the subsequent development of a functional luteal structure capable of secreting the

necessary pattern of progesterone to maintain a quiescent uterus for the housing and development of the conceptus. However the ovarian changes which occurred during the different synchronization programmes and their relationship to the demonstration of overt oestrus and the timing of the LH surge varied amongst the cows.

In two of the cows (group 3), at the start of the treatment when the progesterone was still relatively high, the absence of luteal structure on ultrasound examination is unexplainable other than by the human error associated with either the ultrasound readings or those from the progesterone assay.

The two cows implanted with the progestogen and injected with oestradiol valerate during their luteal phase (group 1), still had a detectable CL on ultrasound and a raised plasma progesterone concentration two days after this treatment. Although circulating plasma progesterone concentrations are known to drop more slowly and variably (up to 96 hours) following both natural and oestradiol-induced luteolysis compared to prostaglandin-induced luteolysis (12 hours; Seguin, 1979), the time factor in these two animals was longer than expected and indeed their CLs only ceased functioning between day four and six after initiation of the treatment corresponding to day 16 to 18 of the cycle. Therefore it is difficult to establish whether luteolysis was induced or occurred naturally.

Such adverse results to treatment with a combination of progestogens and oestrogens in cattle have been reported previously (Jones et al., 1989) and most probably occurred in a number of heifers in the field study described in chapter two and may explain why they did not conceive at the synchronized oestrus. The more frequent scanning and bleeding undertaken in these detailed investigations substantiated the occurrence of a delay in lysis of the functional

CL in cattle treated with oestrogens. As yet the mechanisms associated with lysis of the CL either by PGF2α and its analogues or by oestrogens are not fully understood. However, it would appear from the marked differences in the time taken for the circulating plasma progesterone to decline with the two different methods, that the effect of these two luteolytic agents involve different mechanisms.

The slower and more variable luteolytic action of oestradiol may result in some animals so treated having prolonged variable levels of circulating plasma progesterone. This is known to result in variations in follicular turnover with follicles persisting throughout such treatment in animals where the above baseline circulating plasma progesterone concentration is present. Follicular development is related to the amplitude and frequency of LH release which in turn is controlled by the concentration of circulating plasma progesterone (Savio et al., 1992). In addition the possibility of early CLs outliving the progestogen treatment has been discussed in chapter two and the detrimental effect of such results on the final outcome already highlighted. Therefore oestrogen-induced luteolysis may lead to asynchrony in some treated animals and be partly responsible for the poor conception rate when fixed-time AI is used after this type of synchronization programme.

In the animals treated with PRID the amount of progesterone released into the circulating plasma by the PRID were reasonably constant ranging between 5.8 and 7.7 ng/ml. These concentrations mimic the natural luteal phase and means that these animals were under the effect of high levels of progesterone similar to that experienced during a dioestrus phase and for a similar period of time. However in one animal at the time of removal of the PRID, the circulating plasma progesterone was almost baseline whereas in the other it was still raised

(4.0 ng/ml). Therefore apparently some PRIDs may cease secreting progesterone earlier than expected. Workers in the field use PRIDs for even longer periods than the ten days used here believing that progesterone continues to be released. The results presented here indicate that this might not be so. This aspect of PRID warrant further investigation. Not only has the level of progesterone to remain above 2 ng/ml to mimic the luteal phase and prevent ovulation from occurring during the treatment period but also it is important that this level is maintained as high as possible (> 4 ng/ml) in order to ensure a similar follicular turnover. Natural variability in the endogenous luteal and follicular status when the treatment is initiated, variability of the luteolytic action of oestradiol and release from the device of just above baseline progestogen concentrations (> 2 ng/ml and < 4 ng/ml), may all combine to result in the presence of follicles varying greatly in size and maturity. Hence the possible variation in the timing and amplitude of LH surge and the subsequent timing of ovulation. However it is interesting to note that although there were variations among animals in both the size and number of follicles present on the day of cessation of treatment, and in the amplitude and timing of the LH surge, this was not reflected in the timing of onset of oestrous behaviour. Assuming that the surge of oestrogens is closely associated to the LH surge and to the onset of oestrous behaviour the apparent correlation reported here could be due to the fact that oestrus detection was carried out far less often than intensive bleeding.

The frequency of the scanning undertaken during the treatment period may be thought insufficient for monitoring follicular turnover. However when Savio et al. (1988) successfully studied follicular changes during the oestrous cycle in the bovine by daily ultrasound examination and recorded the appearance of the

large dominant follicles they found that during a three wave cycle the first dominant follicle is present by day four, the second by day twelve and the third by day 16. Therefore using the appearance and regression of the dominant follicles as a means of determining successive waves of follicles ought to be possible by monitoring at the intervals used in the investigations reported here.

The follicular fluctuations observed by ultrasound during the treatment period indicated that except for the cow with cystic ovaries and cow No.19, all cows had at least two waves of follicles during these ten days. Ginther et al. (1989) and Savio et al. (1990) reported that the length of the oestrous cycle reflects the number of follicular waves such that a 21-day and a 24-day cycle consist of two and three waves respectively. In these small groups of cattle it seemed that two waves of follicles occurred during a period equal to less than a half that of a 21-day oestrous cycle. In all animals the first wave was already present when the treatment was initiated meaning that the full length of this wave did not occur during the treatment. Although the number of waves is disputable owing to the frequency of scanning the evidence is that in five of the six cows (the exception is cow No. 19), the dominant follicle present at the beginning of the treatment was replaced by another one from a newly developing wave. This may indicate that the level of progesterone/progestogen released in the blood stream by the devices was high enough to allow for follicular turnover to occur. In one cow (group 3; No. 19) the large follicle observed at insertion of the PRID apparently persisted throughout the treatment period and subsequently ovulated. This animal conceived somewhat unexpectedly when mated by AI. This is a prolonged period of time for the dominant follicle to be present prior to a fertile ovulation. It could be that this dominant preovulatory follicle present in this cow prior to the insertion of the implant had already been selected to

ovulate. Despite the presence of this persistent dominant follicle, growth of other follicles in this animal occurred in one ovary. However none of these subsequently developing follicles reached ovulation revealing an intriguing form of dominant-subordinate follicular relationship. Recently Savio et al. (1992) reported the persistence and ovulation of the dominant follicle of the first wave in cattle undergoing synchronization with low progesterone-containing devices and suggested this resulted in low fertility. These authors explained this phenomenon as being caused by an increase of LH pulses in response to the lower progesterone concentrations. It appears that LH pulses of high amplitude inhibit further follicular growth allowing the dominant follicle of the first wave to persist and ovulate. This could explain the reported good synchronization but the poor fertility associated with the use of long term progestin-releasing devices (Odde, 1990). However in the cow with the persistent dominant follicle, follicular growth appeared to be inhibited only in one ovary and ovulation of the persistent follicle resulted in fertilization and successful conception. Further studies are needed to identify the mechanisms associated with these findings. Obviously it would have been beneficial to know the plasma concentrations of norgestomet during the treatment.

In the five apparently normal cows the interval from removal of treatment to onset of oestrus was approximately 44 hours irrespective of whether the cows were treated with Crestar or PRID. Jones et al. (1989) reported a longer (54 ± 4.1 hours) period from removal to onset of oestrus which could be attributed to these workers undertaking a less frequent schedule (twice daily) for observation of oestrous behaviour.

There were individual differences among cows in the timing and amplitude of the LH surge. However this did not appear to result in differences in terms of

the timing of LH surge and pregnancy rate among groups.

In one cow (No. 23) the amplitude of the LH peak was smaller than in the others. Two possible explanations could be considered: (a) the most likely is that our two-hourly bleeding schedule was not sufficiently frequent to identify the complete duration and magnitude of the peak. Rahe et al. (1980) who reported surges of higher amplitude in the cows they studied, collected blood samples for assay at 15 minutes intervals; (b) that the absence of large follicles (> 1.5 cm) at removal of the progestogen device resulted in the smaller surge of LH. Some credence for this second explanation is given by study of another of the cows (No. 7) which also had only medium-sized follicles at removal of the Implant, and here the LH peak was delayed by approximately six hours as compared to the average timing in the five apparently normal cows. Again this variation in the LH pattern could be associated with the absence of a large follicle at the time of removal of the progesterone device. Thus both these LH findings could indicate a relationship between the follicular status of the ovaries and the amplitude and timing of the LH surge.

Whether the lower amplitude or the delay in the LH peak reported in these studies resulted in delayed ovulation is not known but ovulation occurred within the required period as both of these cows conceived at the synchronized oestrus. Mikeska and Williams (1988) reported poor conception rate (10%) in a group of cattle where the LH surge was delayed for more than 12 hours after onset of oestrus.

The LH surge was closely related to onset of overt oestrus in four out of six cows including the cystic ovary cow. These findings are in agreement with those reported by Mikeska and Williams (1988) who in a study of oestrus synchronization in heifers using progestogen/oestradiol treatment reported that

the mean interval from onset of oestrus to ovulation and onset of LH surge to ovulation were approximately 23.3 and 23.1 hours respectively.

In the cow with cystic ovaries there was apparently suppression of follicular development in the ovary containing multiple cysts but only a partial inhibition in the other ovary until after removal of the progestogen device. Inhibition of follicular growth does occur in normal cycling bovine, when a large dominant follicle suppresses the growth of medium-sized follicles and the appearance of other follicular waves until it has either ovulated or started regressing (Ko et al., 1991; Roche and Boland, 1991). Despite all the extensive work done in this field, the mechanisms governing this action of the dominant follicle are as yet not fully understood. The follicular growth inhibited in the cystic cow may be associated with the same mechanisms.

Both the onset of oestrus and the onset of the LH surge occurred a few hours earlier in the cow with cystic ovaries than in the remaining cows suggesting a possible contribution of the cysts to the surge of oestrogens which may have resulted in both oestrus and the LH surge evolving prematurely and so causing the release of the oocyte before proper maturation. Hence the failure in conception.

The fertile life of fresh sperm in the bovine female genital tract after natural mating ranges between 30-48 hours (Hafez, 1987a). In this experiment frozen semen inseminated 4 hours after onset of overt oestrus achieved conception.

This would tend to suggest that the frozen semen used remained fertile in the female tract for at least 19 hours if ovulation occurred as is reported, at least 23 hours after the onset of oestrus in cows.

In conclusion this study suggested that :

a) Combinations of a progestogen (norgestomet) with oestradiol valerate was equally as effective as that of progesterone with oestradiol benzoate in synchronizing oestrus regardless at what stage of oestrus cycle they were administered.

b) The timing of the LH peak and its amplitude may be affected by the state of follicular development at the time of removal of the progestogen/progesterone treatment.

c) As already reported (reviewed by Ijaz, 1987) the use of progestogens in a cow with cystic ovaries can lead to a normal oestrus following treatment.

d) Frozen semen remains viable for at least 19 hours after intrauterine insemination.

e) There seem to be a substantial credence given in these studies to the results described earlier by Peters et al. 1984. These authors showed that the use of progestogens in synchronizing oestrus in cattle could be improved by using PGF2α immediately prior to the removal of progestogen in order to remove existing luteal tissue. Avoiding the persistence of dominant follicles during the treatment period could also be achieved by improved control of progesterone release from the devices to ensure a consistently high circulating plasma progesterone concentration (> 4 ng/ml; Savio et al., 1992) throughout the treatment.

The use of only two animals per group was a major impairment in these investigations and constitutes a limitation to any conclusive statement. Much of the variability observed between individuals can be eliminated by using large groups of animals. This was not possible under the prevailing circumstances.

Part (ii): Prostaglandin group

In this small group of cattle the response to a double injection of a prostaglandin analogue luprostiol was monitored by looking at both oestrous behaviour and hormonal changes including timing and amplitude of the LH surge around synchronized oestrus.

Although on the day of the first prostaglandin injection all animals treated had, on rectal examination, a palpable CL of a size corresponding to a functional CL, three cows out of the four treated (Nos. 115, 17 and 156) were not subsequently seen in oestrus. Two possible explanations for this finding are: (a) the cows were in early dioestrus when injected and the developing CL did not respond to the prostaglandin treatment. It is now established that CLs less than five days after ovulation do not respond to this type of treatment, possibly because the necessary receptor-sites are not yet available on the membranes of the luteal cells. If in these cows the CLs were less than five days old then the original rectal findings would appear faulty. Recently a series of rectal findings in cattle were subsequently checked by ultrasound and or post-mortem and it was established that while mid-cycle CLs are as accurately detected by rectal examination as by ultrasound, both techniques were inaccurate for the detection of young and old CLs (Pieterse et al., 1990). However among the results from the field trial presented in this thesis (chapter two), there were a few occasions where a sizeable CL did not produce the corresponding circulating plasma progesterone concentration and/or did not respond to the prostaglandin injection. It was postulated that there may be occasions where growth of the structure does not go hand in hand with its maturity and so early CLs may become palpable before they are capable of responding to the prostaglandin treatment.

The other possible explanation for consideration is (b) some of the cows did

respond to the treatment and came in oestrus but were missed due to the infrequent oestrus detection or had 'silent heats'. Most authors accept that silent oestrus or 'silent ovulation' as is called by some of them can occur in cattle but tends to do so in the first ovulation after parturition (Savio et al., 1990).

Indeed the only cow which apparently responded to the first injection (No. 94) may well have had a silent oestrus following the second prostaglandin injection. Despite this absence of oestrous behaviour, cow (No. 94) did respond with a surge of LH, which was somewhat attenuated. This along with the clinical signs of approaching oestrus demonstrated suggest an abnormal pattern of oestrogens secretion by the maturing follicle possibly with a smaller peak than normal. However in the absence of an oestradiol assay no evidence can be provided for this hypothetical explanation.

Comparing the clinical and hormonal parameters in the two different types of treatment described in this chapter (progestin and prostaglandin), some differences appeared in both the average amplitude of LH surge and the intervals between second prostaglandin injection or removal of progestin device to both onset of LH surge and first observed oestrus. However these differences could not be shown as statistically significant probably owing to the small number of animals used. Many authors (Louis et al., 1973; Jackson et al., 1979b) reported similar intervals for prostaglandin-treated animals and drug manufacturers recommend a shorter interval between removal of the progestin-releasing device and AI than between the second prostaglandin injection and AI. This difference could be due to the nature of action of the two treatments. Progestogens act by suppressing the release of both the hypothalamic releasing factors and the pituitary gonadotrophins thereby allowing their build up in the site of their synthesis so that on the removal of the progestogen both GnRH and the gonadotrophins FSH and LH are available in sufficient amounts. Once

released both FSH and LH immediately initiate the related ovarian activity required to produce a normal mature follicle at the appropriate time. Prostaglandins on the other hand seem much more traumatic in that they interrupt the normal dioestrus period shortening the time available for the hypothalamo-pituitary system to build up its reserves of gonadotrophins and/or their releasing factor and thus the level of gonadotrophins present may not be as great as with progestogen treatment and so follicular growth and development including oestrogen secretion takes longer. The association of the longer interval 'second injection to onset of oestrous behaviour' and 'release of the LH surge' to follicular development and maturity appears obvious as the demise of the CL following prostaglandin injections by intramuscular route is known to take approximately 12 hours. However both methods require a responsive gonad with follicular content that can cope with the interruption of the normal mechanisms. The integrated mechanisms for follicular growth and gonadotrophin synthesis and release still require investigations by *in vitro* studies of the capabilities of follicles collected at different phases of the oestrus cycle and at different times during intervals between the end of such treatments and the induced oestrus.

In all cows regardless of whether or not they conceived, the plasma progesterone concentration was raised six or seven days after insemination. The plasma progesterone concentration varied among the four cows and appeared to be correlated to the amplitude of the LH surge. However this apparent relationship remains mere observation in such a small number of individuals and this relationship needs meaningful statistical proof.

The findings described here were detailed enough to highlight the importance of the relationship between the CL the follicle and the hypothalamo-pituitary

axis. Moreover it was also clear from the variations encountered among individual animals that the level of progesterone secreted during the luteal phase is critical to the turnover of the follicular population present. The latter being important for subsequent CL formation and maintenance it becomes difficult to guess which of the two, the follicle or the CL, may initiate a cycle of ovarian malfunction. Therefore the study of key aspects of the structure and function of apparently normal CLs is needed. This will then allow comparison with CLs formed after induced ovulation of dominant follicles from different waves.

CHAPTER FOUR

A STUDY OF THE STRUCTURE AND FUNCTION OF CORPORA LUTEA OF VARIOUS STAGES OF THE CYCLE

I. INTRODUCTION

In both the field study and the detailed investigations presented in chapter three, the need for a better understanding of CL formation, function, maintenance and regression was demonstrated. The importance of this structure was emphasized when poor responses to oestrus synchronization programmes were shown to be the result of unpredictable luteal reaction.

A study of CLs suspected of naturally or artificially induced abnormalities requires detailed knowledge of the normal structure during the different stages of its life-span.

Initially structural and functional aspects of the bovine cyclical CL were studied using histology, histochemistry, cell culture and electron microscopy. The stage of the oestrous cycle was deduced by gross macroscopic examination of luteal structures and associated ovarian follicles. Furthermore circulating plasma progesterone prior to slaughter was used as an additional means of monitoring the functional status of the subsequently collected CLs.

II. MATERIALS AND METHODS

A. COLLECTION OF BLOOD SAMPLES

Blood samples were collected at slaughter from heifers into labelled heparinized tubes (Sarstedt Ltd, Leicester, England). Plasma was separated by centrifugation (1000 x g, for 10 minutes), decanted and stored at -20 °C until assayed.

B. TISSUE COLLECTION

For the macroscopic and histological studies, ovaries bearing a CL were removed from the heifers from which blood samples had been collected and transported to the laboratory in warm physiological saline.

Ovaries destined for histochemical studies were transported to the laboratory in a thermos containing ice after a small block of luteal tissue was removed from the inner parts of the structure and immediately fixed for electron microscopy.

CLs for cell culture studies were dissected from the ovaries and placed immediately in 50-ml plastic bottles containing ice-cold medium 199 supplemented with 25 mM hepes, Earle's salts and glutamine (Gibco, Life Technologies Ltd., Paisley, Scotland, UK).

C. TISSUE PROCESSING

Part (i): macroscopic and histological studies

1. Macroscopic description

Upon arrival at the laboratory gross macroscopic examination of each luteal structure was carried out. The parameters recorded were those described by Ireland et al. (1980) with some modifications and are recorded in table 3.1.

2. Histology

2.1. Fixation

2.1.1. Reagents and preparation of fixative

Bouin's fluid (Culling, 1974) was used as fixative for all histology samples:

Picric acid, saturated aqueous solution	75 ml
Formalin (40% formaldehyde)	25 ml
Glacial acetic acid	5 ml

A stock solution was made up by adding formaldehyde to picric acid followed by the addition of glacial acetic acid. The solution was kept at room temperature for an indetermined period and was used when needed.

2.1.2. Method of fixation

A block of luteal tissue of approximately 5 mm in size was removed from each CL and immersed in Bouin's solution for 24 hours.

Table 3.1 Criteria used during gross macroscopic examination of CLs to determine the stage of the cycle (adapted from Ireland et al., 1980).

Stage of the cycle (days after ovulation)	External appearance			Internal appearance			Associated follicles of >1.0 cm in size
	Colour	Epithelium over apex	vasculature	Tissue consistancy	Colour	Size (*cm*)	
First *(1-4)*	Pale, haemorrhagic point of rupture	Absent	Absent	Friable	Red or brown	<1.6	Absent
Second *(5-10)*	Dark red or brown	Present	Present but not covering apex	Compact soft	Red or brown apex, remainder orange or tan	>1.6	Present
Third *(11-17)*	Orange or tan	Present	Present, covering apex late during this stage	Firm	Tan or orange	>1.6	Present or absent
Fourth *(18-21)*	Light yellow to white	Present	Not visible	Hard	Light orange to yellow	<1.6	At least one present

2.2. Processing for histology

Using an automatic 24-hour tissue processor (Shandon Elliot Ltd) the tissue blocks were taken through the following steps:

70% spirit + 5% phenol	2 hours
90% spirit + 5% phenol	2 hours
Methylated spirit	2 hours
Absolute alcohol + 5% phenol (I)	2 hours
Absolute alcohol + 5% phenol (II)	1 hour
Absolute alcohol + 5% phenol (III)	1 hour
1% celloidin in methyl benzoate	4 hours
Histoclear (I)	1 hour
Histoclear (II)	1 hour
Histoclear (III)	1 hour
Paraffin wax (I)	5 hours
Paraffin wax (II)	5 hours
Blocking out in fresh wax.	

2.3. Cutting

5 μm-thick sections were cut using an optical microtome (Spencer AO 821, American Optical Company, Buffalo 15, NY, USA), mounted on glass slides and dried in an oven at 56 °C for one hour.

2.4. Staining

2.4.1. Haematoxylin and Eosin (H & E)

Haematoxylin and eosin stain was prepared and used according to the method described by Mayer (1903).

a) Reagents and preparation of stain

Heamatoxylin

Haematoxylin	1 g
Potassium alum	50 g
Sodium iodate	0.2 g
Citric acid	1 g
Chloral hydrate	50 g
Distilled water	1 l

Hematoxylin was dissolved in water by gentle heating, then potassium alum was added and the mixture again heated gently. Sodium iodate, citric acid and chloral hydrate were then added and the mixture was boiled for five minutes, then cooled and filtered.

Eosin

Eosin	3 g
Absolute alcohol	100 ml

A saturated solution of eosin was prepared by adding eosin to absolute alcohol.

b) Staining procedure

Sections were agitated in xylene for one to two minutes, transferred to absolute alcohol for one minute, removed and placed in methylated spirit for one minute

and rinsed in water. A few drops of iodine were added and left in the solution for one to two minutes. The sections were again rinsed in water for 30 seconds and hypoed for one minute with 5% sodium thiosulphate, washed again in water and then placed in the haemalum solution for five minutes, rinsed in water and immersed rapidly three times in acid alcohol, washed again in water for 30 seconds and blued in Scott's tap water (2 g potassium bicarbonate and 20 g magnesium sulphate in 1 litre distilled water) for 90 seconds. They were washed in water for two minutes and rinsed in methylated spirit then placed in saturated alcoholic eosin for 20 seconds and rinsed in methylated spirit and then dehydrated, cleared and mounted in DPX.

2.4.2. Martins Scarlet and Blue (MSB)

This stain was prepared and used as described by Lendrum et al. (1962).

a) Reagents and stain preparation

Martius yellow/phosphotungstic acid (0.5%)

Martius yellow (acid yellow 24)	0.5 g
Phosphotungstic acid	2.0 g
95% alcohol	100 ml

Martius yellow and phosphotungstic acid were dissolved in 95% alcohol to make up a stock solution of the stain.

Brilliant crystal scarlet (1%)

Brilliant crystal scarlet (acid red 44)	1.0 g
Glacial acetic acid	2.0 ml
Distilled water	100 ml

Brilliant crystal scarlet was dissolved in glacial acetic acid and water was added to make up this solution.

Methyl blue (0.5%)

Methyl blue (acid blue 93)	0.5 g
Glacial acetic acid	1.0 ml
Distilled water	100 ml

Methyl blue was dissolved in glacial acetic acid and water was added to make up this solution.

b) Staining procedure

Sections were dewaxed and washed in water. They were then taken through the preliminary stages as described for haematoxylin staining up to the acid alcohol treatment stage. Following a good wash in tap water sections were rinsed in 95% alcohol then stained in 0.5% Martius yellow/phosphotungstic acid for two minutes, rinsed in distilled water and stained in 1% brilliant crystal scarlet for ten minutes, rinsed in distilled water, treated with 1% phosphotungstic acid in distilled water for ten minutes, rinsed in distilled water, and stained in 0.5% methyl blue solution, rinsed in 1% acetic acid, rinsed in water dehydrated, cleared and mounted in DPX.

2.5. Examination of sections

Sections were examined under light microscope at magnifications of (x 125, x 250, x 500 and x 1250). For all sections ten randomly chosen fields were examined and cells belonging to the two different luteal cell-types were counted. The type of cell undergoing either mitosis or regression were also recorded. Other details assessed were degree of connective tissue penetration, presence of

spaces between cores of cells and thickness of blood vessel walls. Finally individual cells were closely examined and the following parameters recorded:

(i) cell outline

(ii) cytoplasm: stippling and vacuolization

(iii) Nucleus:

- outline (membrane)
- shape
- intensity of staining
- presence of heterochromatin
- presence of nucleoli

Part (ii): enzyme histochemistry

1. Tissue preparation

In the laboratory, after allocation of the CLs to the corresponding stage of the cycle as previously described, each was divided into four to eight cubes, put into labelled cryotubes and snap frozen by total immersion in liquid nitrogen tanks where they were stored until used.

2. Cutting

Frozen blocks of tissue were transferred to a cryostat (Slee ltd, London, England) and mounted on cryostat block holders using an embedding medium for frozen tissue (Tissue-Tek, Miles Inc., Diagnostics Division, Elkhart, IN 46515 USA). Eight 15 μm-sections were cut at -15 °C from each CL, placed on glass microscope slides (Blue Star Micro Slides, Chance Proper Ltd., Warley, England) and stored at -20 °C until used on the same day.

3. Reagents and preparation of media

All reagents were purchased from Sigma (Chemical Company Ltd, Poole, Dorset, England). The incubation medium was prepared as described by Lobel and Levy (1968). First the following five stock solutions were made up:

Solution (i)

Polyvinyl pyrrolidone (PVP)	6.35 g
0.1 M Phosphate buffer *pH* 7.4	45 ml

Polyvinyl pyrrolidone was dissolved in 0.1 M phosphate buffer *pH* 7.4, using a magnetic stirrer (SM1, Stuart Scientific Co. Ltd., UK).

Solution (ii)

Nitro tetrazolium blue	20 mg
Distilled water	20 ml

Nitro tetrazolium blue was added to distilled water to make up solution (ii).

Solution (iii)

β-Nicotinamide Adenosine Dinucleotide (NAD)	30 mg
Distilled water	5.0 ml

Solution (iii) was made up by dissolving NAD in distilled water.

Solution (iv)

Steroid substrate	15 mg
Dimethyl formamide	5.0 ml

Solution (iv) was made up by dissolving the steroid substrate in dimethyl

formamide. The steroid substrate used for 3β-HSD and 20α-HSD were 5-androsten-3β-ol-17-one (dehydroepiandrosterone or DHA) and 20α-hydroxypregn-4-en-3-one (20α-hydroxyprogesterone) respectively.

Solution (v)

$NADH_2$ (grade III)	30 mg
Distilled water	5.0 ml

Solution (v) was made up by dissolving the reduced form of β-Nicotinamide Adenosine Dinucleotide ($NADH_2$).

These solutions were stored at 4 °C for up to 2 weeks as this was found not to interfere with the reactions when compared with freshly made solutions. Final incubation media contained:

Full treatment medium

Solution (i)	4.7 ml
Solution (ii)	2.0 ml
Solution (iii)	1.0 ml
Solution (iv)	0.3 ml

The full treatment medium was used to detect the enzymes activity. It was made up by first adding solution (ii) to solution (i). Solution (iii) was then added followed by solution (iv).

Control (lacking the steroid substrate)

The control solution was made up as the treatment medium except that solution (iv) containing the substrate steroid was omitted and replaced by 1 ml of dimethyl formamide.

Control of diaphorase activity

The medium used to test the presence of diaphorase activity was made up by first adding solution (ii) to solution (i). Then 1 ml of solution (v) containing reduced NAD was added instead of solution (iii). Solution (iv) was omitted and replaced by dimethyl formamide.

4. Processing for histochemistry

Slides prepared from each CL were placed in open boxes and incubated at room temperature for 90 minutes after adding a few drops of the appropriate staining medium to the slides and covering the box with moistened paper to limit evaporation of the medium. For each CL two slides were used to test for 3β-HSD, two for 20α-HSD, two for control treatment with no substrate and two to test diaphorase activity.

Following incubation slides were rinsed in distilled water then washed in cold acetone for five minutes to remove lipid droplets. Sections were fixed in 15% paraformaldehyde in saline for 15 minutes. Dehydration was carried out by immersing slides in 50% through 70%, 90% and finally absolute alcohol for five minutes each. They were then airdried and mounted in DPX.

In parallel studies, sections were prefixed and/or prewashed in acetone as a variation to the standard method. Initially counter staining was carried out

following fixation of the sections in an attempt to identify the different cell-types in order to assess any variability in enzyme activity from one cell-type to another. The dye used for this purpose was light green (acid green 5, colour index No. 42095).

3. Examination of sections

Sections were observed under light microscopy at magnifications of (x 125, x 250 and x 1250) and scored as follows:

+ + +	intense blue staining
+ +	moderately intense blue staining
+	light staining
-	negative

Part (iii): transmission electron microscopy

1. Fixation

1.1. Reagents and preparation of fixative

FGP fixative was prepared according to the method described by Ito and Karnovsky (1968) and modified by O'Shea et al. (1989):

1.1.1. Reagents

a) Buffer

0.1 M cacodylate buffer *pH* 7.2 at room temperature was made up by adding together the following reagents:

Sodium cacodylate	1.07 g
$CaCl_2$	0.176 g
Distilled water	25 ml

The *pH* was adjusted to 7.2 with addition of small quantities of 1 M HCl.

b) Fixative

Paraformaldehyde	2.5 g
Picric acid	0.5 g
0.1 M Cacodylate buffer	30 ml
25% Glutaraldehyde solution (EM grade)	20 ml
1 M NaOH	drops
Distilled water	100 ml

1.1.2. Preparation

Fixative was prepared within a 24-hour period of its use as follows:

Paraformaldehyde was dissolved in distilled water by heating it to 70 °C. A few drops of 1 M NaOH were then added until precipitate cleared and solution was cooled. Picric acid was added followed by 0.1 M cacodylate buffer and 25% glutaraldehyde solution. The prepared fixative was stored in labelled bijoux bottles after filtering.

1.2. Method of fixation

A small block of tissue (1 to 2 mm) was removed from the inner parts of each CL and put in the fixative for 24 hours.

2. Processing for transmission electron microscopy

After removal from the fixative the tissue blocks were washed by adding fresh cacodylate buffer and shaking by placing the bottles on a bottle rotator for 15 minutes. Then the buffer was replaced with 1% osmium tetroxide in isotonic cacodylate buffer (2 ml per bottle) and the tissue samples exposed to this for two hours. The samples were then rinsed with cacodylate buffer and dehydrated by taking them through a series of alcohols of increasing concentrations (50%, 70%, 80% and 90%; 15 minutes each) followed by immersion in absolute alcohol twice for periods of 15 minutes. The blocks of tissue were then exposed to propylene oxide twice for periods of 15 minutes each and then to propylene oxide/resin 1:1 overnight. Bottles were capped to avoid evaporation of the propylene oxide. Propylene oxide/resin 1:1 was then replaced with propylene oxide/resin 1:3 for a 24-hour period after which bottle caps were removed and propylene oxide was allowed to evaporate. When all of propylene oxide had evaporated, specimens were blocked by fresh resin embedding. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a AEI GB electron microscope at magnifications of x 3000 and x 15000.

Part (iv): cell culture

1. Tissue dissociation

The method described by Simmons et al. (1976) was used with some modifications. Prior to enzymatic dissociation CLs were dried and weighed. Cubes of tissue of 1 to 2 mm in size were obtained by slicing the luteal tissue using two scalpel blades. Physical damage was kept to a minimum by transferring tissue slices into 50 ml-plastic bottles (Greiner Labortechnik Ltd, Dursley) containing medium 199 at frequent intervals during the slicing process.

The tissue slices were washed in fresh medium 199 twice in order to eliminate debris and as many of the erythrocytes as possible. They were then added to medium 199 (5 ml/g of tissue) containing 2000 iu/g type A1-collagenase (Sigma Chemical Co, Ltd, Poole Dorset, England) and 3000 iu/g deoxyribonuclease (DNAse, Sigma Chemical Co, Ltd, Poole Dorset, England) and transferred to a shaking water bath incubator (Gallenkamp, Fisons Scientific Equipment, Loughborough, England) at 35 °C, 80 cycles/minutes. When the suspension became orange in colour (45-90 minutes, depending on the CL), contents were drawn in and out of a Pasteur pipette (Bilbate Ltd, Daventry, England) to free cells which could be still trapped in the blocks of tissue. The undissociated pieces of tissue were left to settle (2 minutes) before decanting the cell-containing phase of the suspension which was then centrifuged (100 x g, 5 minutes). The supernatant was discarded and the pelleted cells resuspended in fresh medium 199 containing no enzymes and stored at 4 °C.
More medium with enzymes was added to the remaining undissociated tissue and dissociation in the shaking incubator was repeated for a further 45 to 90 minutes at the end of which the suspension was decanted, cells pelleted and resuspended as described.

Complete enzymic digestion was carried out in two to three steps depending on the consistency of the CL. For each CL the cell suspensions from all dissociations were pooled together and washed three times by centrifugation and resuspension. The suspension obtained was passed through two mesh prefilters of 80 μm and 45 μm pore diameter (Millipore, UK Ltd, Research Park Riccarton, Edinburgh). The filters were washed with additional medium to free single cells retained in the filters. This cell suspension was pelleted once again and resuspended with an appropriate volume of medium to allow for cell counts and viability assessment.

2. Cell counts and viability

A small volume (10 μl) of the cell suspension made up with an appropriate amount of medium was added to an equal volume of 0.4% trypan blue in 0.9% saline and 10 μl of this solution placed in the chamber of a haemacytometer (Weber, England) and viewed under a light microscope. Cells excluding the dye were considered to be alive and counted (Tennant, 1974). Large (> 25 μm) and small (< 20 μm) luteal cells proportions were determined for each CL.

3. Incubation

Triplicate samples of between 17000 to 25000 viable cells were placed in siliconized round-bottomed glass tubes (Sterilin Ltd, Hounslow, UK) containing 1 ml Hepes-suplemented medium 199 to which 5% donor calf serum, 100 iu/ml Penicillin and 100 μg/ml streptomycin (Gibco BRL, Life Technologies, Paisley, Scotland) were added. Incubation was then carried out in the shaking water bath incubator at 35 °C, 80 cycles per minute under ambient atmosphere.

Sample 1: after 15 minutes one sample was removed and its contents snap-frozen in liquid nitrogen to act as "incubation-time 0".

The other tubes were treated as follows:

Sample 2: 13 ng LH (USDA\bLH\1\1) in 100 μl medium 199.

Sample 3: 13 ng LH in 100 μl medium 199 + 1μg synthetic PGF2α (luprostiol, Prosolvin Intervet UK Ltd) in 100 μl medium 199.

Sample 4: 1 μg synthetic PGF2α in 100 μl medium 199.

Sample 5: 100 μl of medium 199 containing no hormones for control unstimulated baseline progesterone production.

Incubation was continued for samples 2 to 5 for a further period of 3 hours at the end of which the content of each tube was snap frozen in liquid nitrogen and all tubes stored at -20 °C until assayed for progesterone.

4. Progesterone estimation

Progesterone concentration in cell culture medium was determined using the microencapsulated antibody technique (Wallace and Wood, 1984) modified by Jeffcoate and Lindsay (1989).

Progesterone antiserum was raised in rabbits against 11α-hydroxyprogesterone hemisuccinate conjugated to bovine serum albumin. Cross-reaction of this serum with 11α- and 17α-hydroxyprogesterone, 5α-pregnene-3,20-dione and 11-deoxycorticosterone were 82, 3.6, 3.3 and 1.8% respectively and < 1% with other steroids.

Tracer provided by Dr C. E. Gray, The Royal Infirmary, Glasgow, UK, 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).

Progesterone (Sigma Chemical Co., Poole, Dorset, UK) was dissolved in assay diluent to prepare standards ranging from 0 to 20 ng/ml.

Samples and standards (200 μl) were extracted in duplicate with 3 ml diethyl ether (analar grade, May & Baker, Dagenham, Essex, UK) by mixing for five minutes on a multi-tube vortexer (SMI, Alpha Lab., 40 Parham Dr., Eastleigh, Hampshire, UK.). Extraction efficiency was 79 ± 6% (n = 8). The ether phase of each extract was decanted into a glass assay tube after freezing the aqueous phase in a methanol/dry-ice bath, and then dried in air.

Tracer (100 μl, approximately 10000 cpm) and primary antiserum (200 μl, 1:20000) were added and the tubes were incubated in a water bath at 37° C for 45 minutes.

Second antibody (donkey anti-sheep/goat gammaglobulin, 1:20 containing 1:200 normal goat serum, 400 μl; SAPU, Law Hospital, Carluke, Strathclyde, UK.)

was added and incubated at 4°C overnight.

Tubes were centrifuged at 4 °C, 2000 x g and for 15 minutes and the antibody-bound fraction was counted after the supernatant had been aspirated.

Assay sensitivity (2 x standard deviation of total binding) was 0.2 ± 0.02 ng/ml (n = 8). The inter- (n = 13) and intraassay (n = 10) coefficient of variation for two plasma pools containing either high (6 ng/ml) or low (0.5 ng/ml) progesterone concentration were 7.9% and 22% and 6.2% and 17% respectively.

Samples containing incubation medium and killed cells were thawed immediately prior to each assay. Typically each assay included samples from one or two CLs. All samples from a particular CL were done as a single assay to minimize variation.

In an initial assay two sets of samples were prepared by diluting aliquots of incubation medium corresponding to a single CL in assay buffer containing 0.25% bovine serum albumin. The first set was made up by adding 400 μl of incubation medium to 7.6 ml of assay buffer to give a dilution of 1:20. The second set was made up by adding the same amount of incubation medium to 3.8 ml of assay buffer to give a dilution of 1:10. The first set of dilutions (1:20) was found to give better results than the second set (1:10) as it resulted in fewer values being out of range. Progesterone estimation corresponding to a range of 0.5 to 5.0 ng/ml in samples diluted 1:10 and 1:20 were similar (1:20 dilution value ≈ 109% x 1:10 dilution value). Subsequently all samples were made up by a 1:20 dilution of incubation medium in phosphate buffer.

D. STATISTICAL ANALYSIS

Statistical tests were carried out using the version 7.1 of Minitab statistical software (Minitab Inc, State college, PA 16801, USA). A *P*-value ≤ 0.05 was considered as a significant result.

Histology (cell-type proportions): following examination of ten fields per slide (one slide per CL), a mean of the estimated percentage of cells was computed for each of the two luteal cell-types. For each stage a mean (± s.e.m) of the means was then calculated. Changes in cell-types proportions between stages of the cycle were compared using *Student's t-Test*.

Cell culture (progesterone production): three aliquots per treatment per CL were used. The amount of progesterone found in the control aliquot ie. "incubation-time 0" sample, was deducted from the progesterone found in all other samples ie. medium-, LH-, PGF2α- and LH + PGF2α-treated samples. A mean was estimated for each triplicate and a mean (± s.e.m) of the means was used to express progesterone production per type of treatment and stage. Mean progesterone production was compared between types of treatment using paired *Student's t-Test*.

III. RESULTS

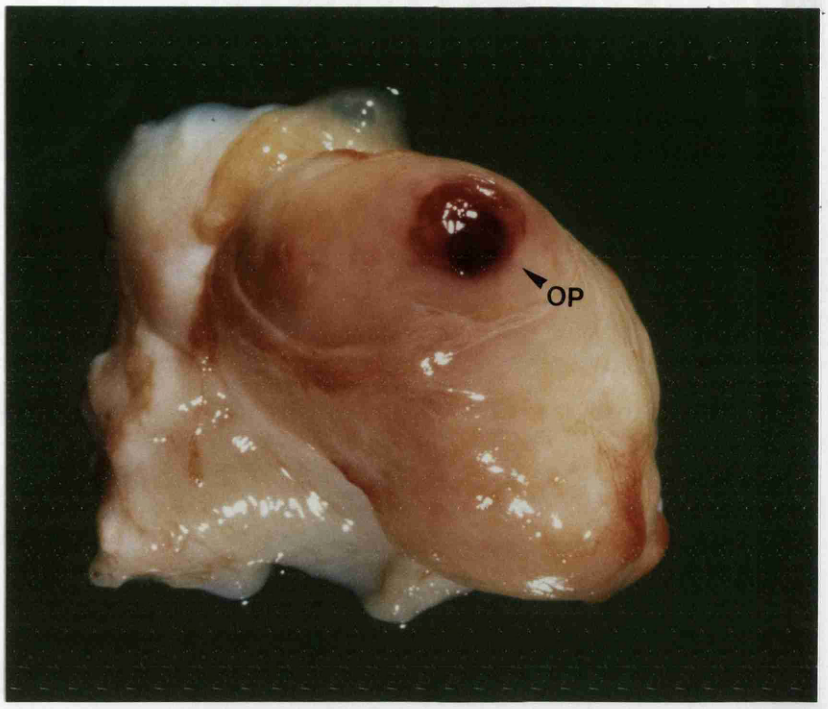
Part (i): macroscopic studies

Plate 3.1a.

The external appearance of a CL from the first stage (day 1-4) is shown in plate 3.1a. Note the obvious presence of coagulated blood at the ovulatory papillum (OP) which is still not covered by the epithelium. The body of the structure is still embedded within the ovarian stroma.

Plate 3.1b.

The internal appearance of the CL shown in plate 3.1a is shown after sectioning. The tissue of CLs at this stage is friable and the whole structure contains numerous empty spaces between folds of luteal tissue.


OP

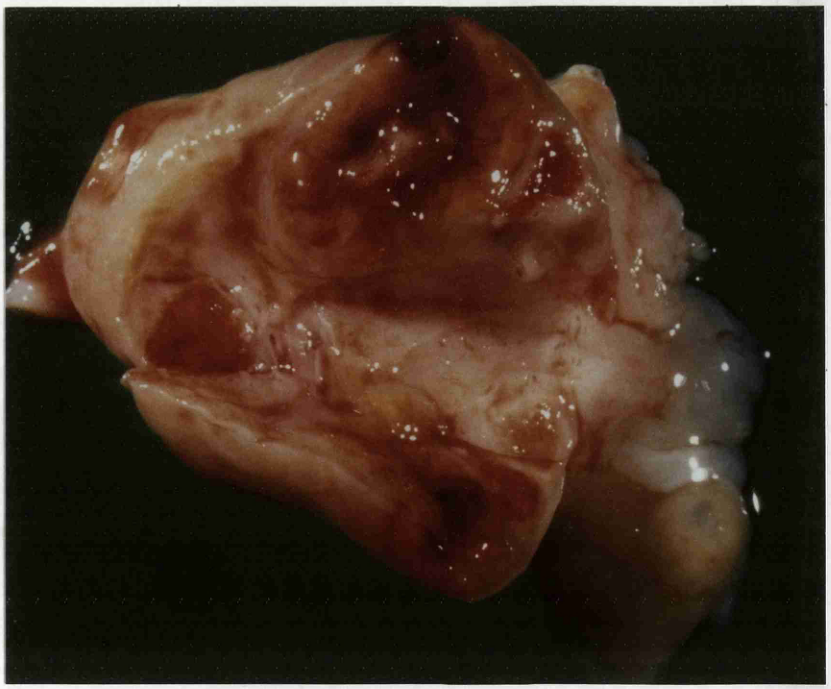
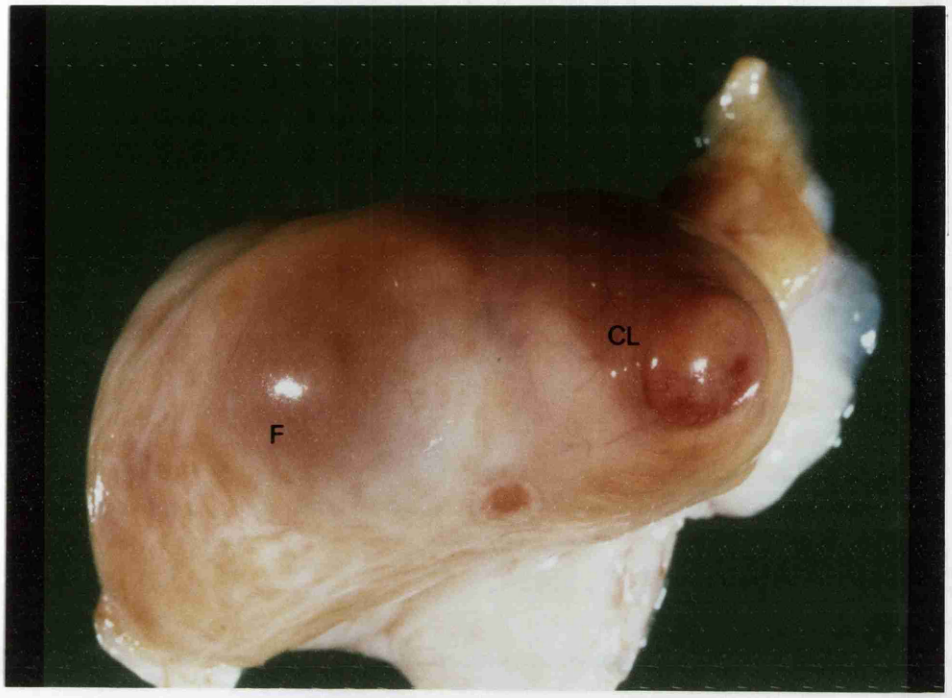


Plate 3.2a.

This plate shows the gross appearance of a CL of the second stage (day 5-10). The ovulatory papillum is now covered by the epithelium and forms a crown-like apex protruding from the body of the structure. The latter is only partially embedded in the ovary and appears as if attached to it. The dimensions of these CLs varied widely but were never more than 2.4 cm or less than 1.9 cm in diameter. Note the presence of a follicle of more than 1.0 cm in size (F).

Plate 3.2b.

An internal view of the CL shown in plate 3.2a is presented in this plate. The difference in the colour of the apex and the remainder of the CL is the most important criterion for the establishment of this stage. A small lacuna is also present.


CL
F

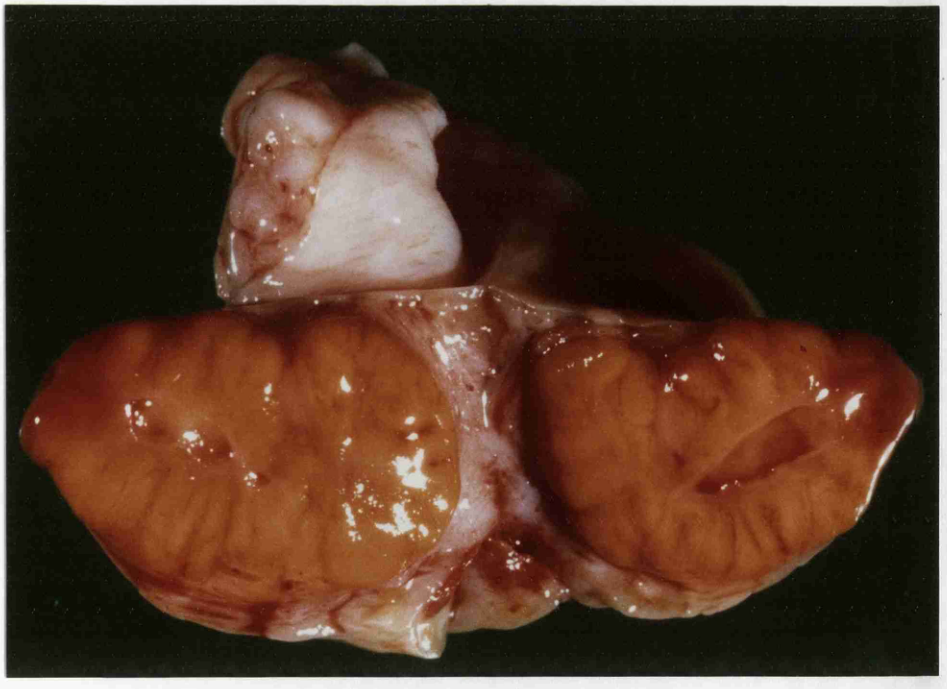
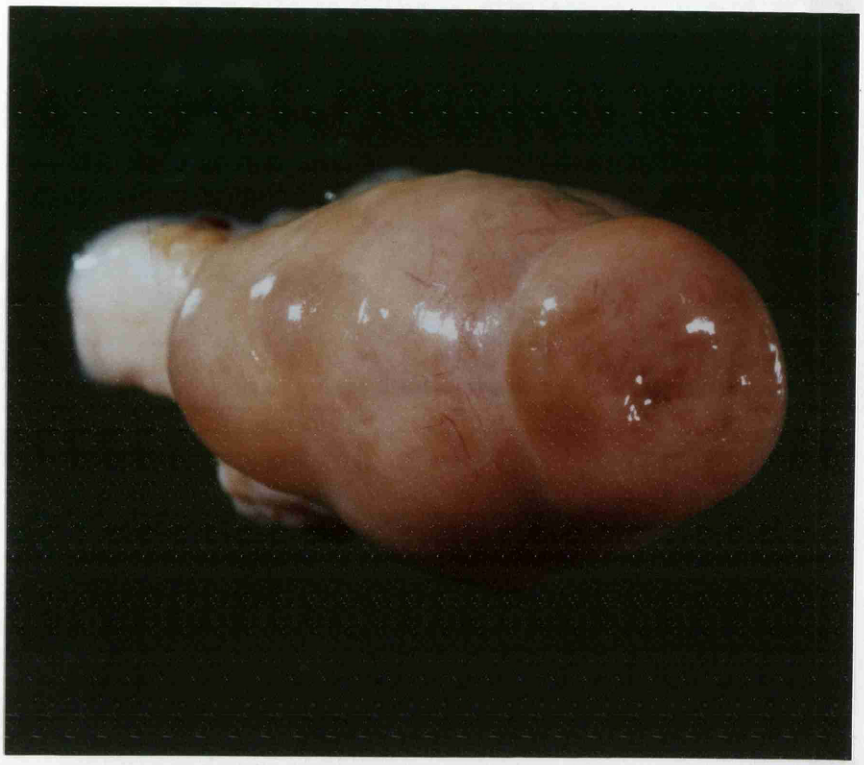


Plate 3.3a.

This plate shows the typical appearance of a CL of third stage (day 11-17). It is worth noting that on external examination little if any differences exist between CLs of this stage and those of the second one.

Plate 3.3b.

The internal view of the CL shown in plate 3.3a is presented here. Note the uniformity of the internal colour. At this stage the apex is no longer red or brown but orange or tan in accordance with the colour of the remainder of the structure.



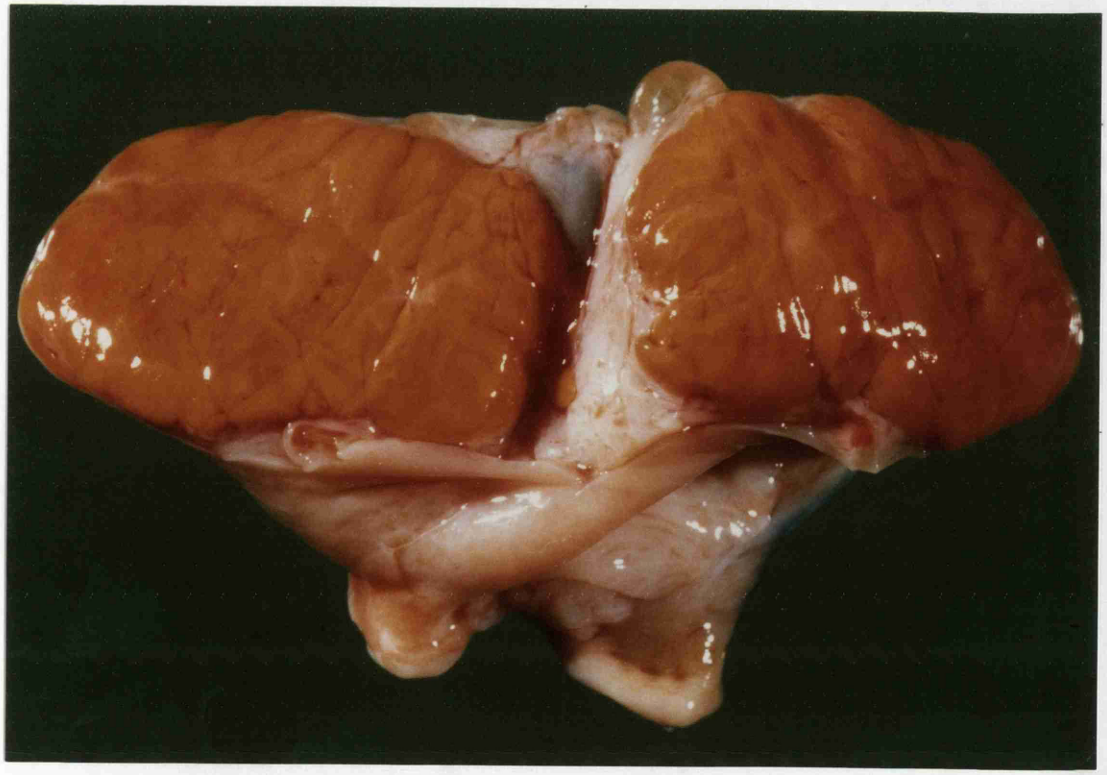
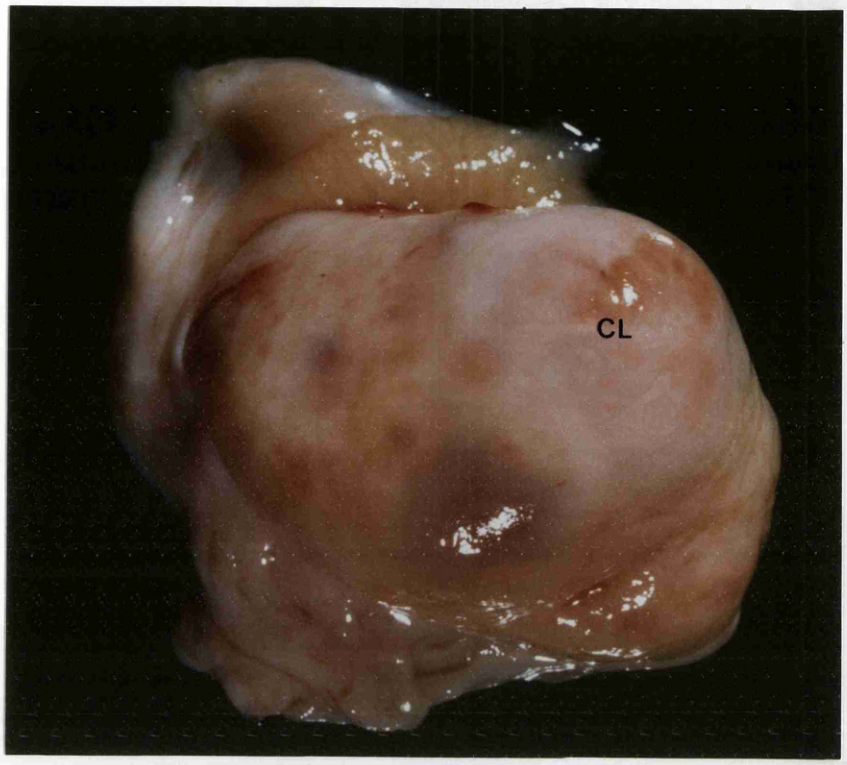


Plate 3.4a.

The gross appearance of a CL of the fourth stage (day 18-21) shows the diminishing size of the structure as it regresses. Note also the pale appearance due to the reduced blood supply.

Plate 3.4b.

The CL shown in plate 3.4a is shown here after sectioning the ovary. The luteal tissue is almost completely surrounded by the ovarian stroma, appears light orange, has a spherical shape, feels hard and is less than 1.6 cm diameter.


CL

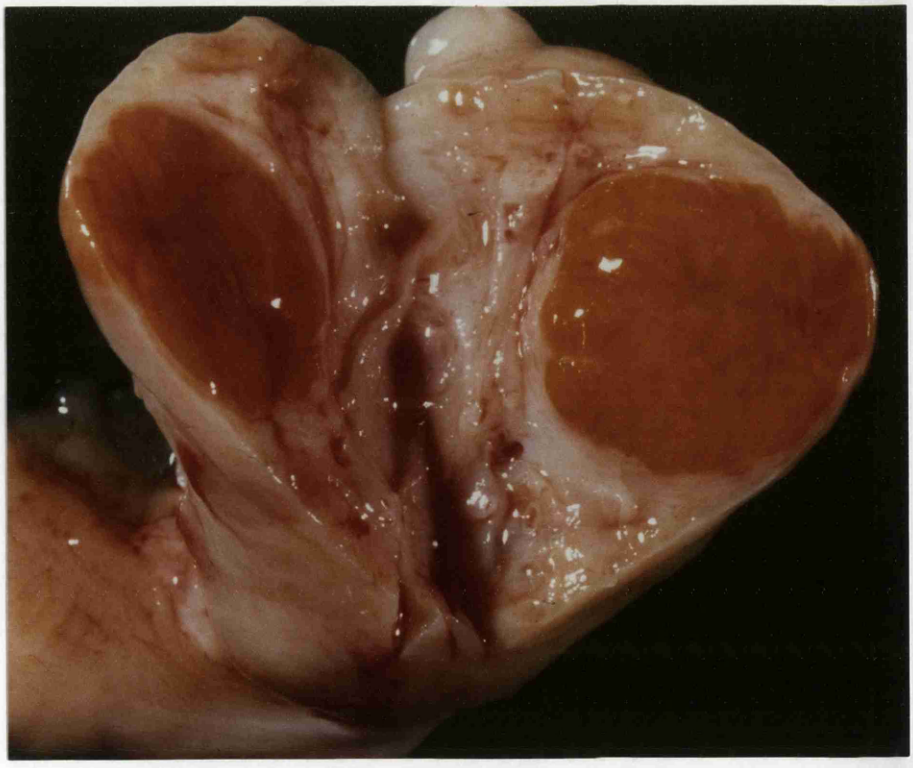


Table 3.2.

This table summarizes the findings of macroscopic examination of CLs of different stages and their associated circulating plasma progesterone concentration. Note that in a proportion of animals the circulating plasma progesterone did not match the stage of the cycle deduced by gross macroscopic assessment of the CL (10% for stages 2 and 3, and 25% for stages 1 and 4).

Table 3.2 Results of macroscopic examination of CLs of different stages and their associated plasma progesterone concentration.

Criteria	*Stage 1*	*Stage 2*	*Stage 3*	*Stage 4*
Number of CLs	4	10	13	4
Epithelium over apex	Absent (4)	Present (9) Absent (1)	Present (13)	Present (4)
External colour	Pale body, red point of rupture (4)	Brown apex, tan or orange remainder(10)	Tan or orange (13)	Orange (2) White (2)
Tissue consistency	Friable (2) Soft (2)	Compact soft (8) Firm (2)	Firm (12) Hard (1)	Hard (2) Firm (2)
Internal colour	Red (2) Red with some blood (1) Red & orange (1)	Red or brown apex, remainder orange or tan(10)	Orange (6) Orange & tan (4) Tan (2) Brown (1)	Light orange to yellow (4)
Vasculature	Visible (2) Not visible (2)	Visible, covering apex (9) Visible on periphery only (1)	Visible, covering apex (13)	Visible, covering apex (2) Not visible (2)
Size (*cm*)	<1.6 (3) 1.7 (1)	>1.6 (10)	≥2 (13)	>1.6 (1) <1.6 (3)
Associated large follicles (>1.0 *cm*)	Absent (2) One (1) Two (1)	Absent (1) One (6) Two (3)	Absent (2) One (7) Two (4)	One (3) Two (1)
Plasma P_4 (*ng/ml*)	<2 (3) >2 (1)	<2 (1) >2 (9)	<2 (1) >2 (12)	<2 (3) >2 (1)

Note: numbers between parenthesis indicate the number of CLs corresponding to the description.
P_4 = circulating plasma progesterone concentration.

Part (ii): histology

Plate 3.5.

A histological section of luteal tissue stained with MSB is presented in plate 3.5. Sections stained with MSB allowed a better definition of the tissue organisation and various cellular features than those stained with H & E. In particular it was easier to distinguish collagen fibres stained in blue (arrows) and estimate the degree of invasion of the luteal tissue by the connective tissue (x 500).

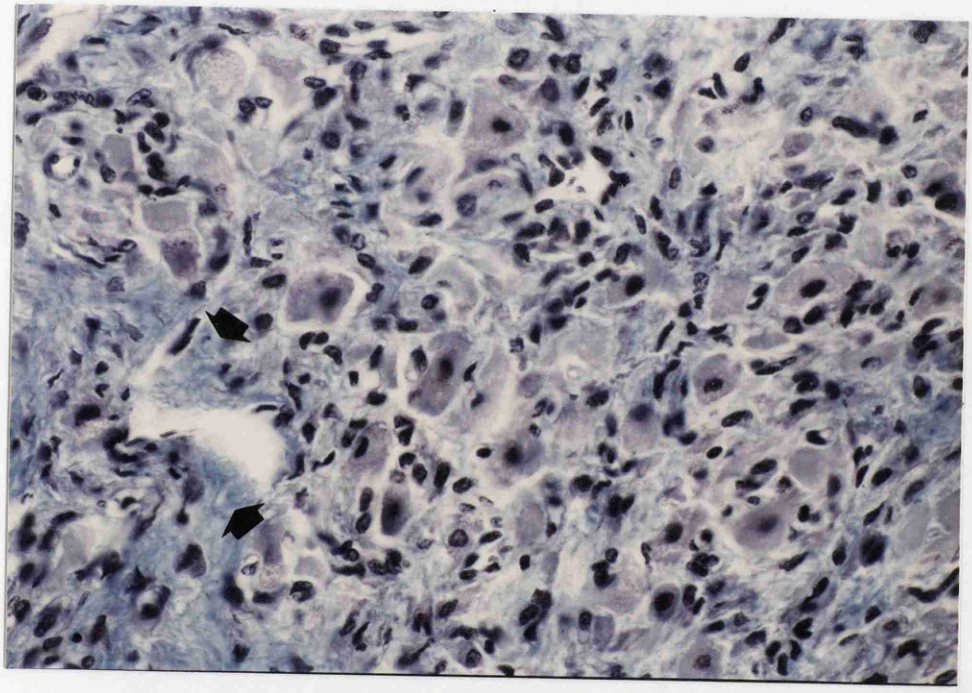
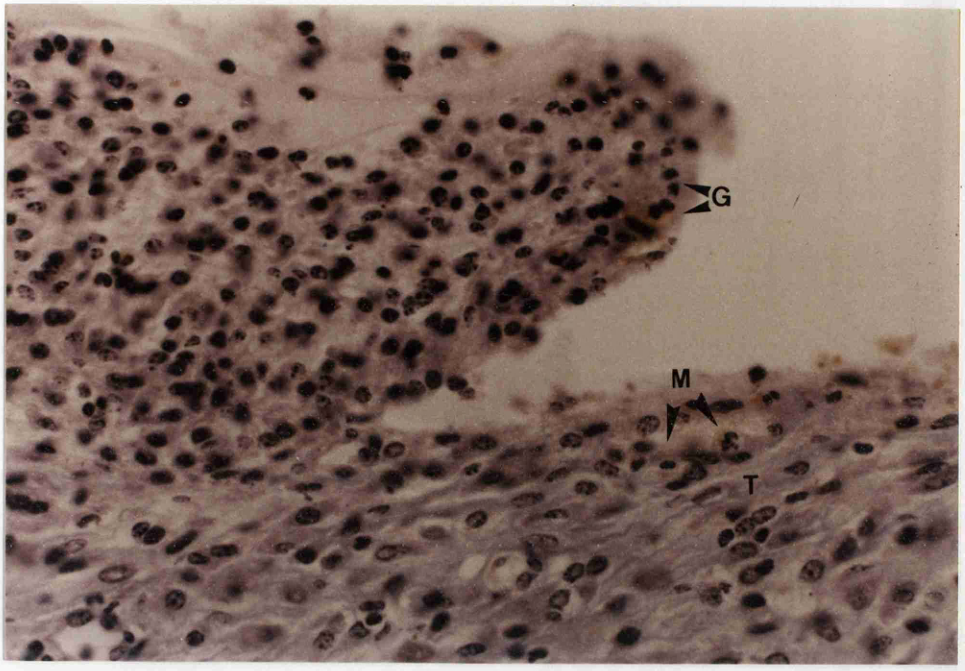


Plate 3.6a.

A section (MSB, x 500) of the wall of a corpus hemorrhagicum is shown (stage 1; day 1-4). At this early stage of formation the tissue organization is not much different from that of a follicle, that is the granulosa (G) and theca (T) layers are still distinct although the basement membrane separating the two layers in the follicle has disappeared. Many cells appear in the process of mitosis (M).

Plate 3.6b.

This section (MSB, x 500) is of a CL of first stage (day 1-4) which is clearly older than the CL shown in plate 3.6a. Note that at this stage it is difficult to distinguish between the size of the granulosa-derived and the theca-derived luteal cells. Both cell-types are intermixed. Connective tissue fibers (cf) are present but not predominent.


G
M
T

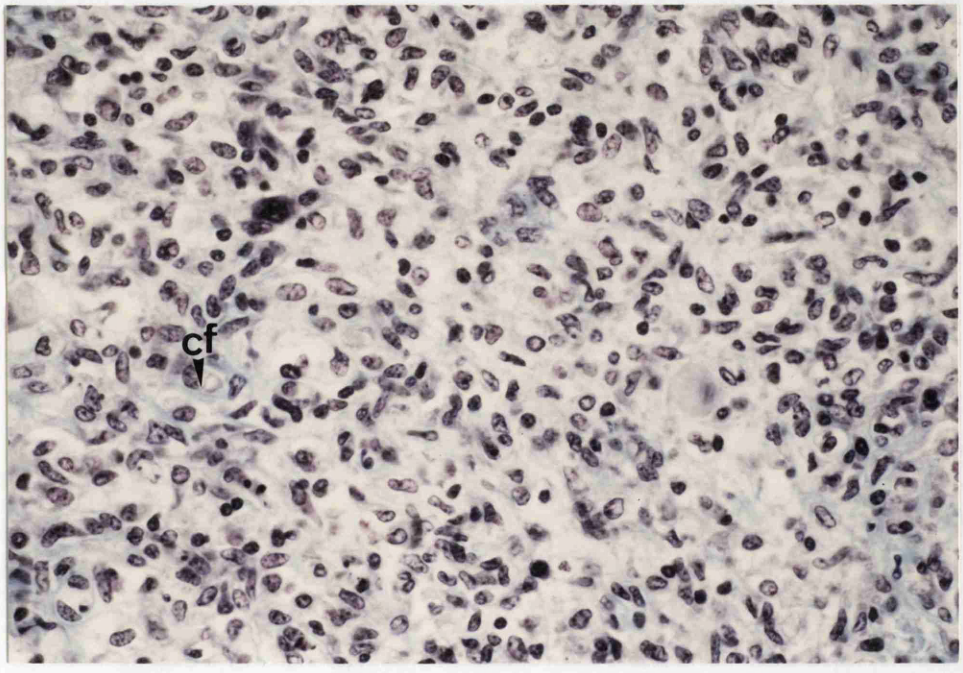

cf

Plate 3.7.

A second stage CL (day 5-10) is shown in this plate. Tissue appears more organized with large (L) and small (S) luteal cells easily distinguishable. Both cell-types have a discrete outline and fine stippling of the cytoplasm (arrows). The nucleus appears round to oval in the large luteal cells and rather polymorph and darker in the small ones. In both cells the nucleus has a very distinct membrane and a densely staining chromatin. Blood vessels are thin-walled and have an open lumen (V). Some connective tissue cells and fibres are present within the cores of luteal cells in addition to the trabeculae (T) that accompany the blood vessels (MSB, x 500).

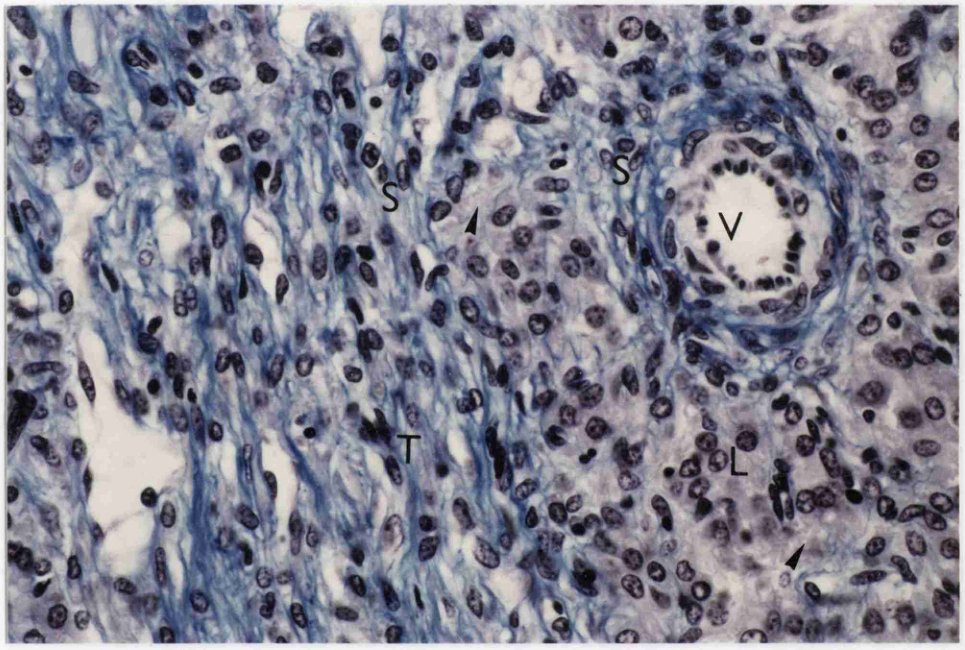
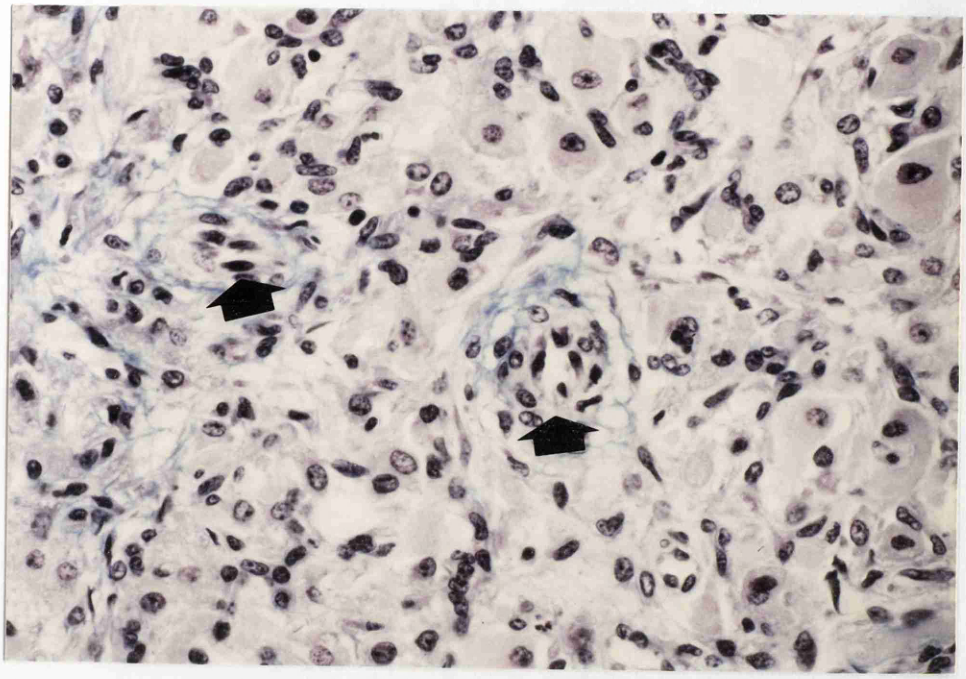

S
S
V
T
L

Plate 3.8a.

A section (MSB, x 500) of a CL of third stage (day 11-17) is shown in this plate. The main difference between CLs of third and those of second stage was the accentuation of the thickening of the walls of the arterioles (arrows). This seems to result in a narrow lumen.

Plate 3.8b.

At this stage both luteal cell-types show a proportion of cells undergoing regressive processes. Such cells have a rounded outline with many vacuoles (vl) in the periphery of the cytoplasm and a nucleus staining lightly. Note that the lumen in the venulae (v) is open as opposed to arteries (MSB, x 500).



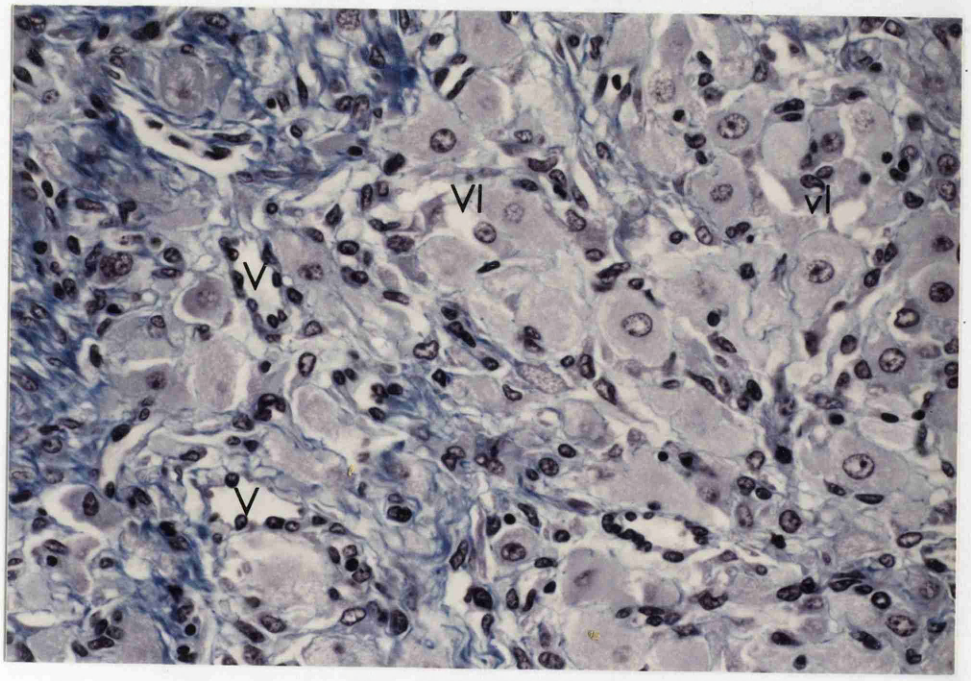
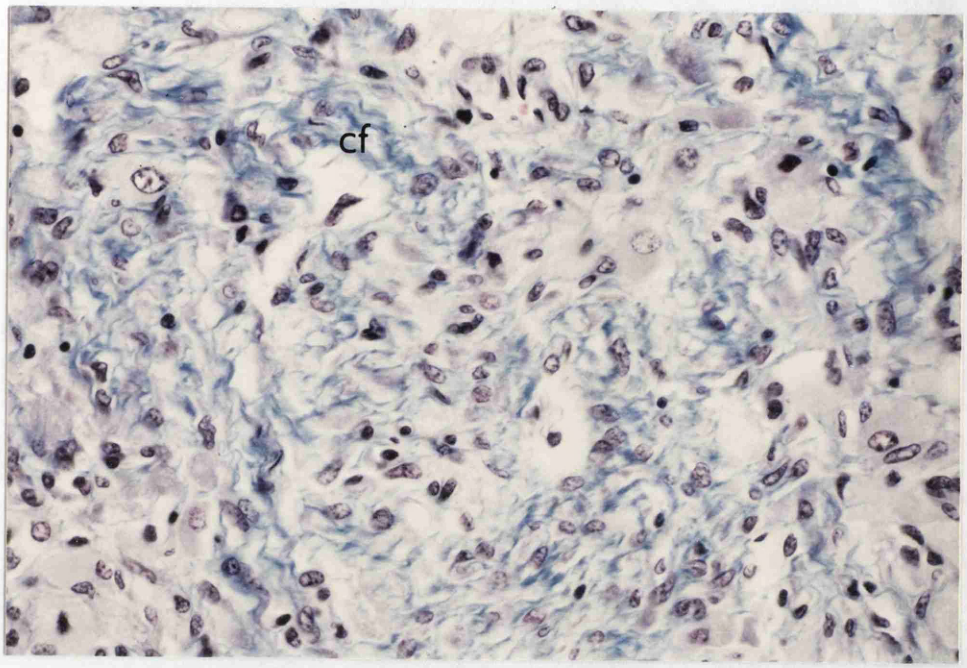

VI
VI
V
V

Plate 3.9a.

This section (MSB, x 500) of a fourth stage CL (day 18-21) shows the abundance of connective tissue fibers. This was one of the specimens which had clearly undergone total luteolysis. When examined macroscopically such structures are as hard as the ovarian stroma. Histologically the luteal tissue is only found in a few isolated small cores surrounded by collagen fibres (cf).

Plate 3.9b.

This section is of a CL of the fourth stage but during the early stages of regression. Luteal tissue is still present and its organization is not disturbed by connective tissue. The cytoplasm in the cells is full of vacuolae and in some cells these appear gathered close to the periphery of the cell (arrows). Most of the nuclei appear either shrinking and dark or normally sized but with a pale nucleoplasm. Some have no nucleoli and the chromatin appears adjacent to the nuclear envelope (MSB, x 500).


cf

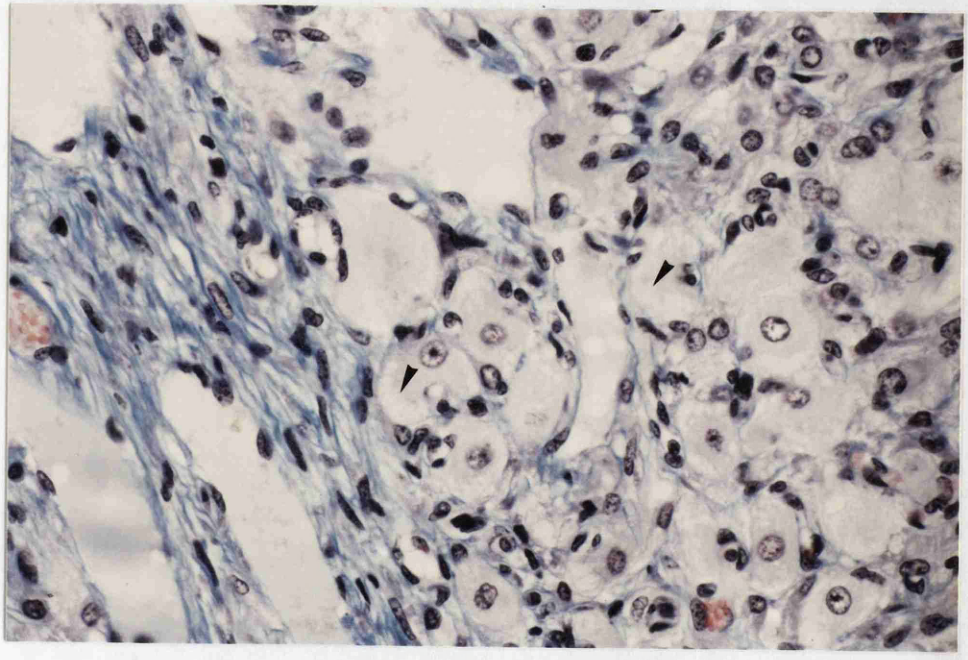


Table 3.3.

During the first stage the two luteal cell-types are difficult to distinguish and so no attempt of counting them was made. During the second stage the total population of luteal cells (excluding the non-luteal cells) was found to be made of approximately 25% of large luteal cells and 75% of small luteal cells. These proportions were found to have significantly changed ($P < 0.05$) in CLs of third stage. Small luteal cell proportions increased to approximately 85% and large luteal cell decreased to approximately 14%. In CLs of the fourth stage which still had luteal tissue left these changes were significantly ($P < 0.05$) reverted as small luteal cells were found to make up 79% of the total luteal population whereas large luteal cells proportions increased to above 20%.

Table 3.3 Changes in luteal cell-type proportions during the four stages of CL life-span.

Cell-type	Cell-types percentage of the total luteal population			
	First stage *(days 1-4)*	Second stage *(days 5-10)*	Third stage *(days 11-17)*	Fourth stage *(days 8-21)*
Large luteal cells	-	25.53 ± 3.4^{a}	14.16 ± 2.0^{b}	20.84 ± 1.1^{a}
Small luteal cells	-	74.46 ± 3.4^{a}	85.38 ± 2.0^{b}	79.16 ± 1.1^{a}
Number of CLs	4	10	13	4

Means in the same row with different superscript differ ($P<0.05$). No estimation was made of cell-types proportions in CLs of first stage as cells were difficult to distinguish. Mean ± s.e.m.

Part (iii): histochemistry

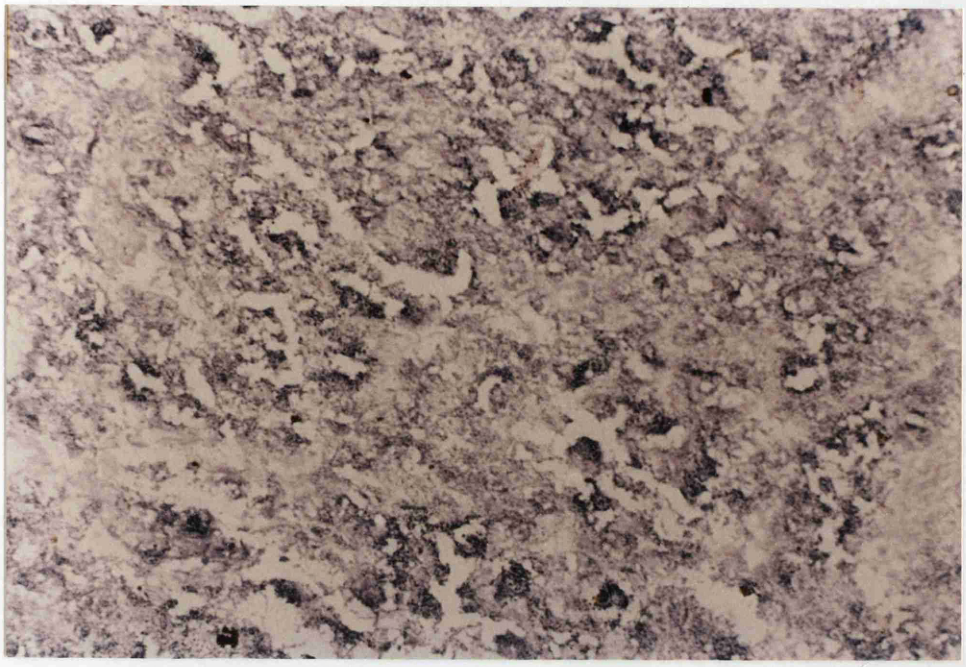
Plate 3.10a.

This is an example of distribution of 3β-HSD activity in luteal tissue. Apart from slight variations in intensity, all CLs regardless of the stage of their life-span showed the presence of this enzyme (x 250).

When sections of luteal tissue stained for 3β-HSD were counter stained with light green cell-types were unable to be identified clearly enough to allow distinction between large and small types and so the variation in the distribution of the enzyme between these different cell-types could not be assessed.

Plate 3.10b.

This is an example of distribution of 20α-HSD activity in a luteal tissue. Note that the intensity of the reaction is moderate (x 250). Prefixing and/or prewashing in cold acetone of sections prior to incubation did not result in any improvements in the intensity of the staining.



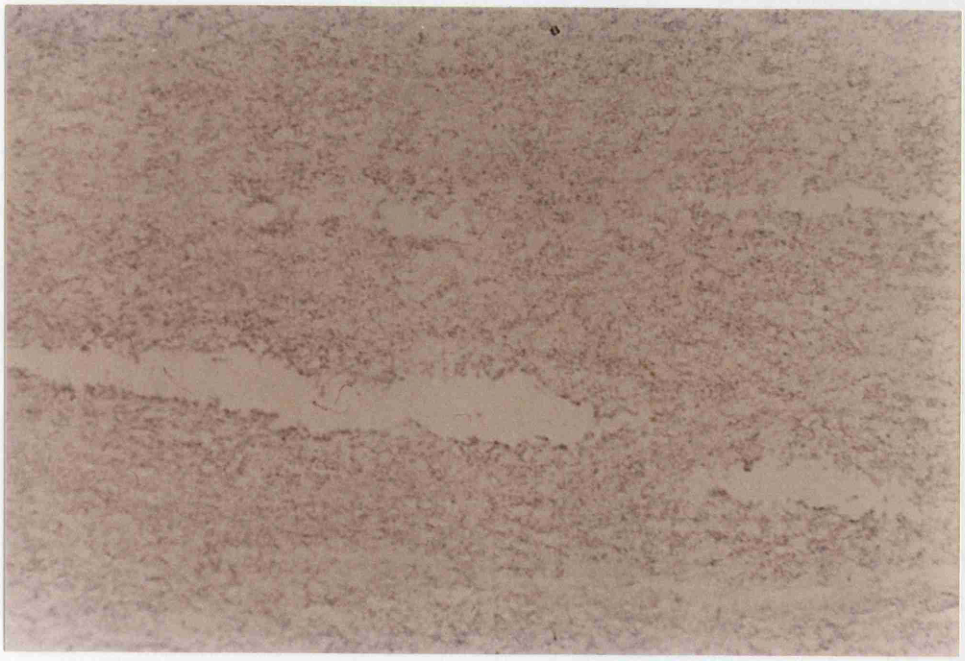
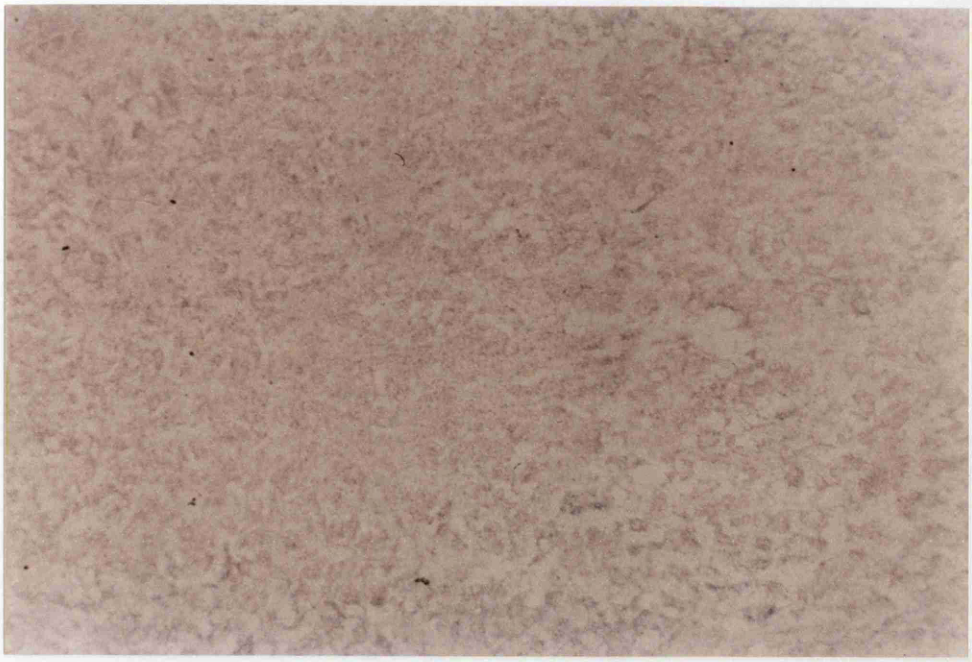


Plate 3.10c.

This plate shows a control section incubated in medium lacking steroid substrate. A light blue stain is observed in many slides. This was lighter than that of most sections tested for 3β-HSD but was equal in intensity to positive sections tested for 20α-HSD (x 250).

Plate 3.10d.

This plate shows the positive reaction of control sections incubated in presence of medium containing reduced coenzyme (diaphorase activity). Note the intensity of dark blue colour compared with that produced in tissue tested for 3β- or 20α-HSD (x 125).



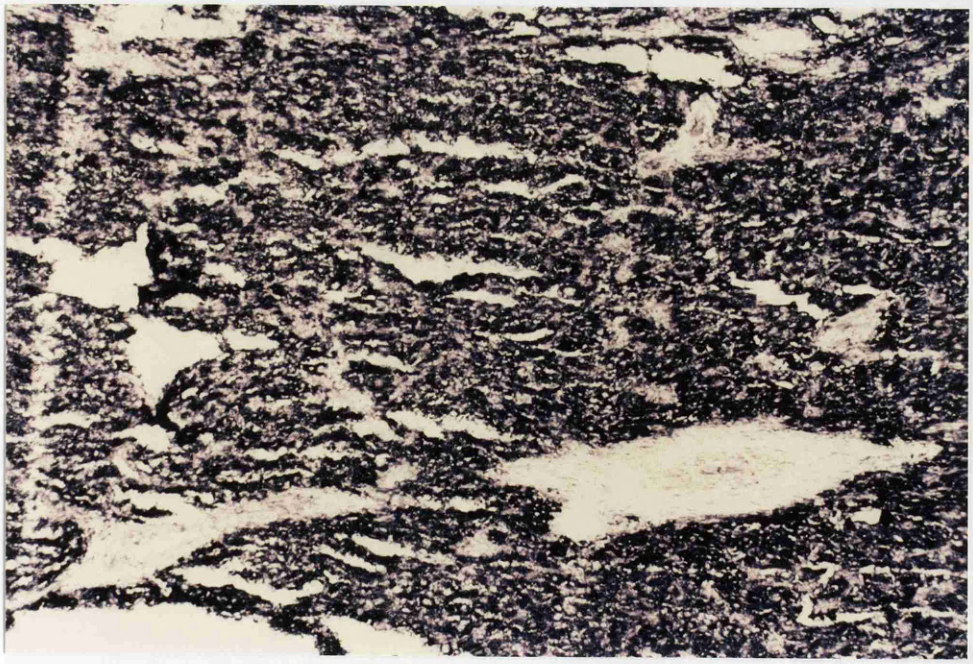


Table 3.4a.

Scoring of the 3β-HSD activity in luteal tissue belonging to CLs of the four different stages is described in this table. Note that the 3β-HSD was present in most CLs regardless of their stage of development. Only two CLs showed no enzyme activity. One was a third stage CL, the other was a fourth stage one. Note also that this enzyme was present in CLs of the first stage.

Table 3.4b.

The scoring of 20α-HSD activity in CLs of different stages is described in table this table. The staining for this enzyme was not intense. The enzyme was either present or absent in CLs of all stages.

Table 3.4a Variation in intensity of staining (reaction) of 3β-HSD during the various stages of CL life-span.

Intensity of staining	Number of samples			
	First stage (1-4)	Second stage (5-10)	Third stage (11-17)	Fourth stage (18-21)
+++	2	3	7	3
++	3	3	2	3
+	0	0	0	0
-	0	0	1	1
Number CLs per stage	**5**	**6**	**10**	**7**

(+++) = intense blue staining; (++) = moderate intensity of blue staining; (+) = light staining; (-) = negative. Note that in some sections incubation in presence of no steroid substrate resulted in a light staining (+). Numbers between brackets = days after ovulation.

Table 3.4b Variation in intensity of staining (reaction) of 20α-HSD during the various stages of CL life-span.

Intensity of staining	Number of samples			
	First stage (1-4)	Second stage (5-10)	Third stage (11-17)	Fourth stage (18-21)
++	2	3	2	2
+	0	1	3	0
-	3	2	5	4
Number CLs per stage	**5**	**6**	**10**	**6**

(++) moderate intensity of blue staining; (+) = light staining; (-) = negative. Note that in some sections incubation in presence of no steroid substrate resulted in a light staining (+). Numbers between brackets = days after ovulation.

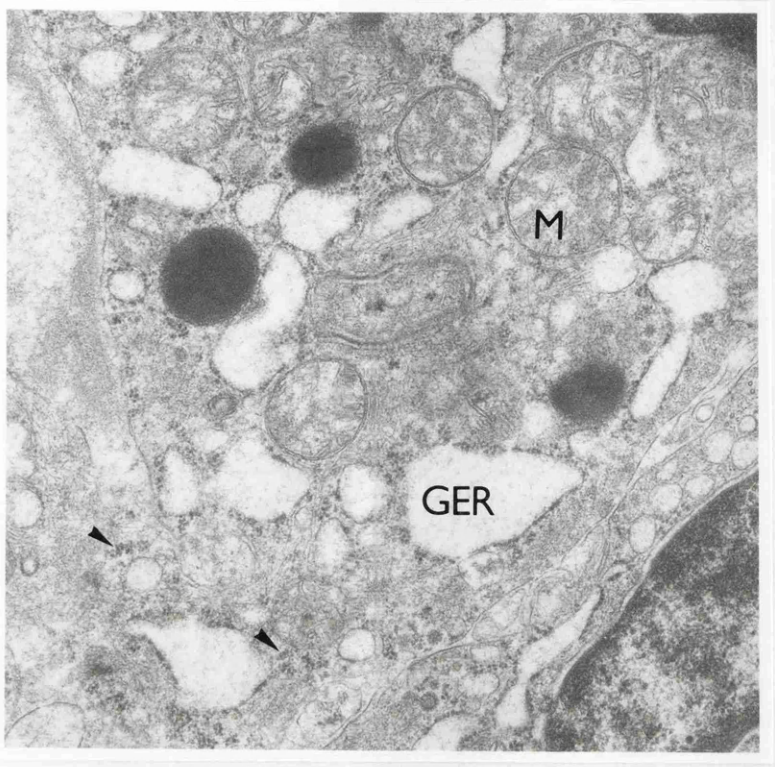
Part (iv): transmission electron microscopy

Plate 3.11a.

A high magnification (x 15000) of two adjacent undifferentiated luteal cells shown in plate 3.11b is presented here. In these cells the granular endoplasmic reticulum (GER) appears as distended vesicles with a light staining lumen containing flocculent precipitates. Note also the presence of several round mitochondria (M) with tubular cristae. Polyribosomes are present both in a free form in the cytoplasm (arrows) and attached to the endoplasmic reticulum. Dense dark staining granules are often observed in large luteal cells.

Plate 3.11b.

An aggregation of undifferentiated luteal cells belonging to a CL of the first stage (day 1-4) is shown, (x 3000). The nuclei in these cells is polymorph and contains a few large fragments of condensed chromatin or heterochromatin (arrows).


M
GER

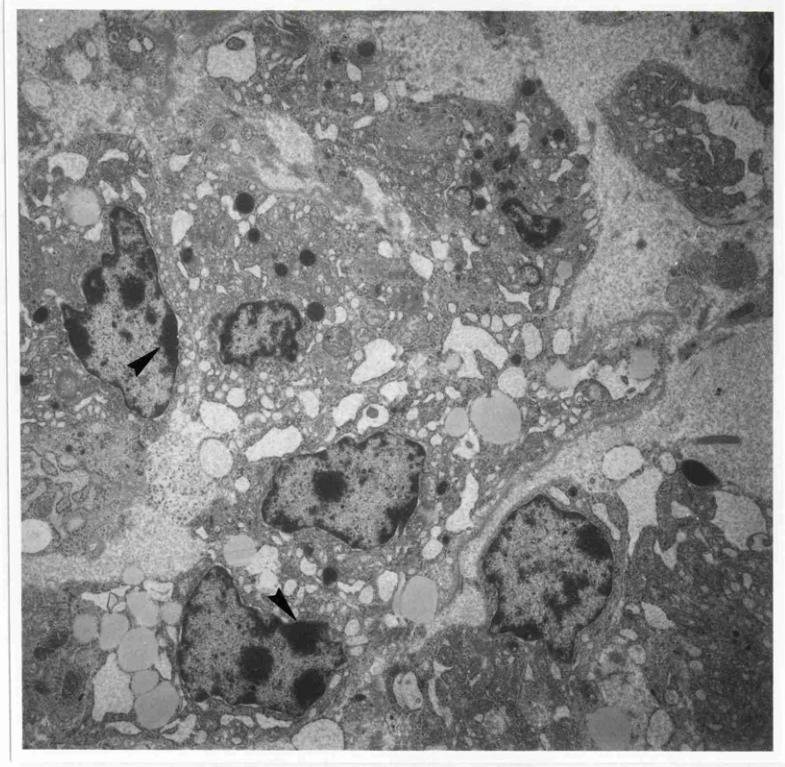
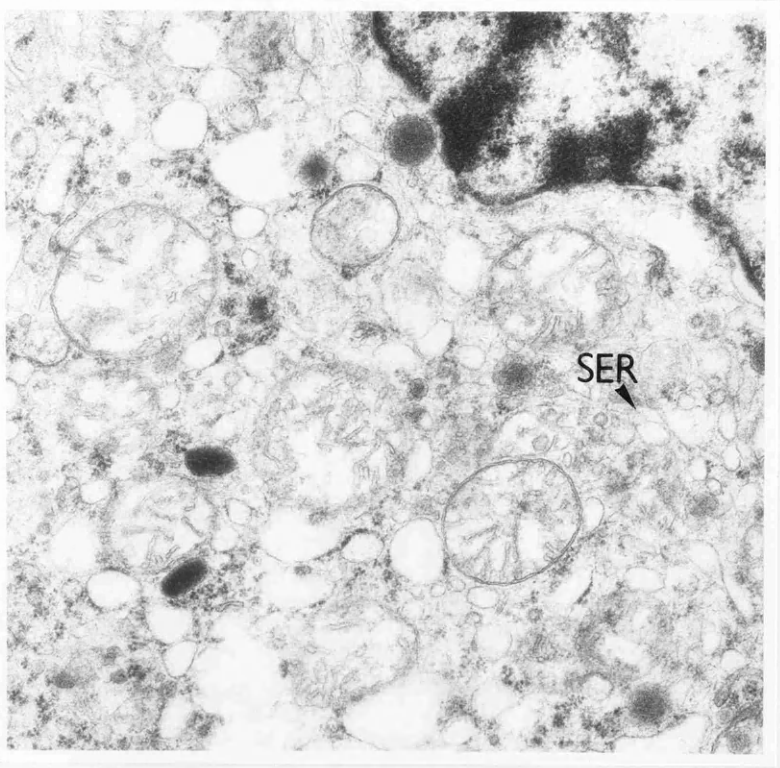


Plate 3.11c.

In this micrograph the cytoplasm of a large luteal cell from a CL of the first stage is shown. Abundant smooth endoplasmic reticulum (SER) is present (x 15000).

Plate 3.11d.

A small luteal cell from a first stage CL is presented in this plate, (x 7500). The nucleus appears elongated, has a large size in relation to the size of the cell and has a peripherally located nucleolus (no). Abundant granular endoplasmic reticulum (GER) is found in the cytoplasm of these cells. A grey precipitate is seen in the lumen of this structure. A few mitochondria are also present in the cytoplasm. Note also the presence of polyribosomes in a free form in the cytoplasm.


SER

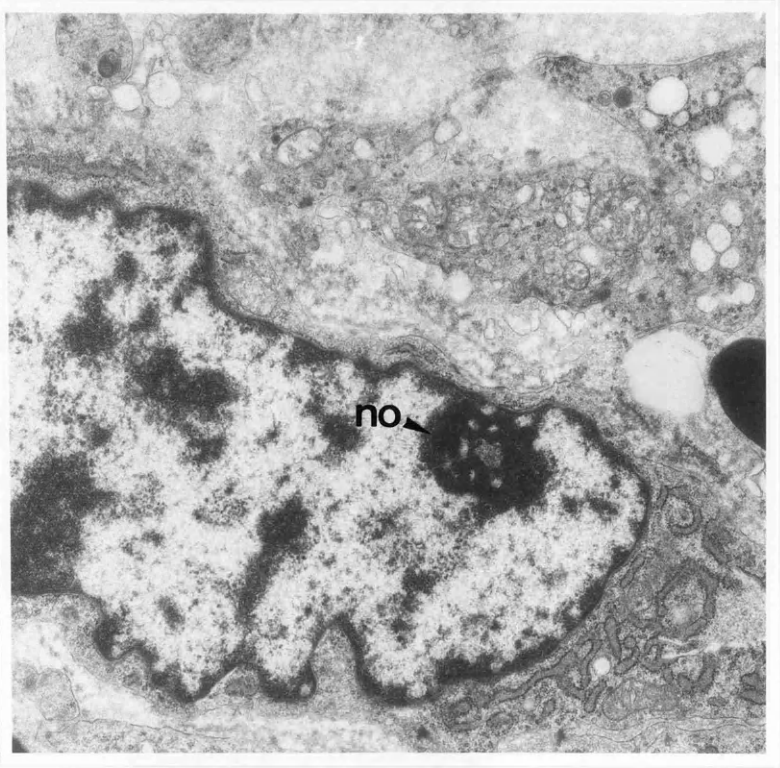
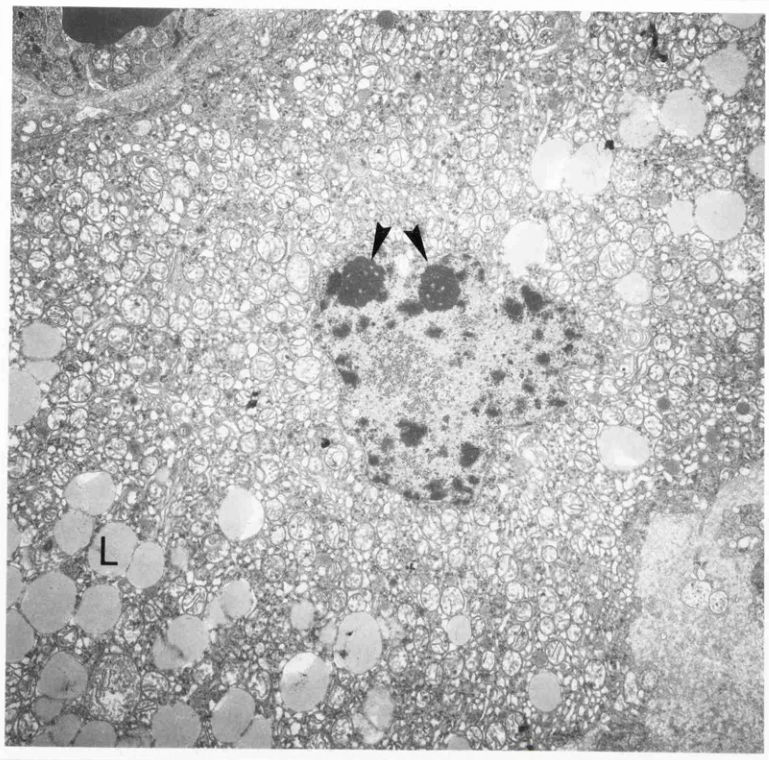

no

Plate 3.12a.

This micrograph shows a large luteal cell from a CL of the second stage (day 5-10). The cytoplasm contains numerous mitochondria, extensive smooth endoplasmic reticulum and large, relatively light staining, lipid droplets (L). This cell is unique in that two apparently distinct nucleolus can be seen in the nucleus (arrows). This could also be due to a section through a single but elongated and curved nucleoli (x 3000)

Plate 3.12b.

In this plate a high magnification (x 15000) of a luteal cell from a CL of second stage is shown. Note the presence of large numbers of dark staining membrane limited granules (G) near the nucleus (N). Close observation of these granules shows that the dark staining is heterogeneous and in many two areas of differing density can be seen.


L

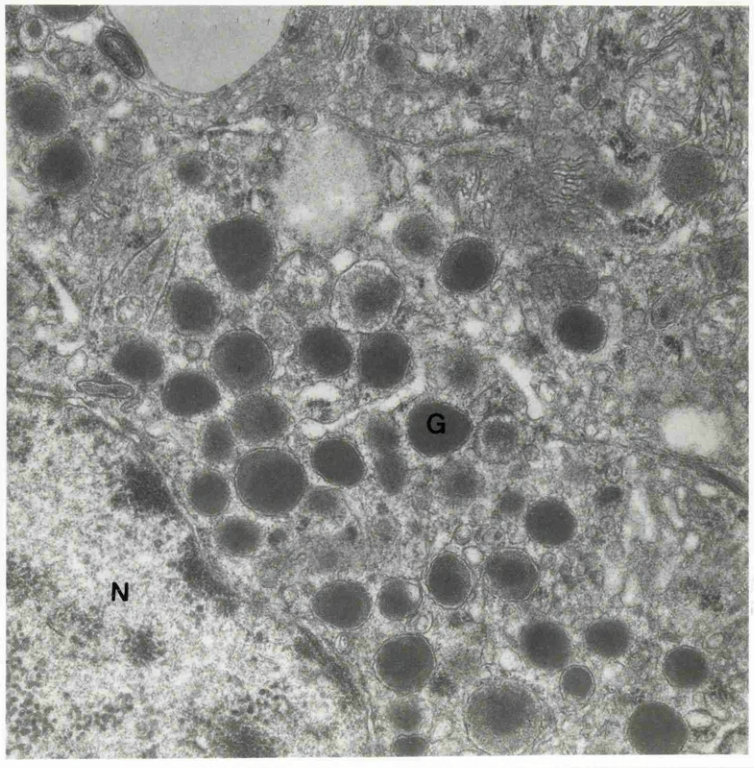

G
N

Plate 3.13a.

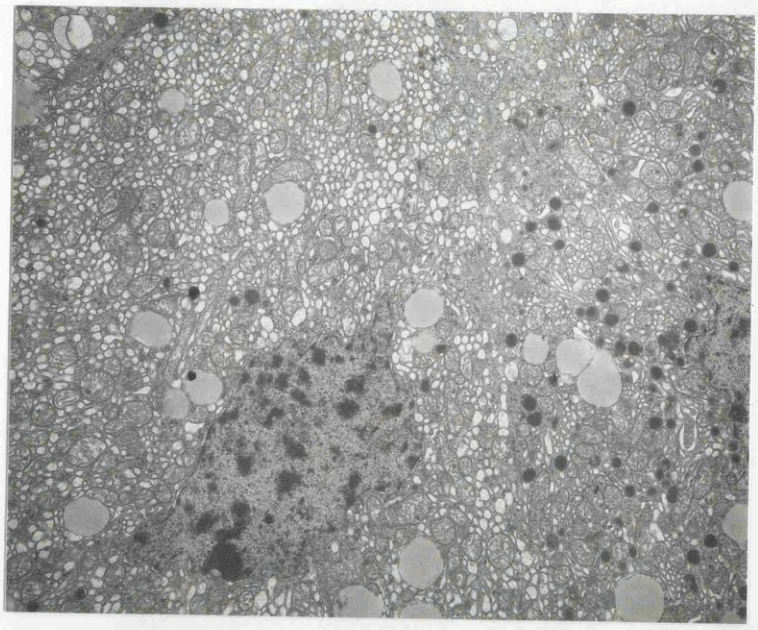
In this low magnification (x 3000) micrograph two large luteal cells from a CL of the third stage (day 11-17) are shown. The cytoplasm is dominated by smooth endoplasmic reticulum. A few lipid droplets are also present. Note that dark staining granules are present in large numbers in only one of the two cells.

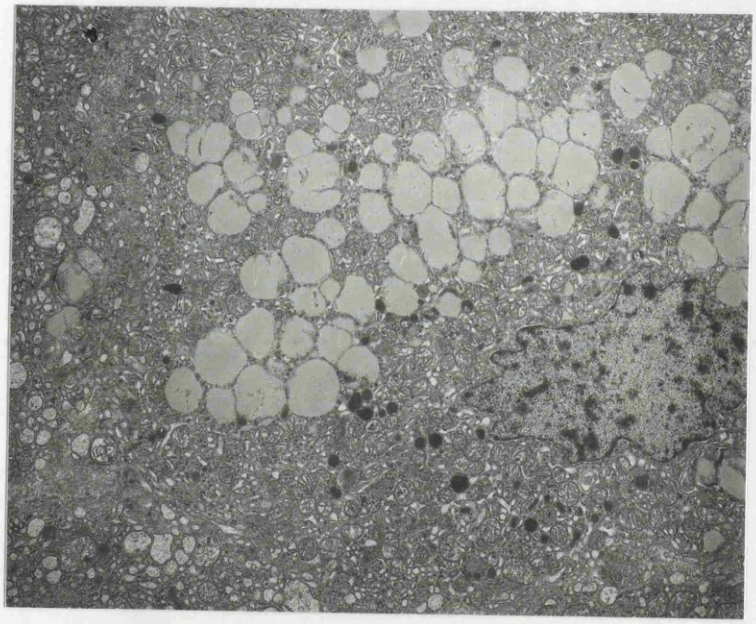
Plate 3.13b.

This luteal cell from a CL of third stage contains large numbers of lipid droplets almost surrounding the nucleus (x 3000).

Plate 3.13c.

A high magnification (x 15000) view of a luteal cell from a CL of third stage is presented in this plate. All the usual constituents of the cytoplasm of a large luteal cell during this stage of CL life-span are present, ie. mitochondria (M), smooth endoplasmic reticulum (SEM), dense staining granules (G), lipid droplets (L) and a few free polyribosomes (P).





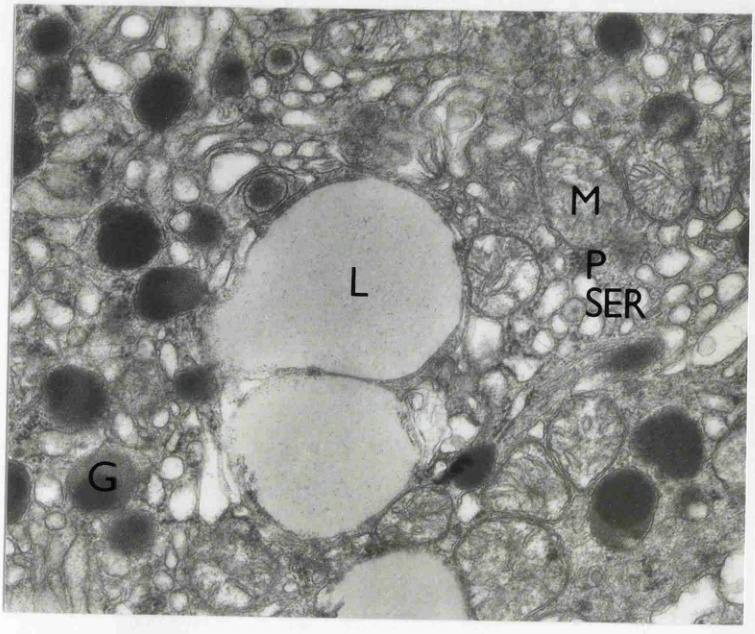

M
P
SER
L
G

Plate 3.14a.

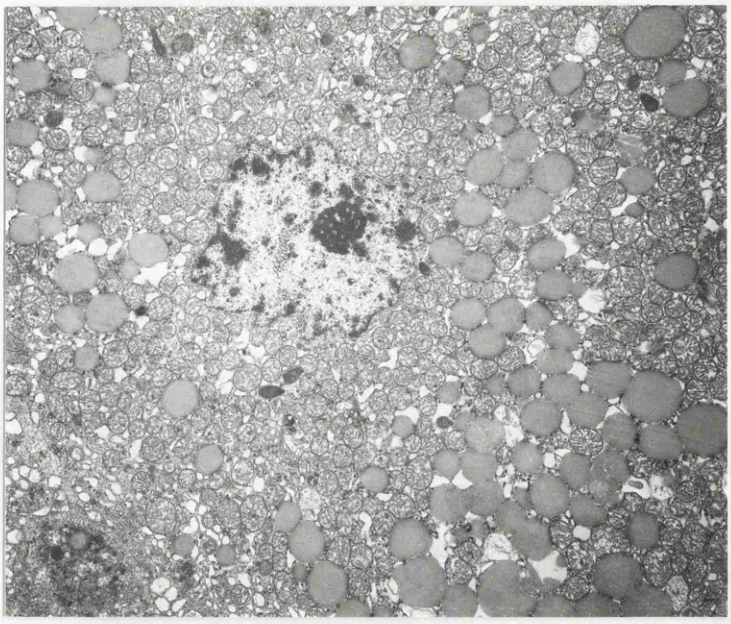
This plate shows a large luteal cell from a CL of the fourth stage (day 18-21). The cytoplasmic contents in this cell are similar to those observed in some luteal cells from CLs of the third stage. In particular there is abundance of mitochondria and smooth endoplasmic reticulum. However lipid droplets appear more dense and some are fusing together. Also note that in the nucleus, the nucleoplasm stains lighter than in previous plates (x 3000).

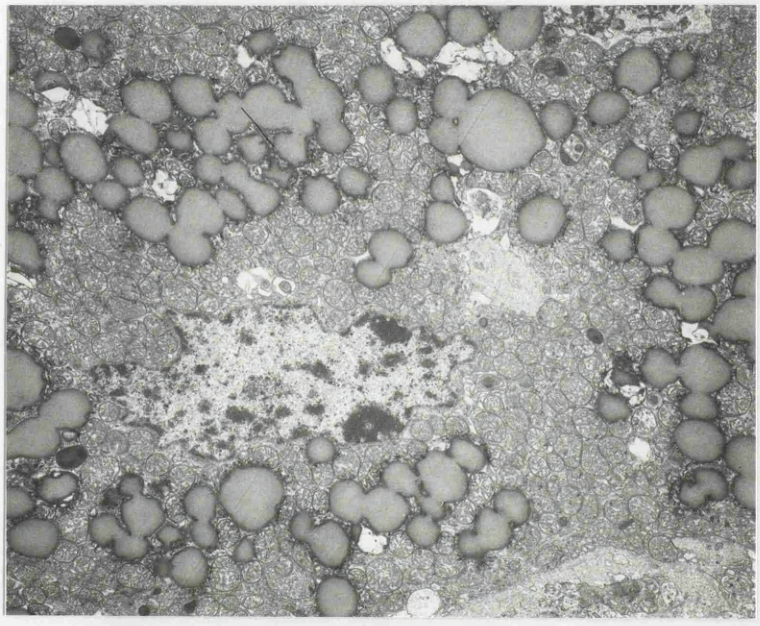
Plate 3.14b.

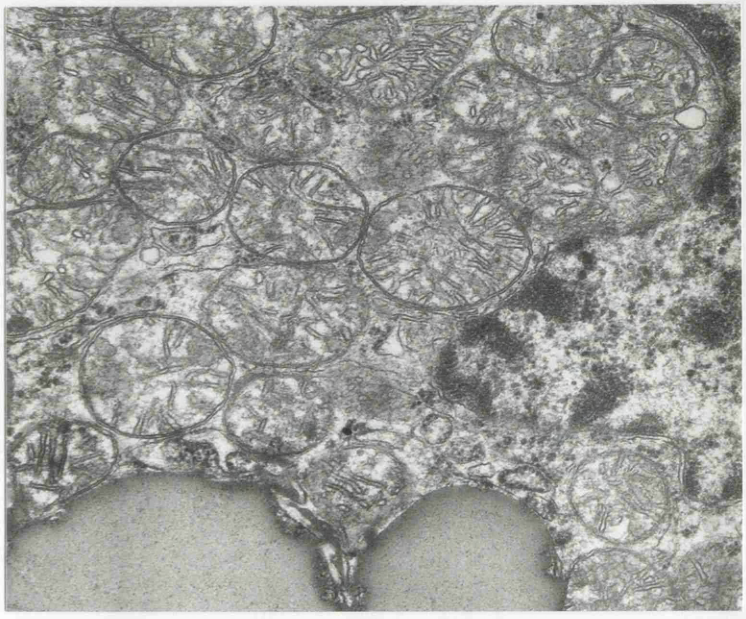
This plate shows a large luteal cell from another CL of the fourth stage. Note the increased number and size of lipid droplets. Most of these vesicles are fused together and appear darker than in plate 3.14a, particularly near the perimeter. The nucleoplasm is stained lightly and the nucleolus is absent (x 3000).

Plate 3.14c.

A high magnification (x 15000) of plate 3.14b is shown in this plate. Smooth endoplasmic reticulum is virtually absent and apart from the lipid droplets mitochondria make up the major part of the cytoplasm.







Part (v): cell culture

Table 3.5.

Some important features recorded before, during and after tissue dissociation process are described. The viability among both large and small luteal cell-types as judged by trypan blue staining was similar and did not appear to vary according to the stage of the cycle. In CLs of the first stage 19 large cells were counted for every 81 small cells whereas only 2 to 7 large cells were counted for every 92 to 97 small cells in CLs of other stages (ratio = 1:4 *vs* [1:12 to 1:45] respectively). CLs of the first stage dissociated faster during incubation with collagenase than CLs of other stages.

Table 3.5 Parameters recorded before, during and after dissociation of luteal tissue.

Stage of the cycle	Number of CLs	CL weight (*g*)	Time required for complete tissue dissociation (*minutes*)	Cell-type percentage of total viable luteal population		Viability	
				%LLC	%SLC	%LLC	%SLC
1	3	1.16 ± 0.16	115.0 ± 48.2	19.0 ± 25.7	81.0 ± 25.7	69-75	53-82
2	6	3.29 ± 1.55	229.3 ± 95.5	2.1 ± 0.8	97.8 ± 0.8	61-90	57-96
3	12	4.05 ± 0.54	209.0 ± 90.9	7.7 ± 6.4	92.2 ± 6.4	43-77	46-79
4	3	3.97 ± 1.58	250 0± 49.5	5.2 ± 4.5	94.7 ± 4.5	67-87	54-81

LLC = large luteal cells; SLC = small luteal cells. Mean ± s.d.

Table 3.6.

In vitro production of progesterone by dispersed luteal cells in response to LH, PGF2α, LH plus PGF2α, and controls is given in table 3.6. The addition of 13 ng/ml LH to dispersed luteal cells significantly increased progesterone production in all but the fourth stage ($P < 0.05$). The addition of PGF2α to dispersed luteal cells from CLs of first, second and fourth stage resulted in similar progesterone production to that found in unstimulated controls. PGF2α increased progesterone production in CLs of the third stage ($P = 0.03$). In CLs of second ($P = 0.002$) and third ($P = 0.01$) but not first or fourth, progesterone production was found significantly reduced when both PGF2α and LH were added simultaneously to luteal cells than when only LH was added.

Table 3.6 In vitro progesterone production by dispersed luteal cells in response to: (a) LH, (b) PGF2α and (c) LH + PGF2α (ng/20 x 10^3 cells/3 hours incubation; mean ± s.e.m).

Treatment	Stage of the cycle			
	1	*2*	*3*	*4*
Control	15.14 ± 1.62[a]	94.10 ± 23.10[a]	27.26 ± 4.10[a]	57.51 ± 4.49[a]
[+13 ng/ml LH]	30.57 ± 2.15[b]	251.30 ± 13.20[b]	65.83 ± 4.75[b]	78.86 ± 8.06[ab]
[+1 µg/ml PGF2α]	32.66 ± 3.75[ab]	95.30 ± 11.50[a]	39.78 ± 5.13[c]	69.41 ± 2.59[ab]
[+13 ng/ml LH +1 µg/ml PGF2α]	32.59 ± 2.61[b]	138.90 ± 12.20[a]	54.25 ± 5.83[c]	101.98 ± 8.72[b]
Number of CLs	**3**	**6**	**12**	**3**

Means with a different superscript in the same column differ (P<0.05).

Figure 3.1a.

Unstimulated progesterone production by luteal cells of different stages of the cycle varied between stages. In particular significant differences were found between stages 1 and 3 ($P = 0.02$), stages 1 and 4 ($P = 0.01$), and stages 3 and 4 ($P = 0.004$). Similarly LH-stimulated progesterone production appeared to vary with the stage of the cycle except between stage 3 and 4 ($P < 0.005$). CLs of stage 1 produced the lowest amount of unstimulated and LH-stimulated progesterone. LH significantly increased progesterone production in all but the fourth stage ($P < 0.05$).

Figure 3.1b.

The response to PGF2α by luteal cells of CLs of different stage is shown in figure 3.2b. In luteal cells from CLs of stage 3, PGF2α-stimulated progesterone production was higher than in unstimulated control ($P = 0.03$).

Figure 3.1c.

The response to both LH and PGF2α, added simultaneously, by luteal cells is shown in figure 3.2c. When PGF2α and LH were added simultaneously to dispersed cells the stimulating effect of LH was reduced in luteal cells from CLs of stages 2 ($P = 0.002$) and 3 ($P = 0.01$) but not 1 and 4.

Fig. 3.1a Basal and LH-induced in vitro progesterone production by luteal cells

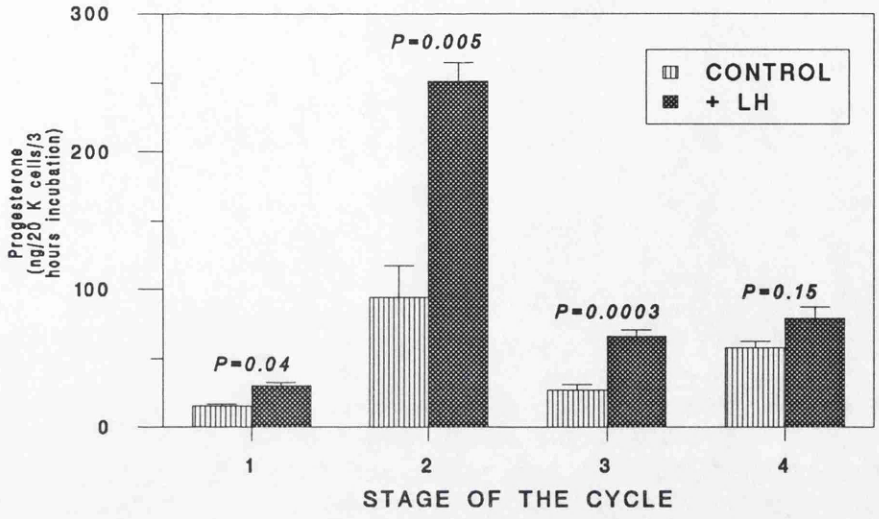


Fig. 3.1b Effect of PGF2α on in vitro unstimulated progesterone production by luteal cells

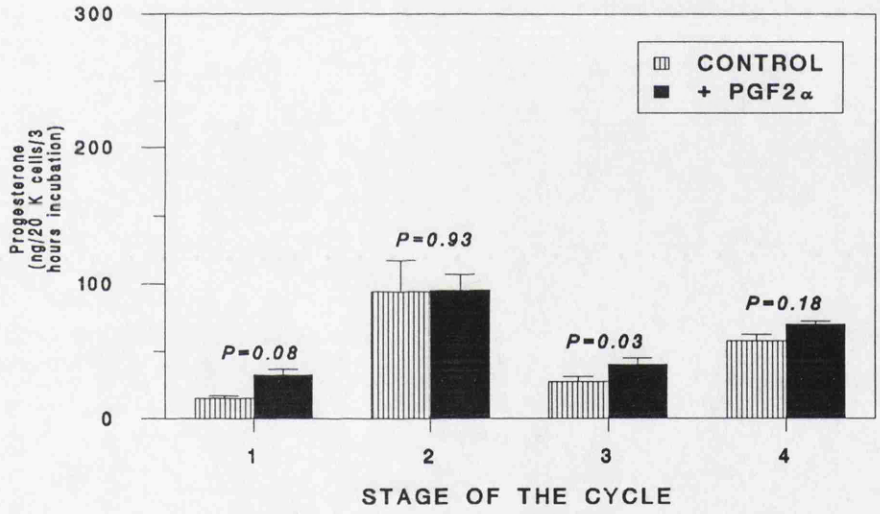
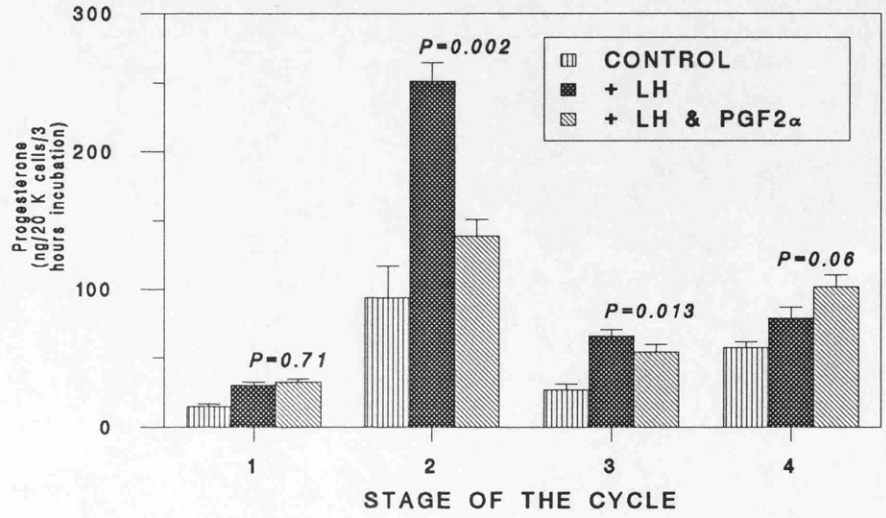


Fig. 3.1c Effect of PGF2α on LH-stimulated progesterone production by luteal cells



IV. DISCUSSION

Parts (i) and (ii): macroscopic appearance and histology

It became very obvious on initial gross examination of CLs that some of the features used by Ireland et al. (1980) to identify the stage of the cycle, were inconsistent. However it must be recognized that whilst the studies published by this group were performed on post mortem material from heifers whose exact day of the cycle was known, the assessment described in this thesis was carried out on ovaries collected from heifers of unknown reproductive status. It cannot be ignored that some of these heifers may have been in an early stage of pregnancy and so the CLs could be exposed to embryo signals. It was not found appropriate to collect uteri and examine or flush them as this would take an exessive length of time which may result in delaying the macroscopic examination and subsequent fixation of the luteal tissue. Although the inconsistency of some of the characteristics listed by Ireland et al. (1980) applied to CLs of all stages, it was most obvious with CLs during the first four days of development. The most likely explanation for this is that during this period the luteal tissue undergoes dramatic increases in both cell numbers and in the size of the cells themselves. The rapidity of these changes is necessary so that by seven days after ovulation the structure is capable of secreting sufficient progesterone to create the proper environment for early pregnancy. The relatively rapid changes observed during this stage on gross macroscopic examination were matched by the finding of dramatic changes when sections of structures of this stage were examined under light microscopy. The histology of structures of this first stage varied greatly. Some appeared to have tissue composition resembling that of a follicle with distinct granulosa and theca cellular components. Others had a more compact form of tissue where both

theca and granulosa cells had become replaced by luteal cells. At this stage some of the luteal cells were seen dividing while others seemed enlarged and appeared to have acquired their final form as luteinized cells. The mechanism regulating such differences in the rate of division and differentiation of the cells comprising the CL are not known but the role of various growth factors such as epidermal growth factor (EGF) and fibroblast growth factor (FGF) are thought to play a role (Carson et al., 1989; Schams, 1989). Receptor-sites for these factors can be induced by gonadotrophins (Hsueh et al., 1984). However the intimate interrelationship of these factors, in particular the possible selective induction of growth factor receptor-sites by gonadotrophins still remains to be substantiated, in particular in the bovine CL.

The relatively small size of the structure at this stage of development was not as expected. In the bovine immediately after ovulation the follicular walls collapse with unfolding of the theca interna so the early CL is a minute structure compared with the 1.7 cm-large preovulatory follicle. The textbook concept of the CL during this period is of the hollow left by the escaping ova and its surrounding follicular fluid becoming almost immediately filled by blood to form the corpus haemorrhagicum which one might imagine would resemble the mature follicle in size. However the majority of the first stage CLs examined were no more than 1.0 cm in diameter. These early CLs were so small that they could easily be overlooked on initial examination and their size was only able to be determined accurately by sectioning the ovary.

The consistency of the luteal structure at this stage of its development ie. day 1-4, was very soft; almost mushy with very little connective tissue. The invasion of granulosa layers by connective tissue strands was reported to occur as soon as the basement membrane separating these cells from the theca interna disappears. Even so an organized network of connective tissue does not become

apparent until several days after ovulation (Donaldson and Hansel, 1965; MacNutt, 1924; Gier and Marion, 1961). The results obtained in the present studies agreed with this as the presence of connective tissue was only observed at a relatively advanced stage of luteinization probably corresponding to day three or four after ovulation. Furthermore the developing connective tissue appeared accompanying blood vessels only and invasive penetration into the cores of luteal tissue was limited. Angiogenic properties have already been attributed to luteal tissue (Redmer et al., 1988). These appear to be related to the secretion of one or several substances which attract vassofactive cells and are involved in capillary formation. The disappearance of the basement membrane of the follicle makes intercommunication between the theca and the granulosa layers possible by forming cell-to-cell contact and by chemical signalling. At the earliest stages of the corpus hemorrhagicum and even before the two cell-types intermix capillaries appear migrating towards the granulosa layers. Therefore it is tempting to suggest that, at this stage at least, these angiogenic properties are a characteristic of the granulosa-derived cells.

None of the early CLs examined had a blood filled cavity. Instead only a tiny pin-point area of clotted blood was found around the point of rupture which appeared as a lesion undergoing healing. Thus bleeding apparently only occurs around the point of rupture and the rest of the structure is made up of luteal cells rapidly undergoing cellular division and growth.

In the CLs of the second stage of the cycle ie. day 5-10 the most consistent differences from the earlier stage were the change in internal colour, the consistency of the tissue and to a lesser extent the size of the structure.

The colour of the region nearest the apex of these second stage CLs was the brown of organising blood with the remainder of the tissue ranging between

orange and tan. An important feature, common to all structures in this category, was the relative compactness of the tissue. Although the tissue was still soft, it was no longer friable. In general the variability in the size, was a helpful tool in the estimation of the stage of the cycle. Despite this a wide range of variation was recorded between individual CLs during the second stage and also during the third stage. It is possible that this difference in size during the second stage is due to differences in the rate of growth between individual CLs. However all the CLs examined must have reached maturity by this stage as the circulating plasma progesterone was more than 2 ng/ml in all animals tested. The fact that most of the CLs of the second stage were only partially embedded in the ovarian stroma compared to structures of the first stage indicates that the growth of the luteal tissue is somehow orientated towards the exterior. This gives the structure an elliptical shape rather than being spherical. This pattern of growth could be dictated by mere physical pressure from the ovarian stroma.

The large follicle invariably found during this stage and the absence of any such follicles in most ovaries of the first stage indicate that this is the dominant follicle of the first wave. The use of ultrasonography to monitor follicular dynamics in the living animal has established the existence of two or three waves of follicles during the interoestrous period in the bovine. It is now accepted that follicles grow and regress continuously during this period and the dominant follicle present at the time of luteolysis is the only one that ovulates. The other dominant follicles of the cycle undergo atresia.

Histological studies of CLs of the second stage indicated that the tissue was more organized with an extensive blood supply and distinguishable luteal cell-types. This indicates that in most normal bovine the luteal tissue is fully formed between day five to ten of the cycle. This is further supported by the fact that luteal cells were not seen in mitotic division at this stage. Conversely some

luteal cells, especially some of the large ones appeared to be undergoing regression as peripheral vacuolization and lighter staining of the nucleoplasm characterized these cells. In one of the CLs the whole tissue appeared to be undergoing regression. Early luteolysis is a relatively common phenomenon in post partum cows and in heifers. However the regressive changes observed in this second stage CL could represent faulty fixation of the specimen as the signs of regression did not include thickening of the wall of small arteries or extensive invasion of the luteal tissue by the connective tissue both of which are associated with normal regression.

By eleven days after ovulation the luteal structures examined demonstrated many of the features of the previous stage except that now the apex was the same colour as the rest of the structure indicating that healing was complete and that the blood had been replaced by luteal tissue.

When examined under light microscopy CLs of the third stage showed even more luteal cells undergoing regression. These included small cells as well as large ones. This suggests that the CL in the bovine begins to lose a percentage of its cells as early as the second stage and this process continues to increase during the third stage.

By the fourth stage (day 18-21) the CLs were invariably firm in consistency, of approximately 1.5 cm in size and appeared pale orange to yellow in colour. These changes were associated with low circulating plasma progesterone concentrations indicating cessation of function. Histologically CLs allotted to the fourth stage appeared either in an advanced state of tissue degeneration or else the luteal tissue had been replaced by connective tissue. The appearance of increased amounts of connective tissue seems to follow the regression of the tissue and was associated with CLs which were very small in size and had the

hardest consistency. Thus the connective tissue apparently plays no role in triggering luteolysis. The replacement of luteal tissue by connective tissue could explain why old CLs are still visible on ultrasound examination even though they are no longer functional.

The thickening of the walls of small arteries, which occurs during the time of luteolysis, resulting in obliteration of their lumen is thought to be caused by increased production of endogenous PGF2α and it is often suggested that this might be the mechanism by which PGF2α achieves luteolysis (Hafez, 1987b). In the histological studies presented here although thickening of the wall of the arteries was observed especially during ageing of the CL, evidence of regression of some large luteal cells occurred as early as the second stage when no thickening of the wall of blood vessels was observed suggesting that this may be rather a consequence rather than the cause of luteolysis. Redmer et al. (1988) observed that luteal cells from ageing CLs exhibited the greatest stimulatory effect on mitogenesis and migration of endothelial cells suggesting that the cells of the regressing CL fully preserve their angiogenic activity. This suggests that the luteal cells are able, unless an inhibitory factor prevents them, to promote vascular growth even during the latest stages of CL life-span.

Among the gross morphological details used by Ireland et al. (1980) as a means of differentiation was the variation in the distribution of the vasculature. In the studies described in this thesis it was found more reliable to assess the vascularization of the tissue by the intensity of bleeding when the tissue was sectioned. CLs bleed easily when sectioned until around the third stage when little bleeding occurred when the structure was incised.

At the cellular level signs of regression were observed even early in the cycle and were associated most frequently with large luteal cells indicating either the targeting of these cells by the luteolytic factor or that some cells were destined

to die after a certain time or following the secretion of a certain amount of progesterone.

The luteal population dynamics were assessed in these studies by counting each of the two cell-types in every one of ten fields chosen at random. Attempts to estimate the proportions of luteal cell-types have resulted in little agreement between the different workers. The estimate of 1:10.2 as describing the proportion of large luteal cells to small luteal cells obtained by Weber et al. (1987) is higher than 1:20-1:40 reported by Hansel et al. (1987). Both of these authors made their observations on dispersed luteal cells which introduces the possibility of selectively losing cells of one type or another during the dissociation and separation process. O'Shea et al. (1989) using morphometric studies found a 1:7.6 ratio. In the studies reported here the ratio of large to small luteal cells was found to vary significantly depending on the stage of the cycle ranging between 1:3 and 1:5.6. During the second stage the large luteal cells make up about 25% of the total luteal population. However as the CL ages towards the third stage the ratio of the large luteal cells to small luteal cells decreases markedly. This is unlikely to be due to the small luteal cells increasing in numbers as they were not seen dividing during the second stage. On the other hand some of both types of cells but mainly the large luteal cells showed signs of regression. The proportions of cell populations were reverted during the early periods of the fourth stage as large luteal cell proportions increased and those of the small ones decreased. This could indicate that the small luteal cells were dying faster than the large ones. On the other hand it is also possible that some of the small luteal cells enlarged to become large ones during the third stage. Alila and Hansel (1984) using monoclonal antibodies showed that such changes begin to occur by day nine of the cycle which corresponds to the late part of the second stage. Large luteal cells have also been shown to increase in numbers in

relation to small luteal cells during pregnancy. In the case of CLs of third stage used in these studies the possibilities are that some of them belonged to mated animals which were in early pregnancy (up to day 25) and so the CL had been influenced by the pregnancy maintenance factor(s). As for the fourth stage-CLs they could also belong to animals which had been pregnant for a few weeks and lost their embryos. Therefore all the regressing CLs examined cannot be assumed to be regressing CLs of the cycle which has not been exposed to embryo signals.

Bovine CLs frequently contain a fluid-filled lacuna. In these studies empty cavities were found consistently in CLs of both the first and second stage. Lacunae could be either present or absent in CLs of the third stage but in most fourth stage CLs studied the lacuna was absent. This suggests that at the beginning of their growth all CLs have empty spaces not as yet occupied by luteal tissue. As the structure develops in some CLs luteal tissue appears to fill the entire empty space whereas in others this does not happen. Hence the persistence of a lacuna which becomes surrounded by a fibrous membrane. As the CL ages the lacuna disappears.

In the absence of any data about the heifers stage of the cycle, collection of blood samples at slaughter was undertaken to support the assessment of the stage of the cycle by macroscopic examination. In the case of second and third stage CLs, the progesterone results were in general agreement with the assessment. However progesterone was raised above 2 ng/ml in heifers which had a CL that could only be classified as a first stage CL. This was also true of CLs rated stage four. This further supports the fact that certain macroscopic characteristic are more reliable than others in that they belong to one stage only. Others can be considered as secondary features.

In the studies described next the following scheme was thought to be satisfactory and so was used to categorize the CLs:

STAGE ONE	Size (cross section) of structure: < 1.6 cm
	Epithelium over apex: absent
	External appearence: red point of rupture
	Consistancy: friable
STAGE TWO	Size of structure: > 1.6 cm
	Epithelium over apex: present
	Internal colour: red or brown apex,
	remainder tan or orange
STAGE THREE	Size of structure: 2 cm
	Internal colour: wholly tan or orange
STAGE FOUR	Size of structure: < 1.6 cm
	Internal colour: light orange to yelllow
	Associated large follicles: at least one
	present

Part (iii): histochemistry

1. Origin and importance of 3β-HSD and 20α-HSD in the CL function

The enzymes 3β-HSD and 20α-HSD are involved in steroid synthesis (Lobel and Levy, 1968). 3β-HSD forms part of the smooth endoplasmic reticulum and in the bovine is first detectable in the follicle at the antral stage ie. when cells

of the early follicle become differentiated and capable of steroid synthesis. Following ovulation and luteinization the enzyme is present in increased amounts (Lobel and Levy, 1968). It is present not only in progesterone secreting tissues (CL, placenta...) but also in tissues where other steroids including androgens and oestrogens are synthesized (Rubin et al., 1963). 3β-HSD is involved in the steroid synthesis pathway at one of the initial steps common to all steroids. It converts the δ^5-3β-hydroxysteroids, pregnenolone and dehydro-epiandrosterone, to progesterone and δ^4-androstene-3,17-dione. The latter is readily secreted in the CL, whereas in the testes and the follicle it is used as a precursor for androgens and oestrogens respectively. Although it has already been shown that this enzyme is present in the bovine CL, to the best of our knowledge, its activity has not been assessed during the various stages of CL life-span. Given its important role in steroidogenesis, this enzyme could prove useful in investigations of CL function.

The enzyme 20α-HSD is found free in the soluble fraction of the cytoplasm (Lobel and Levy, 1968). It is present in the cells of the CL but not the follicle and in the rat its increase is positively correlated to the age of the CL (Pharriss and Wyngarden, 1969). The enzyme 20α-HSD catalyzes, in a reversible reaction, the conversion of progesterone into its 20α-hydroxyprogesterone metabolite also known as 20α-hydroxypregn-4-en-3-one (Wiest, 1959). 20α-HSD is associated with the regulation of progesterone synthesis (Llosa-Hermier et al., 1991). Indeed studies on rat CLs showed 20α-HSD activity only during metoestrus and dioestrus. Newly formed rat CLs have little or no 20α-HSD activity whereas regressing CLs show increased activity (Turolla et al., 1966). There is little information on the localization and quantitative variations of this enzyme in the bovine CL. Both 20α-HSD and 3β-HSD can be regarded as potential targets for the luteolysin PGF2α which by inducing the synthesis of the

former and inhibiting directly or indirectly the function of the latter may play a role in the control of steroidogenesis. Thus it was felt that the identification of any increases and decreases in these two enzymes in bovine luteal tissue could be helpful if correlated to (a) the stage of development of the luteal structure and /or (b) its normality as regards function. Of special interest was the activity of 20α-HSD around the time of luteal regression as it has been suggested from work in the human and the rat that this enzyme works in conjunction with prostaglandins to bring this about.

2. Validity of histochemical procedures

Since the introduction of the Wattenberg (1958) method for the study of 3β-HSD, a considerable amount of work has been done to demonstrate the presence of enzymes catalyzing the oxidation of pregnenolone to progesterone or of the dehydroepiandrosterone to androstenedione (Deane et al., 1962; Deane and Rubin, 1965 and Rubin et al., 1963). These studies were carried out in different tissues and in many domestic and laboratory species including the bovine. However the technique only allows identification of the enzyme within tissues as a whole and is not sufficiently selective to identify the specific cells which contain this enzyme. In addition when the substrate introduced is oxidized, the tetrazolium blue dye acting as hydrogen acceptor is not reduced directly during the reaction. Instead it is reduced by reacting with an intermediate hydrogen acceptor, namely a coenzyme (eg. NAD, NADP) which is present in the incubation medium. This renders the technique unsuitable for exact intracellular localization of the enzymes involved. Finally the reduced tetrazolium blue or formazan tends to adhere to proteins or dissolve in lipids. All these aspects of this technique make identification at cellular level impossible. However under controlled conditions the procedure remains

reliable for the overall investigation of the presence or absence of these enzymes in histological sections of specific structures.

In the preliminary studies using this histochemical approach it was recognized that readily water soluble enzymes such as 20α-HSD can diffuse into the incubation medium where they oxidize the substrate and so the reduced tetrazolium dye (formazan) which produces the staining reaction deposits in sections or sites other than where the enzyme is located (Lobel and Levy, 1968). Consequently in these studies a modified method of incubation from that of Lobel and Levy (1968) was introduced whereby a few drops of the incubation medium were added to individual slides containing a single section instead of using Columbia dishes where many section-containing slides are incubated together.

As discussed above formazan is not formed from the direct oxidation of the supplied substrate and a co-factor is necessary as a hydrogen acceptor which also requires a hydrogen transferring system with an electrode compatible with that of tetrazolium blue (Bjersing, 1977). Therefore if the results are negative it must be ascertained that both the tested enzyme and the hydrogen transferring system also called diaphorase system are present together in the same site. This was achieved by exposing a series of slides (a duplicate for each tissue sample) to a reduced coenzyme ($NADH_2$) and the tetrazolium blue. Only when a positive diaphorase activity was shown was a negative result recorded.

3. Activity of 3β-HSD

There was no clear evidence of any changes in the amount of 3β-HSD present, as demonstrated by staining intensity, in the different stages of the bovine CL. 3β-HSD was present in the bovine luteal tissue regardless of its functional status except in two cases in late dioestrus. These two negative results could either be

due to poor technique or else these two CLs were older than deduced and contained no luteal tissue. All the other CLs in late dioestrus contained the enzyme (day 17 to 21).

The finding of 3β-HSD in the earliest luteal tissue is in agreement with the results of Lobel and Levy (1968) who reported the existence of 3β-HSD in both theca and granulosa cells from the early stage of antrum formation of bovine follicles. The continuous presence of this enzyme from the follicular stage is as expected as progesterone is the precursor for both oestrogens and androgens which are known to be present in increasing amounts during maturation of the follicle. Therefore the enzyme is already available before luteinization and ovulation take place. Unfortunately because the method used in these studies was not sensitive enough, the apparent lack in any differences in the amount of 3β-HSD between the various stages of luteal tissue cannot be assumed to be accurate. The method used only allowed monitoring of the presence or absence of 3β-HSD in CLs and so it can solely be concluded that this enzyme is present in bovine CLs of all stages without any speculations on quantitative changes. The investigations previously undertaken in the cow by Lobel and Levy (1968) identified a difference in the activity of this enzyme between the follicular stage and the early CL. This aspect was not undertaken here. Scwall et al. (1986) made attempts of counting dispersed ovine luteal cells stained for 3β-HSD. They found significant changes between stages of the cycle, in the number of cells showing 3β-HSD activity. The figures obtained by these authors suggest that the numbers of 3β-HSD containing-cells increase between the early and the mid-luteal phase in both large and small cell-types. However during luteal regression the changes in these numbers were reversed in only the small luteal cell-type. Unfortunately these authors did not express their results in terms of proportions of 3β-HSD-containing cells out of the total cells present and so it is

not known whether this enzyme can be induced or inhibited during the life-span of the luteal cell or else the changes are only apparent as a reflection of the of increases and decreases in cell numbers.

As staining was only carried out on intact luteal tissue in the studies described in this thesis, it was not possible to count cells. Counter staining with light green gave little success and cell outlines remained hardly recognizable. If the results obtained by Scwall were to be confirmed in the bovine it could become almost certain that steroidogenesis in the small luteal cells is hormone dependent both for stimulation and inhibition, whereas in the large luteal cells it remains independent of factors like LH and PGF2α. On histology the large luteal cells seemed to have a predetermined life-time as they appear to start regressing at an early stage of the oestrous cycle, when no regressive changes are apparent on the remaining luteal population.

The use of more sensitive methods of detecting 3β-HSD as well as other steroidogenic enzymes within specific cells such as immunocytochemistry, direct biochemical quantification of the enzyme or the introduction of a DNA probe to study the expression of the genes encoding the synthesis of these enzymes require to be undertaken before any increases and decreases can be established. Such procedures could determine the role of 3β-HSD in steroidogenesis in the two different types of luteal cells and so further elucidate the functional capabilities of the bovine CL.

4. Activity of 20α-HSD

There is well documented evidence that, in the rat and human 20α-HSD intervenes in the luteolytic process of the oestrous cycle and at parturition (Llosa-Hermier et al. 1991). In the studies of bovine luteal tissue reported here the enzyme was present, according to the staining intensity, in tissues from CLs

of all stages. However staining of the enzyme was not as intense as that of 3β-HSD and therefore quantitative deductions from this method for this enzyme proved more difficult than for 3β-HSD. This is probably because of the hydrosolubility of the enzyme which is found free in the cytoplasm. Because control sections lacking the substrate showed some slight staining it was impossible to know whether the light staining found in sections incubated in full treatment medium were genuinely positive. Davis et al. (1966) encountered the same difficulties in his studies of rat, rabbit, guinea pig, cow and human ovaries. The light staining produced when the sections were incubated in medium lacking the steroid substrate could be due to the fact that progesterone if present in the cells would serve as a substrate and so the reaction would be expected to occur. The variations introduced to the standard technique aimed at overcoming the problem of solubility of this enzyme and included prefixing and prewashing of the tissue prior to incubation. However the results did not differ from those obtained following the usual procedure. Therefore no real deduction could be made as to whether or not there were changes in the amounts of 20α-HSD in the CLs of the different stages of the cycle even though it can be stated that the enzyme does exist in the bovine CL. However the results previously published regarding the presence of this enzyme in rat and human luteal tissue using the same method did identify an increase in the 20α-HSD around the time of luteolysis. This would tend to suggest that as no such increases were apparent in the studies in the bovine the role of this enzyme may be somewhat different from that of other species.

Again if a more sophisticated method was available by which accurate quantitative assessment could be made of the changes in the amounts of this enzyme during the various stages of both follicular development and luteal

formation and regression this would be of great benefit. Recently Llosa-Hermier et al. (1991) successfully prepared and purified monoclonal antibodies against the 20α-HSD enzyme. This method has great potential for future studies of the role of both these enzymes in the function of the CL both during the oestrous cycle and parturition.

Part (iv): transmission electron microscopy

In the living cell the shape of the nucleus, and the amount of condensed chromatin (heterochromatin) it contains are reliable indicators of the functional status of a cell. A multilobulated nucleus with a large surface area in association with the cytoplasm allows the increased nucleus-cytoplasm interaction necessary for protein synthesis. Furthermore as only the transcriptionally inactive chromatin is stained in the nucleus the presence of little heterochromatin in the nucleoplasm indicates that the cell is synthesizing large amounts of several types of proteins (Fawcett, 1981a). This was indeed the case with luteal cells at all stages. Variable amounts of rough or granular endoplasmic reticulum were present during the first and second stages. This feature along with the consistent observation of free polyribosomes in the cytoplasm of all cells at all stages is further evidence that the luteal cell synthesizes large amounts of proteins. The fate of these proteins produced by the luteal cells appears to depend on the stage of the cycle. It is widely believed that ribosomes require to be attached to granular endoplasmic reticulum for storage of the synthesized proteins to occur. The presence of free polyribosomes in the cytoplasm is indicative of proteins being produced for cellular use (Fawcett, 1981b). Granular endoplasmic reticulum was evident in bovine luteal cells of both stage one and two but was much reduced in other stages. Polyribosomes, by contrast, were constantly present at all stages and only appeared to be reduced in number in extreme

cases of cellular involution. This along with the fact that the number of what was assumed to be secretory granules (electron dense granules) appeared to dwindle during the later stages of luteal life span, suggests that the luteal cell synthesizes proteins for secretion via the rough endoplasmic reticulum, the golgi apparatus and finally the secretory granules only during the early phases of the cycle. On the other hand protein synthesis for local use is continuous and lasts until the cell regresses. There is now evidence that the large luteal cell secretes both oxytocin (Kruip et al., 1985; Fehr et al., 1987) and neurophysin (Guldenaar et al., 1984) and both of these peptides have been localized immunohistochemically, in the electron-dense secretory granules in these cells (Fields et al., 1992). The free polyribosomes present in the cytoplasm of luteal cells may be involved in synthesis of enzymes necessary for both steroid and protein synthesis and so do not need to be attached to rough endoplasmic reticulum as their products are not stored for later secretion but are used continuously.

In the studies reported in this thesis, the changes observed in bovine luteal cells during various stages of CL life span were in agreement with findings of previous published work (Parry et al., 1980; Koos and Hansel, 1981). During the first and second stages both rough and smooth endoplasmic reticulum are present. However as the CL ages the latter becomes the predominant feature and the amounts of rough endoplasmic reticulum lessens. In many instances the small amounts of granular endoplasmic reticulum observed appears as large distended vesicles containing a flocculent precipitate thought to be the remains of storage products which are lost during the fixation process (Fawcett, 1981b). The smooth endoplasmic reticulum appeared as membrane-limited tubules devoid of ribosomes and in steroid-secreting cells is correlated to the extent to which the cell synthesizes its own cholesterol (Christensen and Gillim, 1969).

This organelle is known to contain the enzymes necessary for synthesis of cholesterol from acetate and other enzymes involved in the later steps of steroid synthesis, eg. hydroxylase, hydroxysteroid dehydrogenases. Luteal cells also contained numerous pleomorphic mitochondria with tubular cristae. This type of mitochondria was found to be the most prominent organelle in the fully functional CL. They are known to contain the important cholesterol side chain cleavage enzyme, cytochrome P450, which converts cholesterol into pregnenolone. It is worth noting that even in CLs from the latter stages of the cycle these mitochondria were still present although their number appeared to decrease probably as a result of autophagy. Indeed in many instances luteal cells showed the presence in the cytoplasm of autophagic vacuoles with a mitochondria-like structure inside. Despite this, mitochondria were still present at the end of the cycle whereas the smooth endoplasmic reticulum was almost completely absent by this time. 3β-HSD which is attached to the membranes of the smooth endoplasmic reticulum and which is also thought to be a target for PGF2α, was present in luteal tissue of all stages according to the histochemical studies described in this chapter. As the rate limiting enzyme, cytochrome $P450_{scc}$ is present in the mitochondria, the question is raised as to why the mitochondria and not the smooth endoplasmic reticulum are still present in the regressing cell.

Others features probably associated with regression of luteal cells is the increase in the number and size of lipid droplets which by the later stages almost completely occupy the cytoplasm. This is thought to occur due to the accumulation of cholesterol precursors following the decrease of progesterone synthesis. On the other hand electron-dense secretory granules become less numerous and totally disappear from the cytoplasm of the large luteal cells during the latter stage of CL life-span indicating either a decrease in the

synthesis of protein destined for secretion or the disappearance of the granulosa-derived large luteal cells and their replacement by theca-derived luteal cells. It is worthwhile noting that in these studies the densely staining bodies were only assumed to be secretory granules. Indeed secretory granules, peroxisomes and lysosomes are difficult to distinguish on purely morphological grounds and special staining is necessary to establish which of these three structures are present in a cell (Parry et al., 1980). In fact some of these electron dense bodies had a heterogeneous appearance whereas other appeared evenly stained and so there could be a mixture of secretory granules, peroxisomes and lysosomes. The assumption that these were secretory granules was based on their manner of aggregating close to the nucleus which was described by Parry et al. (1980).

Part (v): cell culture

1. Tissue dissociation, cell counts and viability

In every cell culture system care should be taken to ensure that cells are subjected to the minimum damage possible during both the dissociation process and the washing process. Dissociation of bovine luteal tissue involved two steps: - first, a physical slicing of the tissue and, - secondly, enzymatic digestion. The time taken for the tissue to become a soup of dispersed cells was less for CLs of the first stage compared to CLs of other stages. This was probably due to the relatively small amount of connective tissue found in CLs of the first stage. For every CL the luteal cells were tested for viability by trypan blue staining. The viability was similar in both large and small luteal cells and did not vary according to the stage of the cycle. So the proportions of large and small luteal cells present after dissociation were regarded as representative of the proportions of viable large and small luteal cells included in every incubation

aliquot. Thus the results of progesterone production can be assumed to be representative of the different types of luteal tissue examined. When cell-type proportions were estimated during the histological studies the ratio of large to small luteal cell was found to range from 1:3-1:5.6 depending on the stage of the cycle of the luteal tissue. However when cells were counted following dissociation and washing this ratio was considerably lower (1:12-1:45) during the second, third and fourth stages. These values are similar to those reported by Hansel et al. (1987) and suggest heavy losses of large luteal cells during the dissociation process. Therefore it is worth considering this factor when transposing results of luteal function from *in vitro* models to the *in vivo* situation. In CLs of first stage, the estimation of proportions of large and small luteal cells following dissociation is only an approximation as most granulosa luteal cells are still enlarging and have not fully acquired their final size.

2. In vitro progesterone production

In the cow progesterone production by the large cells is known to be much greater than that by small luteal cells. However large luteal cells respond poorly to small doses (10 ng/ml) of LH to which the small ones are known to be six-times more sensitive (Koos and Hansel, 1981). This is particularly true of the cells from the CL of the oestrous cycle as in the pregnant cow large luteal cells appear to be as responsive to LH as the small ones (Chegini et al., 1984). One of the explanations for this discrepancy is the richness of the CL of the pregnant cow in large luteal cells which are thought to be derived from small luteal ones and during their transformation keep some of their functional characteristics such as responsiveness to LH. Large luteal cells from CLs of the cycle only increase their progesterone production when high doses (100 ng/ml) of LH are administered (Alila et al., 1988). It is not known whether this response is by the

granulosa-derived large cells or by the theca-derived large cells which were originally small luteal cells.

The addition of 13 ng/ml of LH to the isolated bovine luteal cells significantly stimulated progesterone production in all but cells from CLs of the fourth stage. Because unstimulated progesterone production was maintained in CLs of all stages, the loss of responsiveness to LH in the late-stage CL could indicate luteolysis in the small luteal cells. The addition of PGF2α to LH-treated luteal cells resulted in the reduction of progesterone secretion in CLs of second and third stage. This may suggest that during the third stage of the cycle, PGF2α acts on the small luteal cells to cause a loss of responsiveness to LH. However Alila et al. (1988) claimed that with highly purified preparations of small and large luteal cells PGF2α stimulated LH-induced progesterone production in the small cells and inhibited LH-induced progesterone production in the large luteal cells. These conflicting results could be due to the following: (a) the dosage and type of synthetic PGF2α used; (b) the variation in the age of CLs used in these studies such that a CL allocated for example to the third stage could be between 11 to 17 days old and so the progesterone produced varied widely from one CL to the other; (c) large and small luteal cells are known to synergize to promote progesterone production during the mid-luteal phase (day 10-12) and may inhibit one another during luteal regression (Hansel et al., 1991). Therefore results from *in vitro* studies with either dispersed luteal cells or separated cell-types would be expected to vary depending on the contents of the two different populations of luteal cells. Koos and Hansel (1981) reported that when the two types of luteal cells were separated, PGF2α had no significant effect on progesterone production by either type of luteal cell whether LH was present or absent. However as mentioned above seven years later they contradicted these previous results (Alila et al., 1988). Short-time culture is the method used to

assess the response of the cultured material (cells, tissue or organs) to various stimulating or inhibiting factors. However it is not always safe to extrapolate results from the living animal as the immediate response to a certain treatment may change with increasing time of exposure to this treatment. An example of this are the results described by Plate and Condon (1984) who plated cells and showed that the lack of effect of PGF2α on dispersed luteal cells was only temporary and that following the first 24 hours of exposure to PGF2α LH-stimulated progesterone synthesis was inhibited.

By the fourth stage the regressing CL has very few functional luteal cells left and these do not appear to respond to either prostaglandins or LH. Indeed isolated luteal cells from many CL of this stage produced no detectable progesterone. In others no luteal cells were found after enzymatic dissociation.

When PGF2α alone was added to dispersed luteal cells there was no alteration in progesterone production except with cells from CLs of the third stage where progesterone production was increased. This result has been reported previously. When Hixon and Hansel (1979) treated dispersed luteal cells with PGF2α they observed an increase in progesterone production which in later studies was found to be an attribute of the small luteal cells (Hansel et al., 1988).

The CL of pregnancy is thought to become made up exclusively of theca-derived luteal cells some of which enlarge and replace the granulosa large luteal cells. The latter may only be present during the earliest stages of pregnancy prior to the signal for maintenance of pregnancy and the theca-derived cells are designed to support pregnancy subsequently. The large cells are known to produce oxytocin (Fields et al., 1992) and relaxin (Fields, 1980). However it was shown that only a proportion of the large luteal cells contain the secretory granules in which oxytocin was found (Hansel et al., 1987) and it is already

thought that these are the granulosa-derived large luteal cells. The other fraction of large luteal cells which is derived from the small theca-derived cells are proposed as the source of relaxin found mainly in the CL of pregnancy (Fields, 1980). If these new observations were to be supported by further evidence it would greatly explain the replacement of granulosa derived luteal cells by those derived from the theca during CL formation and early pregnancy. If these changes in luteal cell populations were to be established and if a way of separating the sub-populations of large luteal cells according to whether they are derived from granulosa cells or the theca cells was found and the effect of PGF2α on each type of cell determined, it would be of great benefit in our understanding of the maintenance of pregnancy. Looking at differences between periods of the cycle there seemed to be an increase during the second stage in both basal and LH-induced progesterone production. This appeared to be followed by an attenuated secretion during the following stages. Possible LH-receptor desensitization cannot explain this type of changes as a response to LH stimulation although attenuated was noted during the third stage. Desensitization by receptor-site loss is not an acceptable explanation for reduction in basal progesterone secretion during the third and the fourth stage. However luteotrophic agents other than LH have been reported to act on luteal cells and cause increase in progesterone production. These are mainly prostaglandins such as PGE-2 (Shelton et al., 1990), PGI-2 (Hansel and Dowd, 1986) which can be produced by the luteal cells themselves. The loss of sensitivity to such luteolytic factors may occur between the second and the third stage of the cycle resulting in reduced basal progesterone production.

The studies reported here constitute a method for preliminary investigations of the normality of the composition and function of CLs. By using gross

macroscopic examination, histology, electron microscopy, enzyme histochemistry and cell culture CLs suspected or expected to be subfunctional or inadequate may be monitored. Comparing changes in structure and function could allow a better understanding of what might be interfering with the normal function of the bovine CL during synchronization programmes or during early pregnancy resulting in embryonic death.

CHAPTER FIVE

STUDIES OF CORPORA LUTEA INDUCED BY AN INJECTION OF PROSTAGLANDIN DURING EITHER EARLY OR LATE DIOESTRUS AND CORPORA LUTEA INDUCED BY AN INJECTION OF A GnRH ANALOGUE DURING MID-DIOESTRUS

I. INTRODUCTION

The established procedures described in chapters three and four were used to investigate CLs from cows which had been synchronized during either early or late dioestrus with a prostaglandin analogue (luprostiol). In addition two cows one in mid-dioestrus and the other in a nonluteal state were treated with a GnRH analogue and their subsequent luteal function investigated.

II. MATERIALS AND METHODS

1. Animals and treatment

1.1. Experiment 1

The protocol of the first experiment is described in table 4.1. Six multiparous non-lactating Friesian cows were selected on the basis of their normal cyclicity (behavioural records of the immediately previous two successive oestrous cycles). Four of the cows were then injected intramuscularly with 15 mg luprostiol (Prosolvin, Intervet UK Ltd) on day six or seven of the oestrous cycle and the other two cows received the same treatment on day 14 of the oestrous cycle. All cows were slaughtered between days six to nine of the subsequently induced oestrus or if no oestrous behaviour was detected, six days following luprostiol injection. Immediately after slaughter the CLs were removed from the ovaries.

1.2. Experiment 2

The protocol for the second experiment is described in table 4.2. Two multiparous Friesian cows were given an intramuscular injection of 10 μg buserilin (a synthetic GnRH analogue; Receptal, Hoechst Animal Health UK Ltd) in 2.5 ml on day twelve after the cows showed signs of oestrus (in one cow only signs of prooestrus were observed at the expected time of oestrus). One of the two cows was slaughtered six days after the injection was given and the ovaries were collected and CLs removed.

Table 4.1 Protocol for the first experiment.

Days of the cycle / Groups	0 (natural oestrus)	6-7	14	0 (induced oestrus)	6-9*
Group 1	LH peak monitored	Prosolvin injection		LH peak monitored	Cows slaughtered and ovaries collected for in vitro studies
Group 2			Prosolvin injection		

** Five of the six cows used were slaughtered on day 6 or 7 of the induced cycle. One cow in group 2 was slaughtered on day 9.*

Table 4.2 Protocol for the second experiment.

Days of the cycle	0 (natural oestrus*)	12	18
Protocol	LH peak monitored	GnRH injection LH monitored	One cow slaughtered and ovaries collected for in vitro studies

** One of the two cows used only showed signs of prooestrus.*

2. Behavioural studies

For animals of both experiments, checking for oestrous behaviour was carried out as described in chapter three.

3. Ultrasound examination

Ultrasound examination was carried out as described in chapter three. Criteria used to distinguish between various ovarian structures and follicular classification according to size are also described in chapter three.

Experiment 1: cows were scanned on the day of Prosolvin injection, 24 hours following Prosolvin injection, 24 and 48 hours following induced oestrus.

Experiment 2: cows were scanned on the day of spontaneous oestrus, on the day of buserilin injection, 24 and 72 hours after buserilin injection.

4. Blood sampling

4.1. Sampling for plasma progesterone

Collection of blood samples for progesterone estimation and plasma separation were undertaken as described in chapter three.

In *experiment 1* sampling for progesterone was carried out once daily during periods of expected oestrus, on the day of luprostiol injection and on the day of slaughter (day six to nine following the induced oestrus).

In *experiment 2* sampling for progesterone was carried out once daily throughout the trial.

4.2. Sampling for LH

Collection of blood samples for plasma LH was undertaken using method 2 described in chapter three. Plasma separation was carried as for plasma progesterone samples.

The frequency of bleeding around oestrus period was identical to that described in chapter three.

In the first experiment all six cows were bled for LH during the natural oestrus period immediately preceding the cycle when treatment was given. In addition the LH surge was monitored during the induced oestrus period.

In the second experiment bleeding for LH was also carried out during the oestrus period immediately preceding the cycle of the treatment. In addition the LH pattern was monitored from 240 minutes before and for up to 240 minutes after the injection of buserilin.

5. Hormonal studies

5.1. Plasma progesterone

Plasma progesterone was measured in triplicate as described in chapter two.

5.2. Plasma Luteinizing Hormone

Plasma LH concentration was estimated using the method described in chapter three.

6. Tissue collection and fixation

For all slaughtered animals, ovaries were collected and CLs incised and small blocks removed and immediately fixed, for either light or transmission electron microscopy as described in chapter four. The remainder of each CL was

transported to the laboratory in ice-cold medium.

7. Macroscopic examination

Upon arrival to the laboratory macroscopic features of each CL were recorded according to the method described in chapter four. Then CLs were weighed blot dry and a small block was put in a cryotube and frozen until used for enzyme histochemistry.

8. Histology

Fixed blocks of CLs were processed for histology and sections were stained with MSB as described in chapter four.

9. Histochemistry

Frozen blocks were transferred to the cryostat, sections cut and stained for 3β-HSD as previously described in chapter four. Enzyme histochemistry was only performed for samples of the first experiment.

10. Electron microscopy

The same procedure described in chapter four was used in these studies. Only samples from experiment 1 were subjected to electron microscopy.

11. Cell culture and estimation of progesterone in the medium

Tissue was sliced and cells enzymatically dispersed according to the method detailed in chapter four. The same procedure for cell culture was followed in order to determine the response of luteal cells to LH and PGF2α *in vitro*.

Progesterone production *in vitro* was estimated in the medium using the radioimmunoassay technique described in chapter four.

III. RESULTS

Experiment 1

Table 4.3a.

In this table the ovarian changes and the plasma progesterone concentration during critical periods of the trial are presented in animals of both group 1 and group 2. Following a spontaneous oestrus all cows but one (cow No. 57, see table 4.3b) formed a functional CL (raised progesterone in day seven or 14). On the day of Prosolvin injection (days seven and 14 for group 1 and group 2 respectively) three cows in group 1 and the two cows of group 2 had a functional CL and at least one medium-sized follicle. On the following day the CL was still present on ultrasound but the plasma progesterone decreased to less than 2 ng/ml in three of the four cows of group 1 and in one of the two cows of group 2. Ultimately three cows of group 1 and the two cows of group 2 were seen in overt oestrus and their circulating plasma progesterone was less than 2 ng/ml. Plasma progesterone concentration and post-mortem findings indicated that in all five cows a functional CL was present by day seven to nine.

Table 4.3a Ovarian and plasma progesterone changes during critical periods of *experiment 1* in cows treated with one injection of Prosolvin during either early *(group 1)* or late dioestrus *(group 2)*.

Group	Cow's identity	Day of spontaneous oestrus	Day of Prosolvin injection[1]			24 hours after Prosolvin			Day of induced oestrus	24 hours after oestrus		48 hours after oestrus		Day 7 to 9	
			ULSD			ULSD				ULSD		ULSD		Post mortem	
		P_4 *(ng/ml)*	CL	Follicles	P_4 *(ng/ml)*	CL	Follicles	P_4 *(ng/ml)*	P_4 *(ng/ml)*	CL	Follicles	CL	Follicles	CL	P_4 *(ng/ml)*
1	6	<0.5	+ (LO)	One large (RO)	4.8	+ (LO)	Three medium (LO) & one large (RO)	0.6	<0.5	+ (LO)	One medium (LO) & one large (RO)	-	One medium (LO)	+ (RO)	7.2
	8	1.2	+ (LO)	One medium (LO)	>10	+ (LO)	Two large (LO)	1.3	<0.5	-	One large (LO) & one medium follicle (RO)	-	One large (RO)	+ (LO)	2.1
	90	<0.5	+ (LO)	One large & one medium (LO)	2.4	+ (LO)	Three medium (LO)	1.6	<0.5	+ (LO)	One medium (LO) & four medium (RO)			+ (LO)	3.9
2	93	1.5	+ (LO)	Two large (RO)	>10	+ (LO)	One large & one medium (RO)	1.5	0.8	-	-	Data not available		+ (RO)	2.3
	158	0.8	+ (LO)	One medium (LO) & one large (RO)	>10	+(LO)	One large (LO) & one large (RO)	2.2	0.8	-	One large (LO) & one medium (RO)	Data not available		+ (LO)	8.6

([1]) Day of Prosolvin is day 6 or 7 for cows in group 1 and is day 14 for cows in group 2. Note that only three cows are included in group 1. The missing cow (#57) did not respond to the Prosolvin injection (see table 5.3b). (+) = CL present. (-) = no structures observed. (RO) and (LO) = right and left ovaries respectively. Large follicles = follicles of ≥1.5 cm in size. Medium follicles = follicles of ≥0.8 cm in size. P_4 = circulating plasma progestrone. ULSD = ultrasound examination.

Table 4.3b.

This table shows the ovarian and plasma progesterone changes during the trial in cow No. 57. Following an apparently normal spontaneous oestrus, a luteal cystic structure developed in the right ovary of this animal. This structure was secreting normal mid-luteal plasma concentrations of progesterone on the day of Prosolvin injection. On the day following the injection the structure appeared to have responded as the progesterone decreased to 1.7 ng/ml. This cow showed no oestrous behaviour and during the next five days of the trial the progesterone concentration increased to 4.7 ng/ml.

Table 4.3b Ovarian and plasma progesterone changes during critical periods of *experiment 1* in **cow #57** (treated with one injection of Prosolvin during early dioestrus).

Group	Cow's identity	Day of spontaneous oestrus	Day of Prosolvin injection			24 hours after Prosolvin			P_4 *(ng/ml)*				
		P_4 *(ng/ml)*	ULSD		P_4 *(ng/ml)*	ULSD		P_4 *(ng/ml)*	Days after Prostaglandin				
			Luteal Structure	Follicles		Luteal structure	Follicles		Two	Three	Four	Five	Six
1	57	<0.5	Luteinized cyst (RO)	-	8.0	Luteinized cyst (RO)	Four medium (LO)	1.7	1.8	1.9	3.0	2.34	4.74

(+) = CL present. (-) = no structures observed. (RO) and (LO) = right and left ovaries respectively. P_4 = circulating plasma progesterone. ULSD = ultrasound.

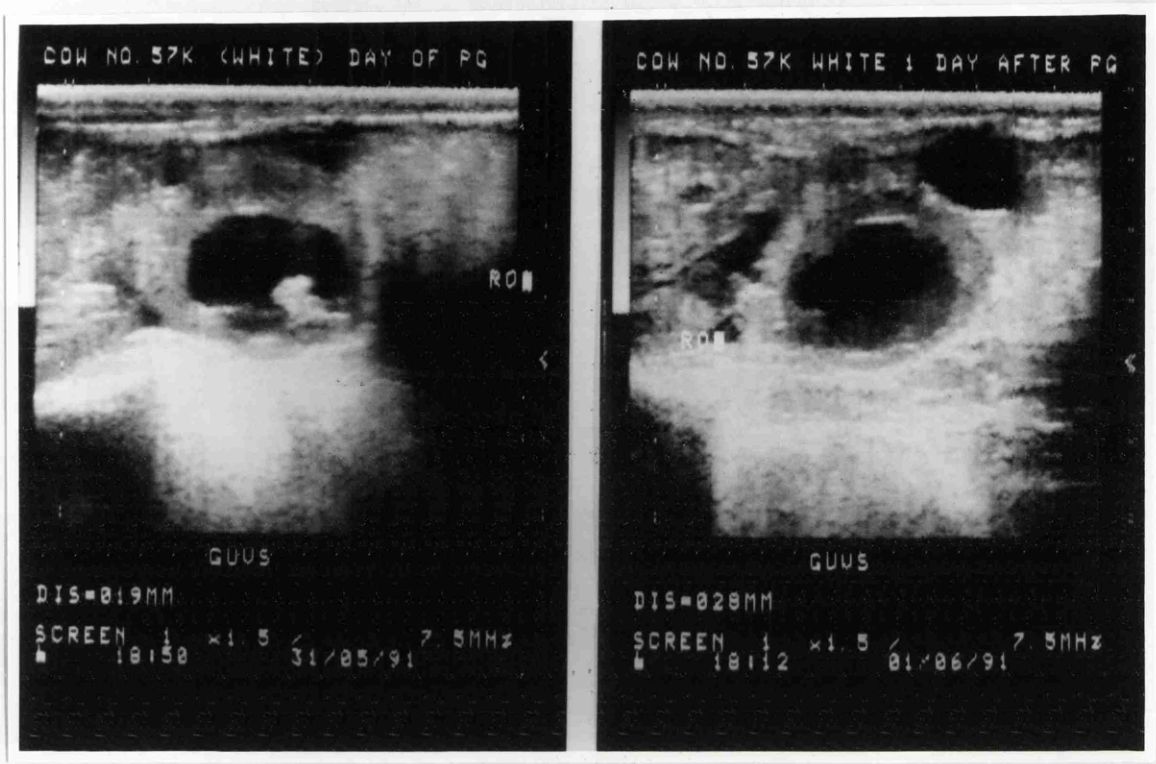
Plate 4.1.

a) This image shows a scanner section of the luteinized cystic structure observed in the right ovary of cow No. 57 on the day of Prosolvin injection. Note the highly echogenic material protruding into the cavity of the structure.

b) One day after Prosolvin injection the structure was still present with the same size and appearance.

Plate 4.2.

A gross macroscopic view of the cystic structure which formed in cow No. 57 is shown following sectioning. The cow was slaughtered on day twelve of the oestrous cycle (six days after Prosolvin injection). Note that the structure is in the right ovary and that no other luteal structure was observed in the ovaries of this animal. Note also the presence of white membranous structures observed on ultrasound inside the cavity of the luteal cyst.


COW NO. 57K (WHITE) DAY OF PG
GUUS
DIS=019MM
SCREEN 1 ×1.5 7.5MHz
18:50 31/05/91
COW NO. 57K WHITE 1 DAY AFTER PG
GUUS
DIS=028MM
SCREEN 1 ×1.5 7.5MHz
18:12 01/06/91

a b

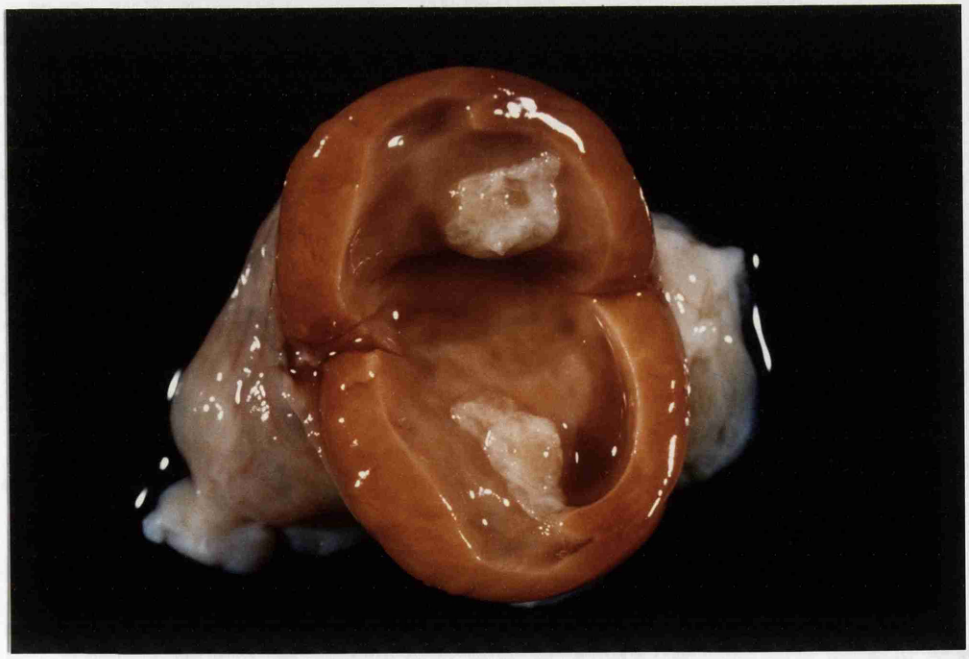


Figure 4.1.

Plasma LH concentration around spontaneous oestrus in cows of both group 1 (a) and group 2 (b) is presented. In group 1, cows (Nos. 57 and 6) showed a peak of LH of more than 30 ng/ml. The peak in cow No. 90 was missed as the bleeding stopped at 10 hours following the first observed oestrus. In cow No. 8 which was bled every six hours throughout the oestrous period, the peak reached a little more than 10 ng/ml. Note the variation in the timing of the onset of LH peak in relation to the onset of oestrus. In group 2, one cow only showed the LH peak. The other one (cow No. 158) only showed a slight rise at approximately 2 hours before oestrus.

Fig. 4.1 Plasma LH concentration around spontaneous oestrus

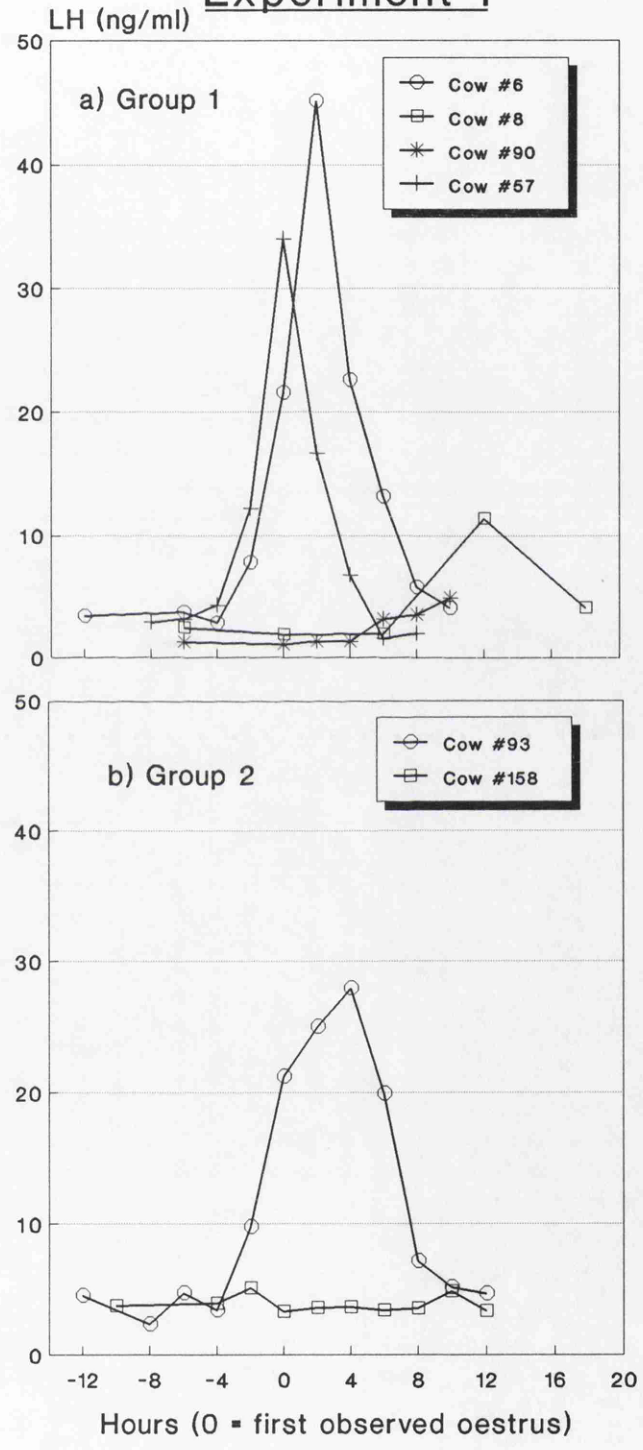


Figure 4.2.

Plasma LH concentration around induced oestrus in cows of both group 1 (a) and group 2 (b) is presented. In group 1 the three cows which responded to the Prosolvin injection had a peak of LH. The timing in relation to oestrus and the amplitude vary widely between individual animals. In both cows in group 2 the LH peak was detected but in cow No. 96, the peak was late and so was partly beyond the bleeding period.

Fig. 4.2 Plasma LH concentration around induced oestrus

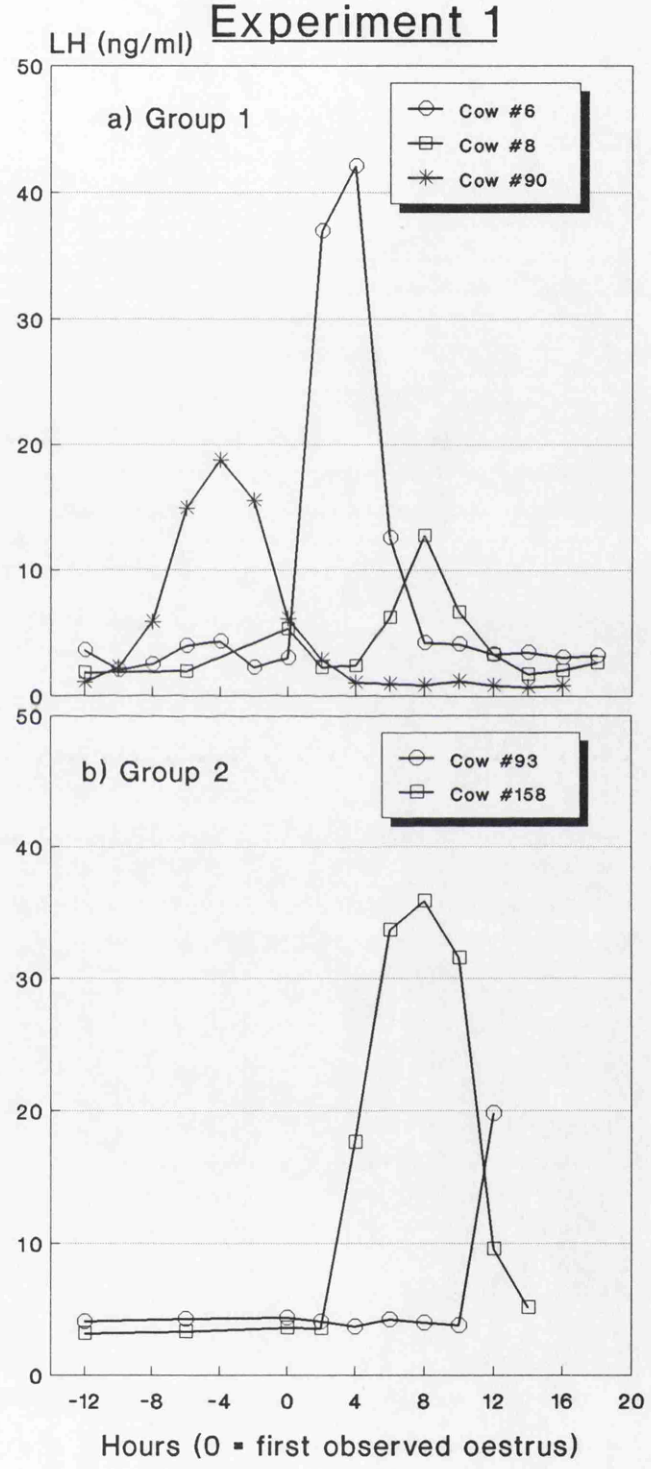


Table 4.4.

A few parameters related to the LH peak are compared in cows of group 1 and group 2 during both spontaneous and induced oestrus. Variation within each category in the timing and the amplitude of the LH peak was such that no difference was found between types of oestrus or treatment groups.

Table 4.4 Timing of LH and oestrus during *experiment 1*.

Group	Cow's identity	Spontaneous oestrus		Induced oestrus			
		Amplitude of LH peak *(ng/ml)*	Interval from onset of oestrus to onset of LH peak *(hours)*	Amplitude of LH peak *(ng/ml)*	Interval from onset of oestrus to onset of LH peak *(hours)*	Interval from injection of Prosolvin to onset of LH peak *(hours)*	Interval from injection of Prosolvin to onset of oestrus *(hours)*
1	*6*	45.1	-3	42.0	+1	55	54
	8	11.3	+9	12.8	+5	81	76
	90	4.9	>10*	18.7	-11	51	62
	57	34.0	-3	No induced oestrus (no response to Prosolvin)			
Mean ± sd for group 1		**23.8 ± 18.8**	**3.2 ± 7.2**	**24.5 ± 15.4**	**-1.6 ± 8.3**	**62.3 ± 16.2**	**64 ± 11.1**
2	*93*	27.9	-3	19.8	+11	71	60
	158	5.11	-3	35.8	+3	83	80
Mean ± sd for group 2		**16.5 ± 16.1**	**-3.0 ± 00**	**27.8 ± 11.3**	**7.0 ± 5.6**	**77.0 ± 8.8**	**70.0 ± 14.1**
Mean ± sd for all animals.		**21.4 ± 16.7**	**1.1 ± 6.4**	**25.4 ± 12.0**	**1.8 ± 8.0**	**68.2 ± 14.6**	**66.4 ± 11.0**

* *Assumed to be 10 in computation of means.*

Table 4.5.

Both gross macroscopic and microscopic features of the luteal tissue collected following slaughter of cows of group 1 and 2 are presented. Macroscopically, the CLs of the three responsive cows (Nos. 6, 8 and 90) in group 1 had similar features to those found in CLs of the second stage of the oestrous cycle (days 5 to 10, see chapter four). The luteal structure of cow No. 57 had no apparent vestiges of the ovulatory papillum (usually a depression point centrally located on the apex) and the internal colour of the tissue was the yellow of the late third stage. In cows of group 2, the CLs were slightly larger than in group 1. In particular cow No. 158, which was slaughtered on day 9 as opposed to day 6 or 7 for the other cows, the CL showed features of the third stage.

At the light microscopy, CLs of the three responsive cows of group 1, had very similar proportions of large and small luteal cells. These proportions were different from those of CL of the second stage studied in chapter four. This difference is even larger in cow No. 93 (group 2). In cow No. 158, the proportions of large luteal cells were the highest so far. There was no particular difference noted between treatment groups, in the general appreciation of the luteal tissue. However sections from all responsive cows showed evidence of a variable but abnormal degree of regression. Note that blood vessels were thin-walled and had an open lumina.

Table 4.5 Gross morphology and histology of CLs induced by an injection of Prosolvin during either early or late dioestrus.

Group	Cow's identity	Gross macroscopic appearance	Histology		
			%LLC[1]	%SLC[1]	General appreciation
1	6	CL of 1.7 cm in size, present in RO. The ovulatory papillum was covered by epithelium and the internal colour was tan with a red apex.	10.4	89.6	In some areas, collagen fibers were present within cores of luteal cells. Blood vessels had an open lumina and a thin wall. In most large luteal cells small lipid droplets were present in the cytoplasm. Small luteal cells appeared normal.
	8	CL of 1.6 cm in size, present in LO. The ovulatory papillum was covered by epithelium and the internal colour was tan with a red apex.	10.7	89.3	Large amounts of collagen fibers were present in most areas of the section. Blood vessels had an open lumina and a thin wall. Both large and small luteal cells stained light due to the presence of lipid droplets of variable size and in some cells vacuoles were present. A few cells had a light staining nucleoplasm.
	90	CL of 2.0 cm in size, present in LO. The ovulatory papillum was covered by epithelium and the internal colour was tan with a red apex.	11.3	88.7	Large amounts of collagen fibers were present in most areas though not as extensively as in cow #8. Presence of vacuolae in the cytoplasm and light staining nucleoplasm were common among all cell-types.
	57	Luteal cyst of 2.1 cm in size, present in the RO. The ovulatory papillum was not visible and the internal colour was yellow.	14.7	85.3	Fibers of collagen appeared invading cores of luteal tissue. Some small arteries had thick walls but were open. Many large luteal cells had no nucleolus. The nucleus stained light and vacuoles were present in the cytoplasm of most cells.
Mean ± sem for group 1 (excluding cow #57):			**10.8 ± 0.26**	**89.2 ± 0.26**	
2	93	CL of 2.2 cm in size, present in RO. The ovulatory papillum was covered by epithelium and the internal colour was tan with a red apex.	7.4	92.6	Collagen fibers were present in most areas of the section. Blood vessels were open and had a thin wall. Many luteal cells of both types showed regressive signs similar to those described in cows #6 and 8.
	158	CL of 3.2 cm in size, present in LO. The ovulatory papillum was covered by epithelium and the internal colour was entirely tan.	34.4	65.6	Abundant collagen fibers almost separating single cells. Presence of small connective tissue cells was also observed. Blood vessels were open and had a thin wall. Luteal cells presenting similar signs of regression as those described in sections from cows #6 and 8.
Mean ± sem for group 2			**20.9 ± 13.5**	**79.1 ± 13.5**	

([1]) *Cell-type percentage of the total luteal population. This does not include other non-luteal cells. SLC = small luteal cells and LLC = large luteal cells.*

Table 4.6.

The ultrastructure of luteal cells from CLs induced by an injection of Prosolvin indicates that the regressive changes observed in histology are real. The presence of lipid droplets in most cells is consistent in luteal cells of all responsive cows.

Staining for the presence of the enzyme 3β-HSD was intense in all but cow No. 90 where it was moderate.

Table 4.6 3β- Hydroxysteroid dehydrogenase (3β-HSD) activity and ultrastructural features in CLs induced by an injection of Prosolvin at either early or late dioestrus.

Group	Cow's identity	Staining for 3β-HSD	Cell ultrastructure (general appreciation)
1	6	+++	The granular endoplasmic reticulum was the most important organelle in the cytoplasm of both large and small cells. Both cell-types had fewer mitochondria than expected in a CL of the second stage. Smooth endoplasmic reticulum was scarce. Many cells had lipid droplets in the cytoplasm. The electron dense secretory granules were absent.
	8	+++	The smooth endoplasmic reticulum and mitochodria were more abundant than in cow #6, but still less than expected. Lipid droplets were present in the cytoplasm of almost all cells observed. The granular endoplasmic reticulum was quite discrete. Secretory granules were present.
	90	++	As cow #8 but more lipid droplets were observed in the cytoplasm of luteal cells.
	57	+++	The cells of this structure were identical to those seen in CLs of the third stage. Both mitochondria and smooth endoplasmic reticulum were dominant features of the cytoplasm. A few lipid droplets were present in the cytoplasm.
2	93	+++	Both smooth endoplasmic reticulum and mitochondria were present in abundance. In virtually all cells, lipid droplets were found in the cytoplasm in more or less large numbers.
	158	+++	

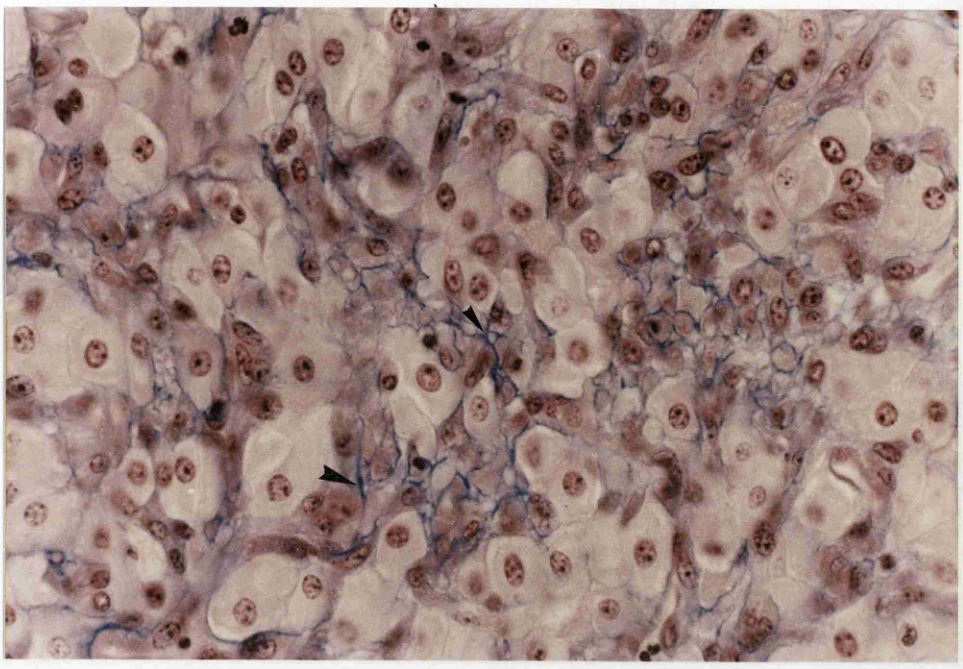
(+++) = intense blue staining, (++) = moderate blue staining.

Plate 4.3a.

Histological section (MSB, x 500) of luteal tissue from cow No. 8 (group 1). Note the presence of large vacuoles in the cytoplasm of luteal cells and the presence of large amounts of collagen fibres infiltrating between luteal cells (arrows).

Plate 4.3b.

Histological section (MSB, x 500) of luteal tissue from cow No. 158 (group 2). Note the presence of connective tissue between cores of luteal cells. Many cells have light staining nucleoplasm with no nucleolus.



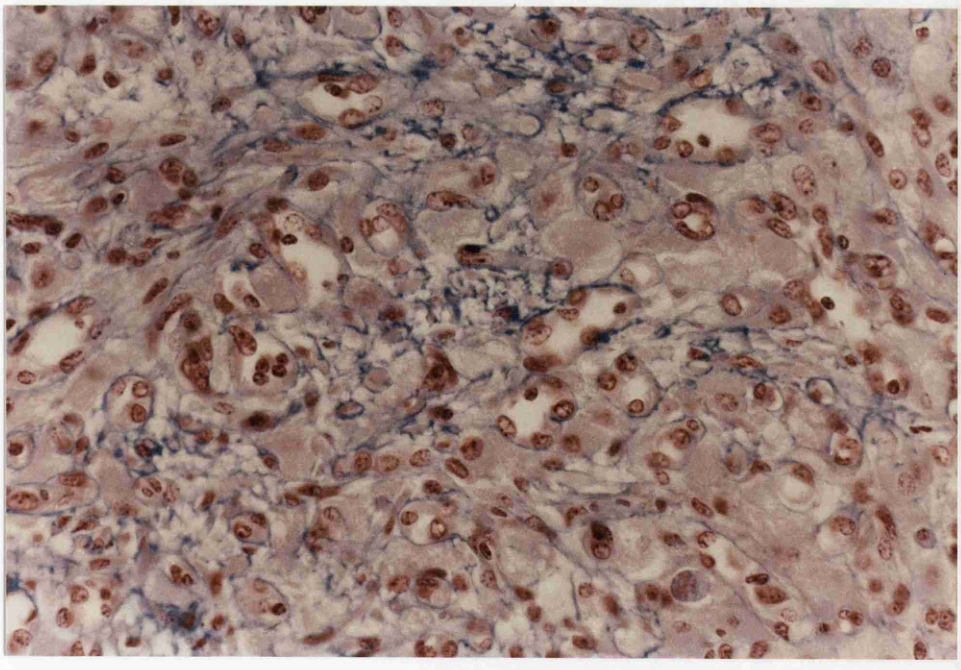


Plate 4.4.

The ultrastructure of a large luteal cell from cow No. 90 (group 1). Lipid droplets (L) are abundant and constitute the main feature of the cytoplasm (x 3000).

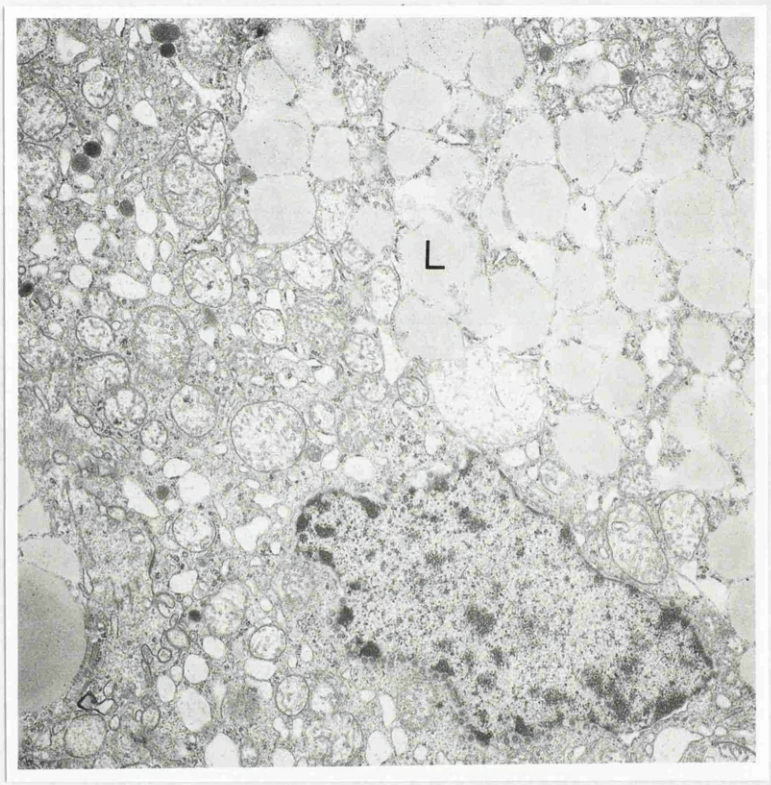

L

Table 4.7.

The response of dispersed luteal cells to LH and PGF2α *in vitro* is shown. Progesterone production was stimulated in luteal cells of two of the responsive cows of group 1 (Nos. 6 and 90). In cow No. 8 the cells produced small amounts of progesterone. The luteal cells from the cystic structure in cow No. 57 were still functional as progesterone was produced in comparable amounts to luteal cells from cows Nos. 6 and 90. In group 2, luteal cells from both cows produced little if any progesterone.

In group 1, the addition of LH, to the dispersed cells stimulated progesterone production ($P = 0.01$). This did not appear to be the case in cow No. 158, in group 2. When results from all animals treated with Prosolvin (except cows Nos. 57 and 93) were pooled together, the stimulation of progesterone by LH was found also significant ($P = 0.02$).

In group 1, the addition of PGF2α, made no significant difference with control and the addition of PGF2α simultaneously with LH resulted in no difference with the addition of LH alone. This result did not change when cow No. 158, from group 2, was included in the statistical tests.

Table 4.7 In vitro progesterone production by dispersed luteal cells from CLs induced by an injection of Prosolvin at either early or late dioestrus.

Group	Cow's identity	CL weight *(g)*	In vitro progesterone production by dispersed luteal cells *(ng/20000 cells/3hours incubation)*[1]			
			Control	+13 ng/ml LH	+1 μg/ml PGF2α	+LH & PGF2α
1	6	2.60	49.1 ± 7.1	168.2 ± 104.8	69.1 ± 8.4	125.7 ± 11.3
	8	2.40	13.7 ± 9.8	14.3 ± 9.4	12.6 ± 13.0	8.5 ± 13.9
	90	3.86	97.3 ± 35.5	255.7 ±15.5	97.1 ± 17.8	150.4 ± 3.3
	57	4.26	47.0 ± 20.2	94.1 ± 26.2	52.4 ± 24.6	72.69 ± 11.93
Mean ± sem for group 1[2]		**2.95 ± 0.46**	**52.0 ± 23.0**[a]	**146.1 ± 70.6**[b]	**59.6 ± 24.9**[a]	**94.9 ± 43.8**[b]
2	93	2.50	-	-	-	-
	158	4.60	12.1 ± 2.9	6.4 ± 5.9	22.3 ± 5.8	2.5 ± 3.4
Mean ± sem for group 2		**3.56 ± 1.06**	-	-	-	-
Mean ± sem for all[3]		**3.37 ± 0.4**	**43.8 ±15.5**[a]	**107.7 ± 47.3**[b]	**50.7 ± 15.4**[a]	**72.0 ± 29.9**[b]

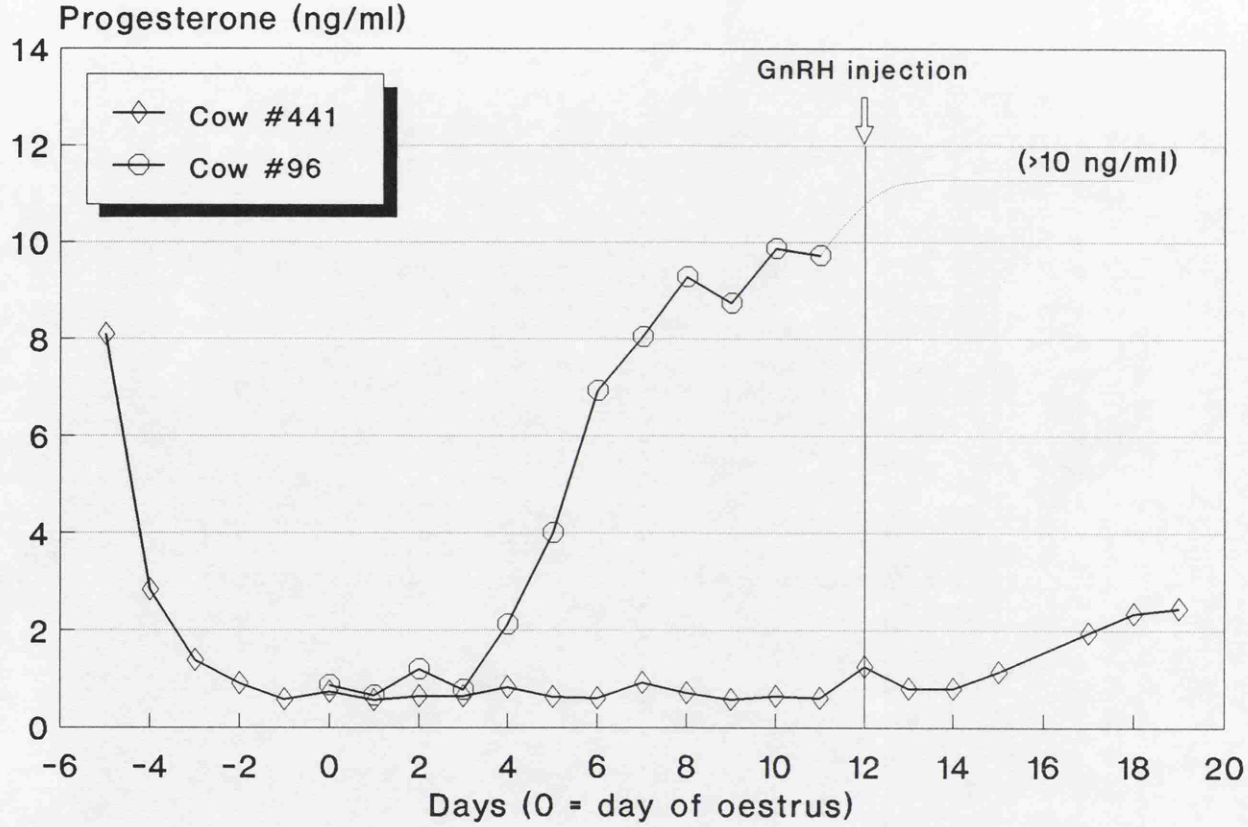
([1]) *Progesterone production for each cow is expressed as mean ± sd of three replicate-tubes per treatment.*
([2]) *Cow #57 is not included in computation of the means.*
([3]) *Luteal cells from cow #93 did not produce any detectable progesterone. Means for groups and for all cows were computed by pooling together the progesterone estimation in replicate-tubes of cows #6, 8, 90 and 158 as appropriate.*
Means in the same row with different superscript differ (P≤0.02).

Experiment 2

Figure 4.3.

Plasma progesterone concentration changes during the trial is shown for both cows Nos. 441 and 96. Cow No. 441 showed signs of prooestrus after an apparently normal cycle but did not show obvious signs of standing oestrus and did not have a raised progesterone during the following cycle until after the injection of the GnRH analogue buserilin when it increased to more than 2 ng/ml. Cow No. 96 was observed in oestrus immediately after being selected for the study and so no history about the previous cycle is available. In this cow plasma progesterone concentration increased to more than 2 ng/ml on day four of the cycle and to more than 10 ng/ml on day twelve of the cycle. It was then maintained above this level until the cow was slaughtered on day 18.

Fig. 4.3 Plasma progesterone changes
in two cows during *experiment 2*



Note that cow #441 showed sign of pro-oestrus only. This is used as 'day 0'.

Table 4.8.

In cow No. 96, both on the day of buserilin injection and on the following day, a CL and three medium follicles were present in the right and in the left ovary respectively. On day 15, a CL and two medium follicles were present in the right and left ovaries respectively. In cow No. 441, on the day of buserilin and on the following day, no structures were identified on either ovaries and on day 15 only a medium sized follicle was present in the left ovary. Note also the increase in progesterone recorded on day 18, in this cow.

Table 4.8 Ovarian and plasma progesterone changes during critical periods of *experiment 2* in cows treated with an injection of buserilin (GnRH analogue) on day 12 of the oestrous cycle.

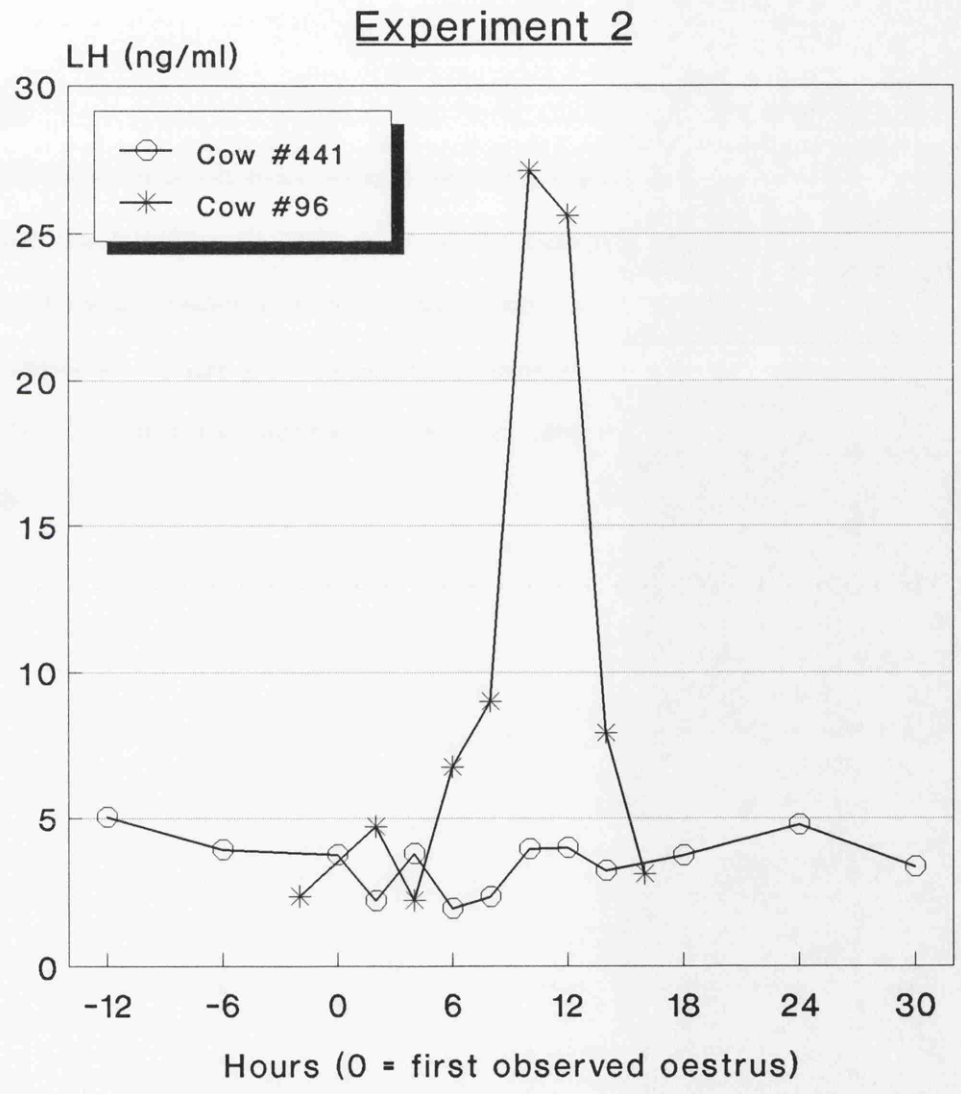
Cow's identity	**Day of oestrus*** (day 0)			**Day 12** (GnRH analogue injection)			**Day 13**			**Day 15**			**Day 18**
	ULSD		P_4	ULSD		P_4	ULSD		P_4	ULSD		P_4	P_4
	CL	Follicles	*(ng/ml)*	CL	Follicles	*(ng/ml)*	CL	Follicles	*(ng/ml)*	CL	Follicles	*(ng/ml)*	*(ng/ml)*
96	+ (RO)	One large (RO)	0.8	+ (RO)	Three medium (LO)	>10	+ (RO)	Three medium (LO)	>10	+ (RO)	Two medium (LO)	>10	>10
441	+ (LO)	one large (LO)	0.7	-	-	1.2	-	-	0.8	-	one medium (LO)	1.1	2.3

**Note that cow #441 only showed signs of prooestrus. (+) = CL present. (-) = no structures observed. (RO) and (LO) = right and left ovaries respectively. Large follicle = follicle of ≥1.5 cm in size. Medium follicle = follicle of ≥0.8 cm in size. P_4 = circulating plasma progesterone.*

Figure 4.4.

The pattern of plasma LH around spontaneous oestrus in the two cows of experiment 2 is presented. In Cow No. 441 the LH peak was not detected. In cow No. 96 the onset of the LH peak occurred approximately seven hours after the first observed oestrus.

Fig. 4.4 Plasma LH concentration around spontaneous oestrus



Note that in cow #441 only signs of prooestrus were observed. This was used as 'time 0'.

Figure 4.5.

Plasma LH concentration prior to and after the injection of buserilin is shown. In the inset, the baseline changes prior to the injection of the GnRH analogue are shown in detail. Baseline values seem higher in cow No. 96. Following the buserilin injection an LH peak occurred in both cows and the main difference between the two animals appear to be the time required for LH to begin increasing.

Fig. 4.5 Plasma LH concentration prior to and after GnRH injection in two cows

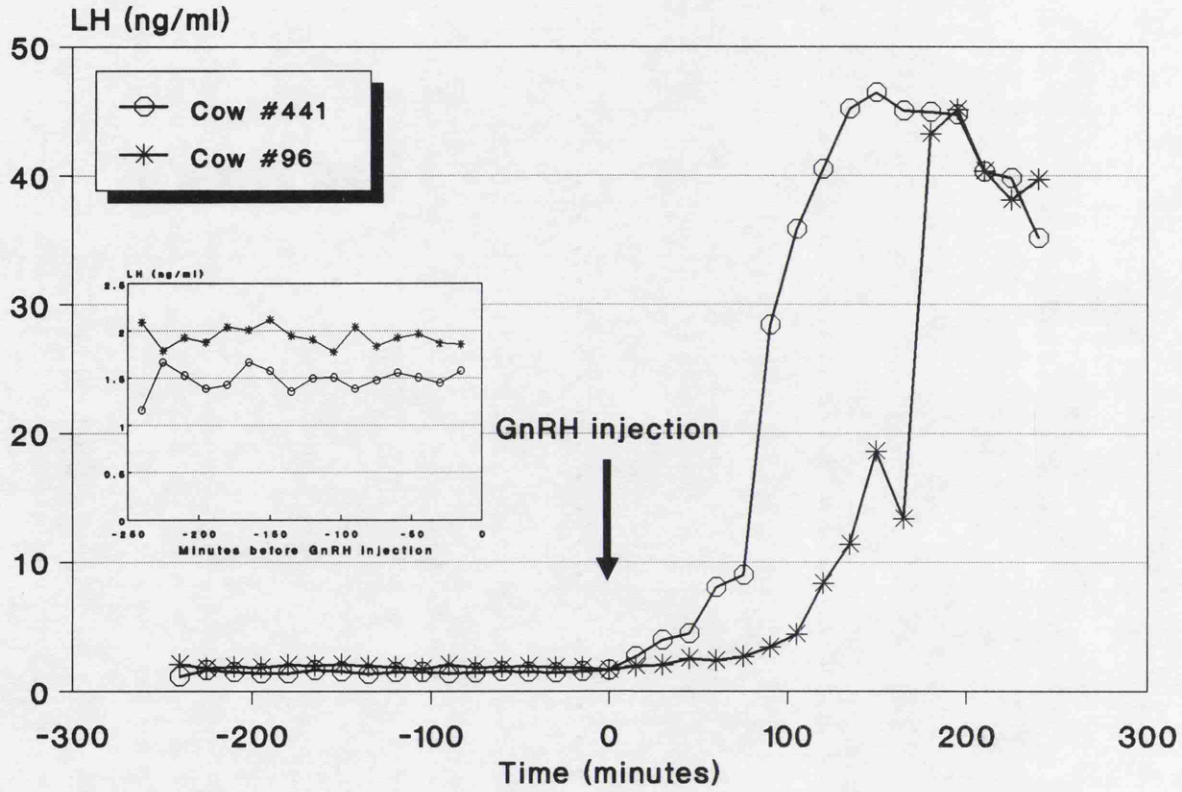


Table 4.9.

The timing and amplitude of the LH peak is given. As no LH peak was noted in cow No. 441, the value equal to two standard deviations more than the mean of baseline values was taken as the amplitude. This was 5 ng/ml and occurred at about 15 hours before the first signs of prooestrus were noted. Following the injection of GnRH analogue LH started to rise in cow No. 441 earlier than in cow No. 96. The LH peak in both cows was of a similar amplitude.

Table 4.9 Timing and amplitude of LH peak during spontaneous oestrus and following buserilin injection (GnRH analogue).

Cow's identity	Spontaneous oestrus*		Day 12 (GnRH analogue injection)	
	Amplitude of LH peak *(ng/ml)*	Interval from onset of oestrus to onset of LH peak *(hours)*	Interval from GnRH injection to onset of LH peak *(minutes)*	Amplitude of LH peak *(ng/ml)*
96	27.1	+7	+82	45.1
441	5.0	-15	+22	46.4

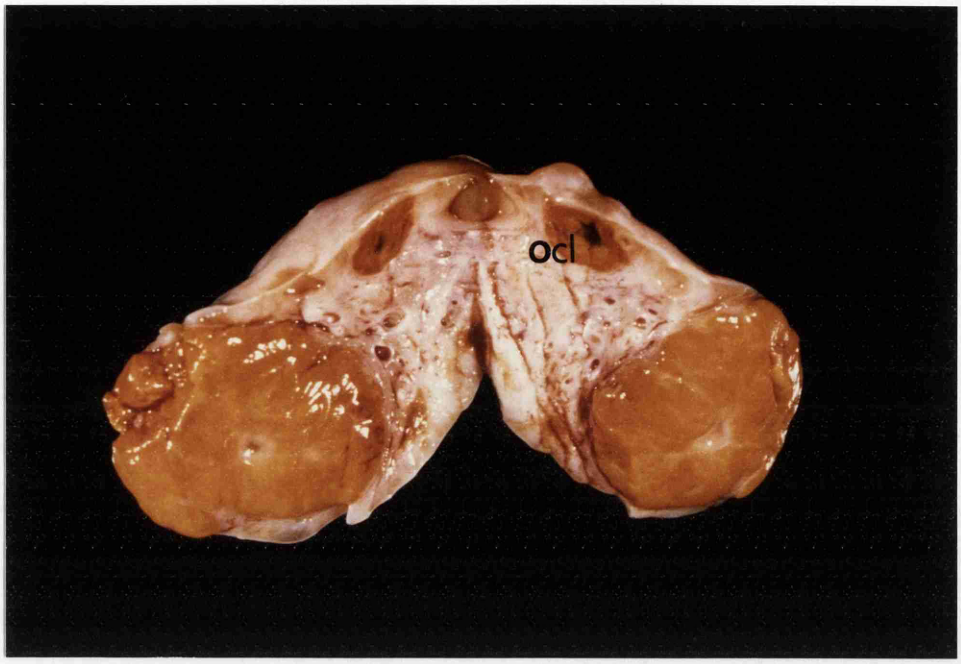
**Only signs of prooestrus were observed in cow #441.*

Plate 4.5a.

The right ovary of cow No. 96 is shown after sectioning. The large CL of the cycle and a small old CL (ocl) of the previous cycle are present in this ovary.

Plate 4.5b.

The left ovary of cow No. 96 is shown after sectioning. A new CL with the features of a second stage CL (< 5 days) is present. Note also that the adjacent follicle (F) appears to have slightly luteinized walls.


ocl

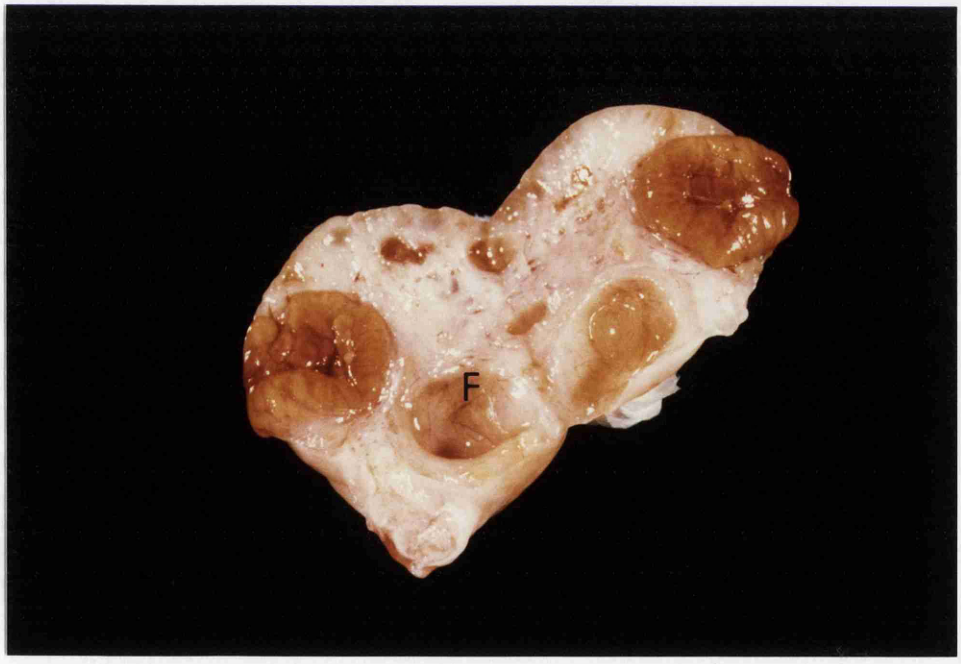

F

Table 4.10.

Both macroscopic and microscopic descriptions of the two luteal structures are given. In addition the *in vitro* response of luteal cells to LH and PGF2α is presented. In the CL of the cycle signs of involution were not obvious. On both gross macroscopic examination and light microscopy the tissue lysis seems to have been delayed. The induced CL appeared normal on macroscopic examination but showed signs of immaturity on histology as many large luteal cells were not enlarged and looked like small luteal cells with granulosa-like nuclei. The presence of connective tissue fibres within cores of luteal tissue resembled that observed in CLs induced by an injection of Prosolvin (experiment 1).

Luteal cells from the two CLs produced low but slightly different amounts of unstimulated progesterone. In both CLs the progesterone production was not significantly influenced by either LH or PGF2α.

Table 4.10 Summary of results of *in vitro* studies of CLs collected five days (day 18 of the cycle) following buserilin injection (cow #96).

CL	Gross macroscopic appearance	CL weight *(g)*	Histology (General appreciation)	In vitro progesterone production by dispersed luteal cells *(ng/20000 cells/3hours incubation)*[1]			
				Control	+LH (13 ng/ml)	+PGF2α (1 μg/ml)	+LH & PGF2α
Cyclic	CL of 2.4 cm present in RO. The internal colour was light orange and the tissue was compact but not hard.	5.09	Signs of cellular regression were observed in only a few large luteal cells. The connective tissue was mainly observed accompanying blood vessels. These had an open lumina and only a few had thick walls.	1.3 ± 1.1	4.7 ± 0.8	2.0 ± 1.2	4.6 ± 2.1
Induced	CL of 1.6 cm present in LO. The ovulatory papillum was covered by epithelium and the internal colour was orange with a red apex.	1.15	Tissue not organized. Small luteal cells appear to have stopped dividing. Some of the large luteal cells have not enlarged yet. No signs of cellular involution were observed in either types. Connective tissue fibers appeared to infiltrate between cores of luteal cells. Blood vessels were open and were thin-walled.	7.0 ± 7.2	17.0 ± 3.5	0.5 ± 0.9	16.8 ± 1.0

([1]) *Progesterone production for each CL is expressed as mean ± sd of three replicate-tubes per treatment.*

V. DISCUSSION

Experiment 1

At the beginning of these investigations the animals were all cycling according to the relevant recorded data. Subsequently they were monitored by behavioural and hormonal studies around the time of next expected oestrus. These findings served as controls for comparison with the findings at the oestrus detected after the administration of the Prosolvin.

The amplitude and the timing of the LH peak varied during natural oestrus from three hours prior to the detection of overt oestrus to nine hours or longer after the demonstration of oestrus. It is possible that the apparent occurrence of the LH peak a little earlier than oestrus, is due to the fact that bleeding for LH was undertaken more often than oestrus detection. However the nine or more hours separating the LH surge and the onset of oestrus in two cows seems long enough an interval to be considered genuine. No differences were identified in the timing and amplitude of the LH peak between the cows in which Prosolvin was administered during early dioestrus and cows injected during late dioestrus. This was also true when, in each cow, spontaneous oestrus was compared to induced oestrus. One particular difference between spontaneous and induced oestrus appeared to be the wider variation in the LH timing in relation to oestrus following induced luteolysis as opposed to spontaneous luteolysis.

During the field trial described in chapter two, Prosolvin was used twice ten days apart as a means of synchronizing oestrus. Evidence was then provided that the initiation of the treatment (first injection) during dioestrus resulted in poorer conception rate at the subsequent insemination than if the treatment was first given during nonluteal periods. However some animals in the first category

did conceive suggesting that either another factor was associated to the stage of the cycle in this detrimental effect, or a specific period within the dioestrus phase was not appropriate for this treatment. Odde (1990) reviewed the use of synchronization programmes in post partum cows and concluded that there was growing evidence that the injection of prostaglandin analogues during the early but responsive period of the cycle resulted in poorer conception rate than their injection during late dioestrus. Evidence from the literature also suggests that CLs formed following prostaglandin analogues may be different histologically and functionally from spontaneous CL (Hardin and Randel, 1982; Hansen et al., 1987).

In the present study the LH peak during induced oestrus was monitored in an attempt to detect variations between treatment groups and between cases of spontaneous oestrus. However, it would appear that the existence of abnormal CL function following prostaglandin injection, if genuine, is not a result of an inadequate pituitary response.

After injecting Prosolvin in both groups of cattle all but one of the cows (No. 57) came into oestrus. Five of the six cows had a functional CL when Prosolvin was injected and afterwards, as expected, a decrease in plasma progesterone concentration occurred. This indicated that in the early dioestrus animals the CLs were mature enough to respond to prostaglandins. An initial drop in plasma progesterone also occurred in the remaining cow (No. 57) in which a luteal cyst was found on ultrasound examination on the day Prosolvin was injected. Subsequently this animal did not show oestrous behaviour although plasma progesterone levels decreased to basal levels. Four days after the Prosolvin injection the plasma progesterone concentration increased to more than 2 ng/ml. When the animal was slaughtered and the ovaries collected the cystic structure was still present in the right ovary and no other structures such

as a new CL or vestiges of regressing luteinized cysts were present, suggesting that in this animal the cystic structure was affected by the Prosolvin injection but somehow managed to reverse the involution process and resume luteal function, hence preventing oestrus and ovulation. This aspect indicates that prostaglandin analogues can also give rise to inconsistent results in particular when an abnormal luteal structure is present. Moreover cows more than heifers have been reported to show this type of result when the injection of prostaglandin is given on day six of the cycle (Momont and Seguin, 1984).

In the normal cycling animals by day six of the cycle some of the CLs may not yet have developed sufficient numbers of receptors to respond adequately to PGF2α. The variation in the results quoted previously in this thesis supports this suggestion that the time taken for the different changes which occur in the first few days of metoestrus can vary greatly between animals. On the other hand in the case of the luteal cyst the deficiency in the number of receptor-sites for prostaglandins may be associated with the overall abnormal formation of this structure.

The five corpora lutea collected from both groups of cattle at post-mortem had the gross appearance of structures of the second stage of the oestrus cycle. This is in agreement with the timing of their previous oestrus. However one of them (cow No. 158) had internal colouring more like that from a third stage CL. This cow was slaughtered a little later than the others and it could be that, recognizing the variability that can occur in individual CLs, some undergo changes in internal colour earlier than others. The cystic structure removed from the remaining cow showed no evidence of an ovulatory papillum despite the occurrence of an LH peak of apparent normal timing and amplitude. Thus it would seem that the cellular composition of the follicle can also be a determining factor as to whether ovulation is achieved or not. It could be that

some factor(s) in the follicular fluid were not sufficiently elaborate to bring about the thinning of the follicular wall in the area of the stigma where rupture of the follicular wall takes place. It is already established that ovulation can be prevented following the injection of PGF2α synthetase inhibitors (Armstrong and Grinwich, 1972).

When the luteal tissue from the other five cows was examined by light microscopy, evidence of regressive changes was seen in all the CLs regardless of whether the Prosolvin had been injected in early or late dioestrus. However one significant feature of the regressive changes noted in third but mainly in fourth stage normal CLs was the thickening of the walls of and in some cases occlusion of the blood vessels (chapter four). This was not evident in the cows treated with Prosolvin. The regressive signs observed could not have been caused by bad fixation or processing as, added to the presence of vacuolae and lipid droplets in the cytoplasm of the cells, there was also presence of connective tissue fibres, infiltrating between cores of luteal cells. This indicates that the process was on-going in the living animal. Furthermore the histology findings were fully supported by electron microscopy in which a different fixative was used. The presence of lipid droplets is thought to indicate a decrease in steroidogenesis as these droplets are known to contain accumulated cholesterol (Flint and Armstrong, 1971).

The plasma progesterone concentration on the day of slaughter was raised above 2 ng/ml in all the cows. However this could represent progesterone synthesized before steroidogenesis ceased. Storage and packaging of secretory products in the cytoplasmic organelles of the cell precede their release in the extracellular matrix and so progesterone may still be present in cells after synthesis has ceased. In addition the regressive changes observed histologically were not affecting all the cells and those remaining functional cells may have

produced the progesterone determined in the circulating plasma.

Histochemical staining for 3β-HSD indicated that this enzyme was present in the luteal tissue of all cows treated. However as previously shown and according to the technique used this enzyme is present in naturally regressing CLs which are producing no progesterone. However the tissue was stained for these enzyme in order to assess the correlation between possible luteal insufficiency and 3β-HSD deficiency. Until more sophisticated investigations as to the role of this enzyme can be undertaken no conclusions can be drawn from these results at present and one can only note the overall observation of 3β-HSD activity in these CLs.

In vitro progesterone production by cells isolated from the CLs from the Prosolvin treated cows varied with the different luteal structures. Luteal cells from cows of group 2 and from one cow of group 1 produced very low levels of progesterone whereas the cells from two cows of group 1 produced relatively high amounts of progesterone similar to those produced by cells from CLs from second stage CLs of a normal cycle (chapter four). These results may or may not indicate a deficiency in the ability to produce progesterone by the cells which gave poor results as some of the cells which apparently were only capable of producing low amounts of progesterone may have recently secreted the levels stored in the cells and those producing high levels may have done so by releasing progesterone already synthesized. However it is an aspect which warrants further investigation as this may be part of the abnormal function that results in an early demise of luteal cells after treatment with prostaglandins.

When the effect of various agents on progesterone production was assessed, it was found that the luteal cells of the present study behaved in the same manner as luteal cells from CLs of the second stage, with regard to response to LH and PGF2α. However the addition of both LH and PGF2α simultaneously did not

result in a difference from aliquots treated with LH alone. These results differed from those obtained when cells from normal CLs were similarly treated. Indeed the absence of an effect of PGF2α on LH-stimulated progesterone production in luteal cells from CLs induced by an injection of prostaglandin does not match the PGF2α-induced reduction in progesterone production in luteal cells from CLs of either second or third stages. Saturation of PGF2α-receptors on the surface of the cells could explain this result. However it would be of some benefit to know whether the cause of this saturation, if genuine, is related to an endogenous release of PGF2α or to the synthetic PGF2α injected and which could be still released from the site of its injection. The fact that in these cows, the induced CL showed regressive changes on histology is not impaired by the response of its cells *in vitro* to added LH. This is because luteal cells from CLs of the third stage were shown to respond similarly with the presence of resembling signs of involution.

In the field study described in chapter two, a proportion of animals treated with the first injection of Prosolvin had basal circulating plasma progesterone concentrations ten days afterwards despite evidence on ultrasound of formation of a new luteal structure. It was suggested that these structures were either still forming as a result of delayed ovulation and/or luteinization or they were regressing prematurely and so had a short life-span. In the study described in this chapter it cannot be determined whether the induced CLs would have definitely regressed prematurely or not. However the regressive processes observed on light and electron microscopy and the poor *in vitro* productivity of progesterone by cells isolated from these structures strongly suggest that these CLs were likely to have a shorter life-span than is normal for an oestrous cycle. The reason for this adverse effect needs further investigation. A possible

explanation could be that the powerful synthetic prostaglandin analogues currently used may not be completely metabolized and remain present in the blood for long enough to cause lysis of the luteal tissue once it became responsive, ie. five days after ovulation.

Experiment 2

The administration of the GnRH analogue buserilin to cattle on days 11 and 13 after insemination has been reported as improving pregnancy rates (Macmillan et al., 1986). However similar studies involving the injection of buserilin on day twelve after oestrus synchronized with PGF2α resulted in comparable pregnancy rates with controls (Drost and Thatcher, 1992). In several other studies involving the use of PGF2α for oestrous synchronization, buserelin apparently extended the timing of the return to oestrus suggesting a delay in luteolysis (Lajili et al., 1991). The capability of buserelin to improve conception rates remains controversial as the number of studies reporting conflicting results is increasing (Drost and Thatcher, 1992).

In the present preliminary investigation details of hormonal and ovarian changes prior to and following the injection of buserilin in two cows were studied.

In one cow (No. 441), buserilin was injected on day twelve after the cow showed signs of prooestrus followed by a failure to ovulate and produce a functional CL. The signs of prooestrus consisted of repeated attempts by the cow to mount other cows followed by her escape from being mounted. The absence of both oestrous behaviour and the LH peak, despite the presence of a large follicle on the day of oestrus suggests a possible lack of oestrogen

secretion from the follicle. This follicle did not ovulate as no CL was found on a subsequent scan on day twelve and plasma progesterone remained low throughout the twelve day period. In addition no follicular activity was observed in this animal during the above mentioned period and a medium follicle only was observed on day 15 after the animal showed signs of prooestrus (three days after GnRH analogue injection).

In the other cow (No. 96) buserelin was injected on day 12 of an apparently normal oestrous cycle and an LH peak and overt oestrous behaviour occurred within the expected time followed by an increase in the circulating plasma progesterone concentrations and identification of a luteal structure on ultrasound. Three medium-sized follicles were present in the ovaries of this cow on days 12 and 13 after overt oestrus. Intensive monitoring of the plasma LH concentrations prior to the injection of buserelin suggested that in the cow which did not ovulate there were lower tonic levels of LH than in the animal which did. The LH assay used was not sufficiently sensitive as to allow differences of less than 2 ng/ml to be identified. However given that all the values in the cow that ovulated were more than 1.7 ng/ml and were consistently higher than those in the cow that did not ovulate, it seems reasonable to assume that the consistently lower levels in this one cow were significant. In a normal, cycling bovine the decrease in plasma progesterone after luteolysis is always accompanied by an increase in the amplitude of LH pulses and so progesterone is considered the main inhibitory factor of LH. In the animal in which lower progesterone concentrations were recorded, the absence of raised progesterone levels should have resulted in high baseline LH secretion. However this was not the case. This could indicate a deficiency in pituitary LH. However when the animal was given buserelin this subsequently produced an LH peak. The onset of this increase occurred even more quickly than in the other cow suggesting

that the deficiency in circulating plasma LH may have been due to a deficiency in the hypothalamic releasing factors rather than in the availability of LH from the pituitary.

Following the administration of the GnRH analogue plasma progesterone concentrations remained high in the normal cow (No. 96) until the day of slaughter. The progesterone profile alone was not sufficient to say whether or not this cow would have had a delayed return to oestrus. However the finding of an accessory CL which was the result of a recent ovulation tends to indicate that this cow was not likely to be in oestrus during the following several days which indeed would have meant a delay in her return to oestrus. It has been reported that an injection of buserelin resulted in a decrease in circulating plasma oestradiol concentrations suggesting that the hypothesized delay in luteolysis may be associated with follicular atresia (Mann and Lamming, 1992). Although the spontaneous occurrence of accessory CLs is not that uncommon in cattle, the results in this animal tend to suggest that this might be one of the mechanisms causing the reported delay in return to oestrus. Recently, Harvey et al. (personal communication) used a relatively large sample of dairy cows and obtained evidence that accessory CL formation was indeed responsible for the delayed return in a considerable proportion of the animals.

After the administration of buserelin to the other cow which did not ovulate (No. 441) the circulating plasma concentrations rose to levels very similar to those found during dioestrus. Ultrasound did not identify the presence of luteal tissue three days afterwards probably because CLs less than five days old are hardly distinguishable from the surrounding ovarian stroma. This cow was turned out to grass at that point and so it cannot be ascertained whether or not she had produced an accessory CL or whether a follicle had luteinized. These concentrations of progesterone are greater than one would expect at this

interval after oestrus in the normal cycle and a CL of this age would not necessarily be identified by ultrasound. Therefore it is possible that this type of treatment causes a rapid response from the pituitary such that the release of LH causes either luteinization or ovulation depending on the capability of the most mature follicle at the time of administration of the drug.

Histological studies of the CLs present in the ovaries of the normal cow indicated that the initial CL of the cycle was not undergoing degeneration. Knowing that this structure was 18 days old it is tempting to suggest that lysis of this structure was delayed. The accessory CL showed signs of immaturity in that many of the luteal cells were not enlarged and resembled small luteal cells with a granulosa cell like nucleus. The presence of fibres of collagen from the connective tissue amongst the luteal cells, although at a less extensive degree of penetration than in the CLs induced by Prosolvin in the first experiment indicates that this structure could have had a shorter than normal life-span. Rusbridge and Webb (1991) reported that CLs induced by an injection of GnRH during the early part of dioestrus had a shorter life-span than CLs formed following natural oestrus. However these authors lysed the cyclic CL by injecting prostaglandins within five days of the administration of GnRH. The injection of prostaglandin was not likely to have caused lysis of the induced (less than five days-old) CL. However again it cannot be ruled out that the use of these powerful analogues which may not be readily metabolized would result in the lysis of the newly formed CL once it achieves maturity and acquires receptors for PGF2α.

In vitro progesterone production by luteal cells of both CLs was low and variable and although LH appeared to increase progesterone production this was not significant between paired aliquots. Similarly it cannot be said that PGF2α caused any effect on basal or LH-stimulated progesterone synthesis

given the variation and the small amounts of progesterone present in individual aliquots.

The little production of progesterone could be the result of desensitized cells to LH. This was already described by Martin et al. (1990) following the injection of GnRH on days 2 and 8 of the oestrous cycle. However this needs to be further clarified as the mechanisms and circumstances of this receptor loss are not clear in newly formed CLs.

CHAPTER SIX

GENERAL DISCUSSION

The in depth studies of the formation function and regression of bovine CLs presented possible explanations as to how this important structure apparently determines the outcome of attempts to synchronize oestrus in cattle and of the pregnancies subsequently established. The results of the field trial suggested that if using prostaglandins (a double injection scheme) as a means of synchronizing oestrus then better results were achieved if heifers are in the nonluteal phase rather than dioestrus when the treatment is initiated (first injection). This is most probably related to the follicular state of the ovaries at this time as it would appear that in some instances the follicles available for maturation and ovulation are not capable of responding to the increased amplitude of the LH pulses so that maturation and ovulation is not achieved or delayed and conception does not result from the fixed time AI which forms part of this type of procedure.

On the other hand ovulation may be achieved but the resulting CL is not normal in either function or composition and so a short life-span ensues and the pregnancy is lost. The components required by follicles to achieve maturation, ovulation and subsequent formation of a normal CL are as yet not fully understood.

The results of the field trial led to further investigations of some of the changes which may contribute to the abnormal set of conditions which result in poor synchronization programmes. The investigations were confined mainly to the CL rather than the follicle. Whilst normal circulating plasma progesterone concentrations were produced by many CLs induced by prostaglandins, microscopic examination of such luteal tissue indicated that some of these structures were not normal in that they showed signs only found in a structure undergoing regression. Luteal structures may stop synthesizing progesterone

long before the existing stocks of progesterone are exhausted and so the secretion of progesterone can continue for a further period of time.

In an attempt to determine what the deficiencies in these abnormal CLs were, the activity of two steroidogenic enzyme (3β-HSD and 20α-HSD) was investigated histochemically, first in presumably normal CLs collected at a local abattoir. However the method used had its limitations and although both 3β-HSD and 20α-HSD were identified in the bovine luteal tissue examined it was not possible to either identify which of the two types of luteal cells which make up the CL in the bovine was concerned with each of the enzymes or if there was any evidence of increases and/or decreases in the amounts of these enzymes at the different stages of the life-span of this structure. Obviously this aspect of 3ß-HSD functions warrants further investigation with more specific techniques eg. a combination of immunochemistry and electron microscopy, as such results would lead to a better understanding of the role of these and other enzymes involved in progesterone synthesis. Such information would be of particular benefit in understanding the relationship between these enzymes and PGF2α which almost certainly acts on the luteal cells at a site distal from LH-receptor-cAMP system and so influences progesterone synthesis by interfering with one or more steps between cholesterol and progesterone synthesis.

Cell culture was used as a means of investigating how the different cell-types interact in either the promotion or the inhibition of progesterone synthesis. The separation of large and small cells proved difficult and tedious and was never carried out as successfully as wished. Separation of the two types is required in order to determine the *in vitro* response of each of these cells to the various known factors involved in the maintenance and function of the CL.

The use of new expensive techniques such flow cytometry are being published at the moment which should with time overcome the problems of separation and

this would be a great step forward in bringing total understanding of this complex structure much nearer.

Another aspect of the luteal cell function which is still open for investigation is the characterization of receptor-sites for both luteotrophic and luteolytic agents and changes in their numbers. The role of growth factors and other peptides such as relaxin, neurophisin and oxytocin some of which are thought to be secreted by luteal cells is of particular importance for the understanding of the regulation of growth and differentiation of the luteal cell. However it would be of benefit if such studies were undertaken in specific types of follicles eg. follicles belonging to different waves, and in CLs induced from the same type of follicles in understanding why some animals and not others can achieve pregnancy when oestrus is artificially induced. In this thesis, although the number of animals used was not sufficiently large to allow for firm conclusions to be drawn it was apparent that an inadequate luteal phase following the artificial induction of ovulation was more to do with short luteal life-span than delay in the maturing of CLs. Obviously, more could be learned by first using larger samples of animals and secondly by monitoring plasma levels of prostaglandins including injected synthetic PGF2α. Therefore although these studies did produce many interesting findings it is obvious that much still remains to be done in this field of research. Studies of the follicle from which the CL originates would obviously complement the findings detailed here and may well prove to be where the main problem arises which results in abnormal CLs being produced.

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